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Evaluation of the effects of biostimulants on the Madeiran maize variety 'Milho de Santana' under drought conditions

MASTER DISSERTATION

Patrícia Raquel Abreu Silva
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
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November 2023

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Dissertation submitted to the University of Madeira in fulfilment of the requirements for the degree of Master in Applied Biochemistry

By Patrícia Silva

Work developed under the supervision of Professor Dr. Miguel Ângelo Almeida Pinheiro de Carvalho, and co-supervision of Dr. David Jiménez Arias

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ABSTRACT

Anthropogenic activities are responsible for the emission of greenhouse gases into the atmosphere, which leads to an increase in their concentration and, therefore, an increase in temperature. Drought is one of the stressors that most disturb crop production. Biostimulants are eco-friendly substances and can be used to enhance stress tolerance. However, weekly treatments are needed to make them effective, which is disadvantageous for larger farms. One solution to overcome this problem is to use nanoencapsulation techniques to prevent degradation or provide controlled release of biostimulants. In this study, the ionotropic gelation technique was used to produce chitosan nanoparticles, which is fast, relatively cheap, and does not require expensive reagents and equipment, or organic solvents. In this study, L-pyroglutamic acid (LPGA) was used as the main biostimulant.

The Madeiran maize variety called 'Milho de Santana' was subjected to drought. Well-watered (WW) plants with 100 % irrigation were compared to water-deficit (WD) plants under a 50 % irrigation. The following experimental variants were assessed: control (C), empty particle of chitosan (EP), entrapped biostimulant in chitosan particles (EBs), and free biostimulant (Bs). Hoagland's nutrient solution and the experimental variants were directly applied to the soil. Five assays were performed: the first three to establish the experimental design and the last two to assess biostimulant action. Parameters such as soil water content, size of chitosan particles, peat microbe analysis, whole-plant biomass dry weight, root, aerial-part and whole-plant lengths, root stress index (SI), proline (Pro), total soluble sugars (TSS) in roots and aerial parts (stems and leaves), and correlations between these parameters were analysed. No significant differences ($p < 0.05$) were detected for most parameters, except for TSS in the roots and aerial parts. Sampling size, maize variability, difficulties in stress and irrigation reproducibility, among other reasons, may explain the obtained results.

Keywords: agriculture; biostimulants; chitosan nanoparticles; drought; microorganisms.

RESUMO

As atividades antropogénicas são responsáveis pela emissão de gases de estufa para a atmosfera, originando o aumento da sua concentração e, consequentemente, o aumento da temperatura. A seca é um dos stresses que mais afeta a produção agrícola. Os bioestimulantes são substâncias ecológicas, que podem ser usadas para melhorar a tolerância das plantas ao stress. Contudo, são necessários tratamentos semanais para estes serem eficazes, tornando o procedimento economicamente ineficaz em grandes explorações. Uma solução é a utilização de técnicas de nanoencapsulamento para prevenir a degradação dos bioestimulantes e promover a sua libertação gradual. Neste estudo, a técnica de gelificação ionotrópica foi usada na produção de nanopartículas de quitosano, dado que é rápida, relativamente barata e não necessita de reagentes e equipamentos caros, ou solventes orgânicos. Neste estudo, o ácido L-piroglutâmico (LPGA) foi usado como bioestimulante principal.

A variedade madeirense "Milho de Santana" foi submetida a stress, sendo as plantas divididas entre milho bem irrigado (WW) com uma irrigação de 100 %, e milho em défice hídrico (WD) com uma irrigação de 50 %. As seguintes variantes experimentais foram utilizadas: controlo (C), partícula vazia de quitosano (EP), bioestimulante encapsulado em quitosano (EBs), e bioestimulante (Bs) livre. A solução nutritiva de Hoagland e as variantes experimentais foram aplicadas diretamente no solo. Cinco ensaios foram realizados: os três iniciais para otimizar o desenho experimental, e os dois últimos para avaliar a ação do bioestimulante. O conteúdo de água no solo, tamanho das partículas de quitosano, análise do solo, peso da biomassa seca de toda a planta, comprimentos da raiz, da parte aérea e de toda a planta, índice de stress (SI) da raiz, prolina (Pro), açúcares solúveis totais (TSS) nas raízes e partes aéreas (caules e folhas), e as correlações entre estes parâmetros foram analisados. Nenhuma diferença significativa ($p < 0.05$) foi detetada para a maioria dos parâmetros, exceto para os açúcares solúveis totais nas raízes e nas partes aéreas. A dimensão das amostras utilizadas nos ensaios, a variabilidade fenotípica do milho, a dificuldade em manter as condições de stress e irrigação, entre outros, podem explicar os resultados obtidos.

Palavras-chave: agricultura; bioestimulantes; microorganismos; nanopartículas de quitosano; seca.

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Table A.9. Pearson correlation between biomass (g), Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts, independent of the presence or absence of stress, on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars. 90

Table A.10. Pearson correlation between biomass (g), Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts in the absence of stress on day 14. Asterisks represent statistical significance ($p < 0.01$). Abbreviations: Pro, proline; TSS, total soluble sugars. 90

Table A.11. Pearson correlation between biomass (g), Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts in the presence of stress on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars. 90

Table A.12. Pearson correlation between biomass (g), Pro (µmol/g FW), and length (cm) in the fifth assay for the whole-plant, independent of the presence or absence of stress, on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline. 91

Table A.13. Pearson correlation between biomass (g), Pro (µmol/g FW), and length (cm) in the fifth assay for the whole-plant in the presence of stress on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline. 91

ABBREVIATIONS

AACP	Amino acid-containing products
Al	Aluminium
AP	Aerial part
Bs	Biostimulant
C	Control
Ca^{2+}	Calcium ion
$Ca(NO_3)_2 \cdot 4H_2O$	Calcium nitrate tetrahydrate
CFUs	Colony-forming units
CH_4	Methane
CO_2	Carbon dioxide
$CuSO_4 \cdot 5H_2O$	Copper(II) sulphate pentahydrate
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DW	Dry weight
EBs	Entrapped biostimulant
EBIC	European Biostimulants Industry Council
EP	Empty particle
EPA	Environmental Protection Agency
FAD	Flavin adenine dinucleotide (oxidized form)
FADH2	Flavin adenine dinucleotide (reduced form)
FW	Fresh weight
GSA	Glutamate-semialdehyde
H_3BO_3	Boric acid
H_2SO_4	Sulphuric acid
HCP	Hormone-containing products
HK_2O_4P	Potassium phosphate dibasic
HS	Humic substances
IBM	International Business Machines Corporation
ISOP	Code used to access the ISOPlexis's germbank
KH_2PO_4	Potassium dihydrogen phosphate

KNO_3	Potassium nitrate
LPGA	L-pyroglutamic acid
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
$MnSO_4 \cdot 4H_2O$	Manganese(II) sulphate tetrahydrate
N	Nitrogen
N_2O	Nitrous oxide
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Ammonium heptamolybdate
NH_4NO_3	Ammonium nitrate
NS	Hoagland's nutrient solution
OAT	Ornithine-δ-aminotransferase
P5C	Δ'-pyrroline-5-carboxylate
P5CS	Δ'-pyrroline-5-carboxylate synthetase
P5CS1	Δ'-pyrroline-5-carboxylate synthetase 1
P5CS2	Δ'-pyrroline-5-carboxylate synthetase 2
P5CDH	Δ'-pyrroline-5-carboxylate dehydrogenase
P5CR	Δ'-pyrroline-5-carboxylate reductase
PDA	Potato dextrose agar
ProDH	Proline dehydrogenase
ProDH1	Proline dehydrogenase 1
ProDH2	Proline dehydrogenase 2
PGPRs	Plant growth-promoting rhizobacteria
POX	Proline oxidase
Pro	Proline
PSII	Photosystem II
QACs	Quaternary ammonium compounds
r	Pearson correlation

R	Root
R^2	Correlation coefficient
ROS	Reactive oxygen species
rpm	Revolutions per minute
Se	Selenium
Si	Silicon
SI	Stress index
SPSS	Statistical Package for the Social Sciences
TPP	Tripolyphosphate
TSS	Total soluble sugars
USA	United States of America
UV	Ultraviolet
UV/VIS	Ultraviolet-visible
WD	Water-deficit
WD-C	Water-deficit control
WD-EP	Water-deficit empty particle
WD-EBs	Water-deficit entrapped biostimulant
WD-Bs	Water-deficit biostimulant
WW	Well-watered
WW-C	Well-watered control
WW-EP	Well-watered empty particle
WW-EBs	Well-watered entrapped biostimulant
WW-Bs	Well-watered biostimulant
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

CHAPTER I.

INTRODUCTION

1.1. Human population and climate change

Human population growth has been accompanied by several modifications in culture, the economy, science, technology, and agricultural production. The latest has experienced multiple changes that have influenced the development of civilisation and technology, allowing general human development. However, the exceptional and unsustainable growth of the last 100 years has undesirable consequences that, along with environmental changes, impact food supply security. This population growth brings demand for food production, and by 2050, global agricultural production will need to double to accommodate this demand (1).

Climate is an important element that determines many characteristics and the distribution of managed and natural systems, including water resources, aquatic and terrestrial ecosystems, forestry, and agricultural systems (1). Climate change is characterised by significant changes in average precipitation and temperature values. Temperature changes are caused by both natural and anthropogenic activity (2). Changes related to anthropogenic influence include an increase in warm temperature extremes events, a reduction in cold temperature extremes, enhanced rates of sea-level rise, and an increase in the frequency of heavy precipitation events in various regions. Extreme precipitation events are expected to be more intense and frequent in certain areas, and heat waves are expected to become more frequent and last longer (1).

Anthropogenic activities are responsible for the emission of greenhouse gases, such as carbon dioxide (CO_2) and methane (CH_4), as well as other substances that lead to an increase in their concentration and ozone depletion in the atmosphere. Since 1750, the concentrations of greenhouse gases, such as CO_2 and CH_4, have increased by 40 % and 150 %, respectively. Since 1975, the average global temperature has risen at an average rate of 0.15 – 0.20 °C per decade (2).

Food production worldwide is threatened by climate extremes, as determined by the increase in temperature, CO_2 and other greenhouse gases, and changes in precipitation patterns (1). The agricultural sector contributes 15 % of total greenhouse gases emissions, primarily CH_4 and nitrous oxide (N_2O). If dietary preferences and the consumption of food energy are kept constant at 1995 levels, global emissions of agricultural non-CO_2 greenhouse gases are predicted to rise until 2055 (2).

1.2. Climate change and agriculture

Agriculture depends on weather conditions to produce food and other goods needed to sustain mankind (1). Agriculture is highly vulnerable to climate change due to its sensitivity to weather parameters, causing significant economic impacts. Crop yield is affected by these changes, such as precipitation variation and rising temperatures, which vary according to the crop, production, and magnitude of the change. Area and irrigation applications are responsible for the varying effects of climate change on crop yields, which can be increased by the expansion of irrigated areas, creating a detrimental effect on the environment. If temperate and tropical regions increase their temperatures by 2 °C, maize, rice, and wheat production is expected to decrease (2).

The loss of crop yield can increase food prices and affect global agricultural welfare (2). The global population is estimated to reach up to 9.8 billion by 2050, increasing the proportion of undernourished people every year. Consequently, an increase in food production is required, which leads to concerns regarding the use and availability of water. Water shortage is one of the main concerns in future climate predictions, affecting agricultural systems, crop yield, and product quality. The need for water irrigation will increase by more than 50 % and 16 % in developing and developed regions, respectively, generating intense pressure on freshwater resources (3).

Plant-water relationships are susceptible to changes, according to precipitation and temperature. Species and plant developmental stages lead to variation in their responses to water scarcity, as species-specific thresholds exist for different plants, and their responses, such as disturbance in the growth angle and elongation of roots, and reduction in yield, vary among different plant species (2). Regarding water deficit, plant responses depend on various factors, such as the duration of stress and the possibility of activating certain tolerance mechanisms. Long-term drought can cause cell turgor loss, decline in gas-exchange parameters, and water imbalance (3).

The presence of microbial populations in the soil is also affected by climate change as well as enzymatic activities. When evaluated from a temperature gradient tunnel with a 4 – 5 °C higher temperature, the microbial population was significantly higher than that observed under field conditions (2).

Climate change also has the potential to increase pest populations and their migration, which can have various effects on agricultural yields and viability, as population size mainly depends on abiotic factors. Infestation by pests has led to high pesticide costs for pest management. Climate change can enlarge the season of pest development and modify crop and pest synchronization (2).

1.3. Maize (*Zea mays* L.)

1.3.1. Description of maize

Maize (*Zea mays* L.) was first described by Linnaeus in 1753. It belongs to the Poaceae family. It is grown in Madeira for its grain or as a fodder crop, and is cultivated in field farms, or in gardens. In Flora of Madeira, Press *et al.* (4) described *Zea mays* as "(…) a robust, monoecious annual; the female inflorescence is axillary, wrapped in several sheaths, with protruding stigmas ('silks'), the numerous spikelets arranged in rows on a thickened, woody 'cob'; the male inflorescence ('tassel') is a terminal panicle" (Figure 1).

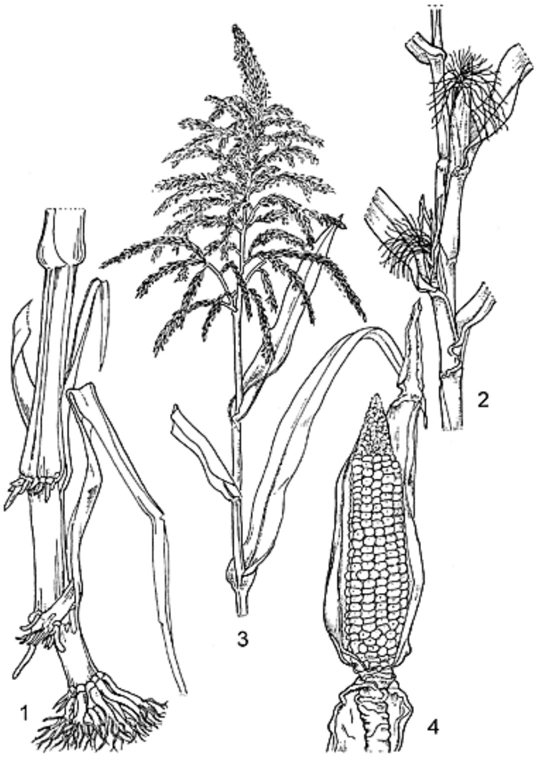


Figure 1. *Zea mays* L. illustration. Legend: 1, basal plant part ; 2, central plant part with female inflorescences; 3, upper plant part with male inflorescence; 4, ripe infructescence. From: Koopmans *et al.* (5)

1.3.2. History of maize on Madeira Island

Zea mays was introduced to Madeira Island in 1760, more than 200 years after the first maize introductions in Europe. Maize was adopted as an agricultural crop only in 1847, promoted by irrigation improvement in local agriculture, and was used for human consumption and animal feeding (6).

Since the beginning of its cultivation in Madeira, maize has been traditionally cultivated by farmers across the entire archipelago in diverse environments ranging from sea level to 1,000 m. Despite the small size of the archipelago, maize is cultivated under a variety of conditions differing in pluviosity, soil, temperature, manure and intercropping conditions, and rotational practices. As a result, open-pollinated cultivars that belong to two main varieties of maize, white ('Branco') and yellow ('Amarelo'), have been acclimated to different environmental conditions because of their geographical isolation, landscape, and farmers' selection criteria. In Madeira, maize is sown from late February to May and harvested from July to the end of October, depending on the geographical location and the specific conditions of the farmer's plot. The successful adaptation to local conditions explains why maize is still used as a crop by farmers and their preferences for old local cultivars, differentiated by several specific morphological and agronomic features. In Madeira, the use of traditional maize cultivars to cook has avoided the replacement of local landraces with commercial hybrids, which had never been successfully used. However, situations such as abandoning traditional agricultural practices and reducing rural populations will lead to irreversible genetic erosion of these maize landraces (6).

1.4. Plant biostimulants

1.4.1. History and definitions

The word 'biostimulant' was coined by horticulture specialists to describe substances that promote plant growth and are not nutrients, pesticides, or soil improvers. In 1997, Zhang and Schmidt, who wrote for a web journal called Ground Maintenance, defined biostimulants as "materials that, in minute quantities, promote plant growth". The authors used the words "minute quantities" to distinguish biostimulants from nutrients and soil amendments, which also promote plant growth but are applied in larger quantities. Humic acids and seaweed extracts are the biostimulants mentioned at the latest. The term biostimulant is not necessarily used in other peer-reviewed papers by the same authors in the same or similar research. Zhang and Schmidt have a paper that describes the use of humic acids and seaweed extracts for increasing drought tolerance of turfgrass, and the term biostimulant was not used. This study focused on the hormone-like activities of the previously mentioned compounds, and the term ‘hormone-containing products’ was used instead of biostimulants. The Environmental Protection Agency (EPA) frees ‘vitamin-hormone horticulture products’ from registration under certain conditions, which can also explain the choice made by the authors. Biostimulation action is explained by hormonal effects and protection against abiotic stress through the antioxidant responses induced by them. In later studies, the term ‘metabolic enhancers’ was also used (7).

When it comes to scientific literature, in 2007, Kauffman *et al.* (8) tried to introduce a classification for biostimulants, saying that they “(…) are available in a variety of formulations and with varying ingredients but are generally classified into three major groups based on their source and content. These groups include humic substances (HS), hormone-containing products (HCP), and amino acid-containing products (AACP). HCPs, such as seaweed extracts, contain identifiable amounts of active plant growth substances such as auxins, cytokinins, or their derivatives”. Biostimulants are different from fertilisers because they act on plant metabolism and their nutrient concentrations are insignificant. These products can alter root conformation and increase root development (9).

Over the years, the term ‘biostimulant” has become increasingly used in the scientific literature, which has expanded the range of substances and modes of action. Biostimulants are defined by what they are not, creating a borderline between them and other categories of substances applied to plants and crops (fertilisers and pesticides). The positive actions of chemical biostimulants with a natural or synthetic origin, such as growth promotion, increased tolerance to environmental stress, and modulation of development and quality traits, can also be applied to bacteria and fungi. ‘Plant growth-promoting rhizobacteria’ (PGPRs) have beneficial effects on plants, without being nutrients, pesticides, or soil improvers. They are diverse in nature and their categories are defined based on their agricultural/horticultural outputs (7).

In 2015, Du Jardin (7) defined plant biostimulants as “any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content”. In 2020, Du Jardin *et al.* (10) added that there is still no stabilised definition for biostimulants, but academic, regulatory, and corporate entities agree that they are modulators of life processes in plants that enhance growth and resource use efficiency under stress or non-stress conditions. The definition of plant biostimulants in the European Union regulation (2019) is: “A product that stimulates plant nutrition processes independently of the product’s nutrient content, with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency; (b) tolerance to abiotic stresses; (c) quality traits; or (d) availability of confined nutrients in the soil or rhizosphere” (3).

The industry has had a huge impact on the definition and promotion of biostimulants. Associations were created, such as the ‘European Biostimulants Industry Council’ (EBIC) in Europe and the ‘Biostimulant Coalition’ in the USA, having conversations with other stakeholders, regulators, and scientists to implement the biostimulant strategy (7).

1.4.2. Categories

Scientists, regulators, and stakeholders recognize seven main categories of biostimulants that include microorganisms and substances (Figure 2) (7).

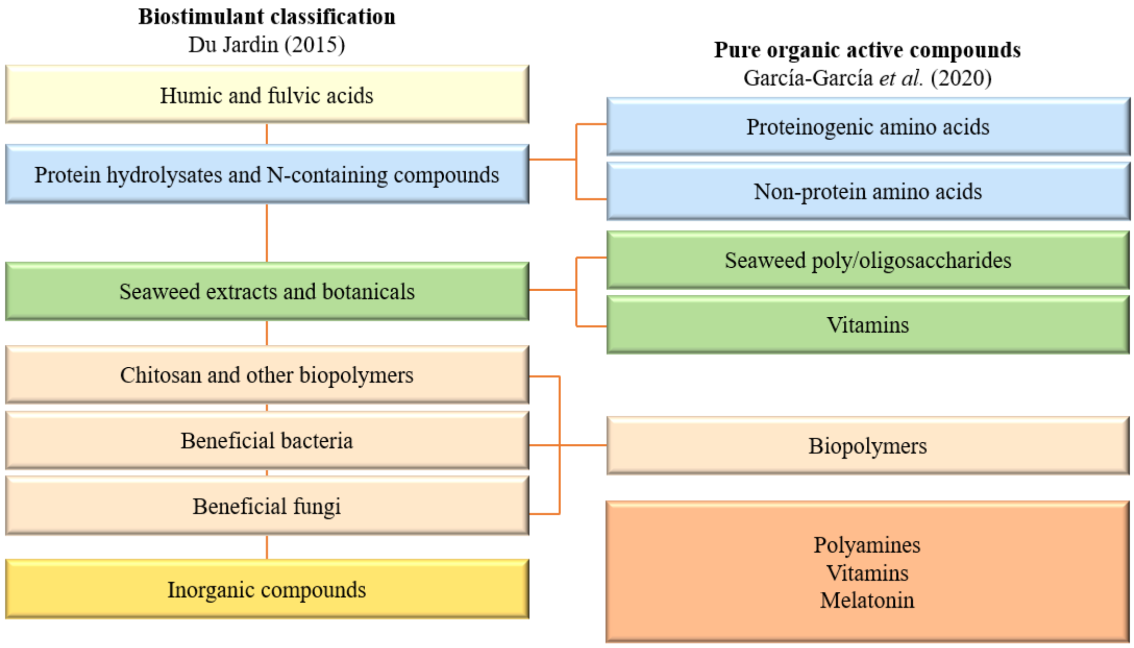


Figure 2. Classification of biostimulants according to Du Jardin (2015) and pure organic compounds, and their relationship with the former classification according to García-García *et al.* (2020). Based on García-García *et al.* (11)

1.4.2.1 Humic and fulvic acids

Humic substances are natural constituents of soil organic matter, resulting from the decomposition of animal, microbial, and plant residues and the metabolic activity of soil microbes using these substrates. They are categorised as fulvic acids, humins, and humic acids, according to their molecular weight and solubility. These compounds exhibit dynamics of association or dissociation into supramolecular colloids, which are influenced by plant roots through the release of protons and exudates. Humic substances and their complexes in the soil result from interactions between microbes, organic matter, and plant roots. The effects of humic substances depend on their source, environmental conditions, receiving plants, and the dose and manner of their application. Humic substances are extracted from compost and vermicompost, naturally humidified organic matter, or mineral deposits. Instead of being decomposed in soil or by composting, agricultural by-products are amenable to controlled breakdown and oxidation by chemical processes, which leads to 'humic-like substances' that are proposed as substitutes for natural humic substances (7).

Treatment with this biostimulant has shown stimulation of root growth and development, which is reflected in better uptake of nutrients and water, and enhanced tolerance to environmental stresses. Due to its molecular complexity and the abundance and diversity of plant responses altered by its application, the effect of humic substances on plant physiology is not completely understood (12).

1.4.2.2.Protein hydrolysates and other N-containing compounds

Hydrolysed proteins are mixtures of amino acids, peptides, polypeptides, and denatured proteins that can be obtained by chemical, enzymatic, and thermal hydrolysis of proteins (or by the combination of different hydrolysis types) from animal and plant sources (12).

These compounds have shown direct effects on plants, including modulation of nitrogen (N) uptake and assimilation, by acting on the signalling pathway of N acquisition in roots and through the regulation of enzymes involved in N assimilation and their structural genes. Some amino acids such as proline have chelating effects that may protect plants against heavy metals and contribute to micronutrient mobility and acquisition. Antioxidant activity is conferred by the scavenging of free radicals by some of the nitrogenous compounds, which contributes to the mitigation of environmental stress. When protein hydrolysates are applied to plants and soils, the indirect effects are important for their detection. Protein hydrolysates increase microbial biomass and activity, soil respiration, and soil fertility. The chelating and complexing activities of specific amino acids and peptides contribute to nutrient availability and acquisition by the roots (7).

Commercial products obtained from plant and animal protein hydrolysates have been released into the market. In many cases, significant improvements in yield and quality traits have been reported for both agricultural and horticultural crops. No ecotoxicity, genotoxicity, or phytotoxicity has been reported based on bioassays using yeast (7).

1.4.2.3.Seaweed extracts and botanicals

Fresh seaweeds have been used as a source of organic matter and fertilisers for a long time in agriculture. However, their biostimulant effects have only recently been reported, which has led to the commercial use of seaweed extracts and purified compounds (alginates, polysaccharide laminarin, carrageenans, and their breakdown products). Hormones, micro- and macronutrients, N-containing compounds (e.g. betaines), and sterols are constituents that contribute to plant growth promotion. Most algal species used in this way belong to the brown algae (*Ascophyllum*, *Fucus*, and *Laminaria* being the main genera). Carrageenans are obtained from red seaweeds and belong to different phylogenetic lines (7).

Seaweeds can be applied to soils, hydroponic solutions, and foliar treatments, where their polysaccharides contribute to gel formation, aeration, and water retention in the soil. Polyanionic compounds contribute to the exchange and fixation of cations, which are of interest in the fixation of heavy metals and soil remediation. One of the positive effects of soil microflora is the promotion of plant growth-promoting bacteria and pathogen antagonists in challenging soils. In plants, through the provision of micro- and macronutrients, nutrient effects show that they act as fertilisers in addition to other roles. Their impacts on seed germination, plant establishment, growth, and development are linked to hormonal-like effects, which are major causes of biostimulation activity in crop plants. Anti-stress effects have been reported due to the presence of protective compounds in seaweed extracts, such as antioxidants and regulators of endogenous stress-responsive genes (7).

'Botanicals' are substances that are extracted from plants, being used in pharmaceutical and cosmetic products, as food ingredients, and in plant protection products (7).

1.4.2.4. Chitosan and other biopolymers

Chitosan is a deacetylated form of the biopolymer chitin that can be produced naturally or industrially (7). Chitosan is composed of N-acetyl-D-glucosamine and D-glucosamine units connected by β-1,4-glycosidic linkages (Figure 3) (13). Poly- and oligomers of variable and controlled sizes are used in the food, agricultural, cosmetic, and medical sectors (7).

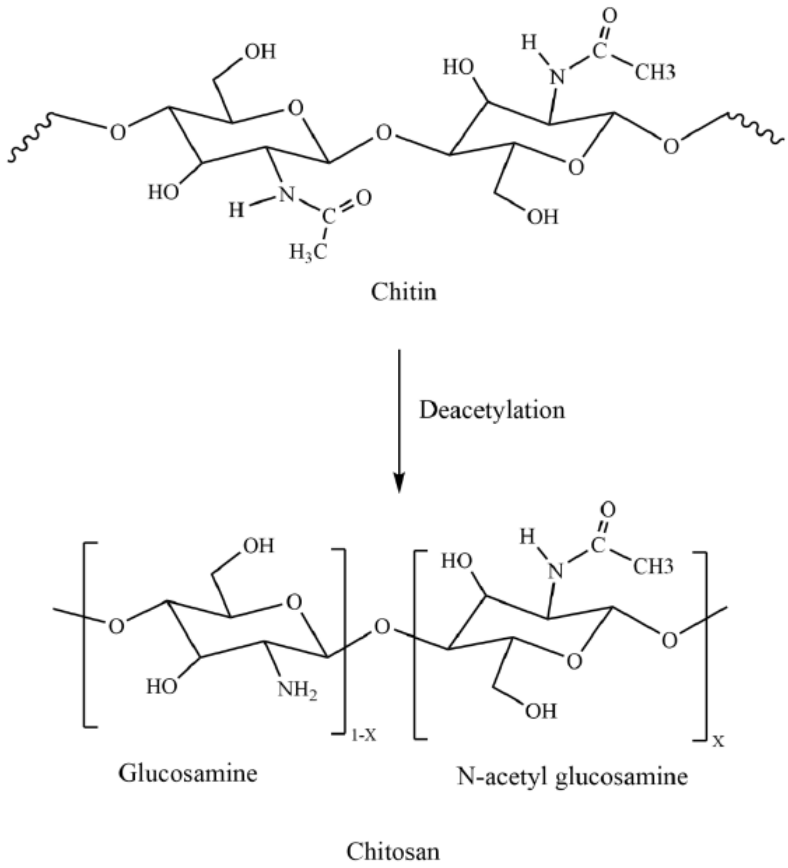


Figure 3. Deacetylation of chitin and subsequent formation of chitosan, and their respective structure. From: Chandrasekaran *et al.* (13)

The degree of deacetylation is one of the most important factors that influence biological (e.g. biodegradability and biocompatibility) and chemical properties (e.g. solubility and high surface area), which change with the process conditions (14). The physiological effects of chitosan oligomers in plants result from the ability of this polycationic compound to bind a large range of cellular components, such as cell wall constituents, DNA, and the plasma membrane, and to bind specific receptors involved in defence gene activation, like plant defence elicitors (7).

Chitin and chitosan use different receptors and signalling pathways. Hydrogen peroxide accumulation and Ca^{2+} leakage into the cell have been shown to be among the cellular consequences of the binding of chitosan to specific cellular receptors and are expected to provoke physiological changes, as they are important in the developmental regulation and signalling of stress responses. Agricultural applications of chitosan have been developed over the years, with a focus on plant protection against fungal pathogens. However, broader agricultural uses bear on tolerance to abiotic stress and quality traits associated with primary and secondary metabolism (7).

Chitosan has been widely studied as a plant biostimulant because of its properties against abiotic stress, as it can increase crop yield, thus making it an interesting carrier material. This material can be used as an agrochemical carrier for fungicides, herbicides, and pesticides. Chitosan has been studied as an encapsulation material that includes biostimulants. It can easily entrap oils and hydrophobic compounds. Chitosan particles have also been used to improve plant fertilisation, showing how chitosan-enclosed fertilisers can increase maize crop yield (15).

Chitosan is normally used to develop nanoparticles owing to its biocompatibility and biodegradability. It can also be absorbed by plant metabolic surfaces, increasing the contact time between enclosed substances and plant tissues. Chitosan nanoparticles can also facilitate the transfer of biostimulants through the cell membrane. Ionotropic gelation is the most commonly used method to produce chitosan nanoparticles, which is accomplished by the inter- and intramolecular cross-linking of polycationic chitosan with an anionic cross-linking agent such as tripolyphosphate (TPP) (15).

1.4.2.5.Inorganic compounds

Beneficial elements are chemical elements that promote plant growth and may be essential to certain taxa but are not required by all plants. The three main forms are aluminium (Al), selenium (Se), and silicon (Si), which are present in soils and plants as different inorganic salts and insoluble forms. The definition of beneficial elements is not limited to their chemical nature but also refers to contexts in which positive effects on plant growth and stress response may be observed. Several beneficial elements promote plant growth, quality of plant products, and tolerance to abiotic stress, including antioxidant protection, cell wall rigidification, interactions with symbionts, and plant hormone synthesis (7).

Inorganic salts of beneficial and essential elements (carbonates, chlorides, phosphates, phosphites, and silicates) have been used as fungicides. These inorganic compounds influence osmotic, pH and redox homeostasis, hormone signalling, and enzymes involved in the stress response (7).

1.4.2.6.Beneficial fungi

Interactions between fungi and plant roots result from mutualistic symbioses when both organisms live in direct contact with each other and establish mutually beneficial relationships, and parasitism. Arbuscular mycorrhiza are a type of endomycorrhiza related to crop and horticultural plants, where fungal hyphae of Glomeromycota species penetrate root cortical cells, forming branched structures called arbuscules. There has been an increased interest in the use of mycorrhiza to promote sustainable agriculture, considering the benefits of symbioses to nutrition efficiency, water balance, and biotic and abiotic stresses protection of plants. There is also information regarding hyphal networks that interconnect fungal and plant partners and individual plants within a plant community, which could have important ecological and agricultural implications. Therefore, crop management practices and plant cultivars should be adapted for interactions with microorganisms (7).

When applied to plants to promote crop yield, nutritional efficiency, product quality, and tolerance to stress, fungal-based products should be considered as biostimulants. The main limitations associated with their use are the technical difficulty of propagating arbuscular mycorrhiza on a large scale due to their biotrophic character and the lack of understanding of the determinants of host specificities and population dynamics of mycorrhizal communities in agroecosystems. Other fungal endophytes such as *Trichoderma* species (Ascomycota) and Sebacinales (Basidiomycota, with *Piriformospora indica* as a model organism), different from the mycorrhizal species, can live at least part of their cycle away from the plant, colonise roots, and transfer nutrients to their hosts through poorly understood mechanisms. They are important plant inoculants because they are easy to multiply *in vitro* and are model organisms to demonstrate the mechanisms of nutrient transfer between fungal endosymbionts and their hosts. There is evidence that many plant responses are induced, including increased tolerance to abiotic stress, nutrient use efficiency, and organ growth and morphogenesis. Considering these effects, these fungal endophytes may be viewed as biostimulants, even though there are claims to support their agricultural use as biopesticides (7).

1.4.2.7. Beneficial bacteria

Bacteria interact with plants in different ways, forming bacterial niches that extend from the soil to the interior of plant cells, in intermediate locations called the rhizosphere and rhizoplane. These associations may be temporary or permanent, with some bacteria vertically transmitted through the seeds. Bacteria influence plant life through their participation in biogeochemical cycles, nutrient supply, increase in nutrient use efficiency, induction of disease resistance, enhancement of abiotic stress tolerance, or modulation of morphogenesis by plant growth regulators (7).

Regarding the agricultural use of bacteria as biostimulants, two main types should be considered: (a) mutualistic endosymbionts of the genus *Rhizobium*, and (b) mutualistic, rhizospheric PGPRs. *Rhizobium* and related taxa have been commercialised as biofertilisers. PGPRs have a variety of functions and influence all aspects of plant life, including interactions with other organisms in agroecosystems, morphogenesis and development, nutrition and growth, and responses to biotic and abiotic stresses. The agricultural uses of PGPRs depend on the variable responses of plant cultivars and their accepting environments. Technical difficulties related to the formulation of inoculants have resulted in inconsistent results in practice. Regardless of these reasons, the world market of bacterial biostimulants is growing and PGPR inoculants are included as a kind of plant 'probiotics', that are efficient contributors to plant nutrition and immunity (7).

Bulgari *et al.* (12) grouped beneficial fungi and bacteria into microorganisms and created another category that included biostimulants derived from extracts of food waste or industrial waste streams, composts and compost extracts, manures, vermicompost, aquaculture residues, sewage treatments, among others. Juárez-Maldonado *et al.* (16) proposed the classification of nanoparticles and nanomaterials as biostimulants. García-García *et al.* (11) emphasised the other components present within the Du Jardin classification (7), such as amino acids, biopolymers, vitamins, and melatonin.

1.5. Pyroglutamic acid

Pyroglutamic acid (or 5-oxo proline pyrrolidone 2-carboxylic acid) is a non-protein amino acid derivative synthesised when glutamic acid or glutamine are cyclised as lactam, becoming a metabolite in the glutathione cycle (17,18). It was first discovered in 1882 by Haitinger, who found that when heated to 180 °C, glutamate is converted into pyroglutamate by losing a molecule of water (18).

In 1978, Mazelis and Pratt demonstrated *in vivo* that L-pyroglutamic acid (LPGA) (Figure 4) is converted to glutamate in plants. The application of glutamate (ionised form of glutamic acid) increases the antioxidant activity of plants under adverse environmental conditions and the role of reactive oxygen species (ROS) in proline metabolism (17). Jiménez-Arias *et al.* (17) hypothesised that the exogenous application of pyroglutamic acid could act as a precursor of proline under stress conditions to counteract osmotic imbalance.

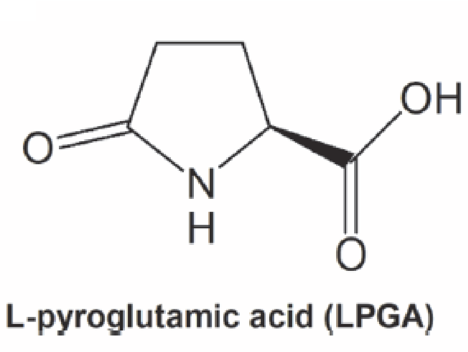


Figure 4. Structure of L-pyroglutamic acid (LPGA). From: Eom *et al.* (19)

1.6. Biostimulant nanoencapsulation

Encapsulation has potential applications in science and technology, where small particles are coated with a specific material to form capsules (20). In nanoencapsulation, in which 'nano' refers to the size of particles with a magnitude of 10^{-9} m, various substances are coated within another material (20,21). The entrapped material is called the core material, internal phase, fill, or filler material. The size range should be approximately 1 – 100 nm in at least one dimension according to the definition of nanoparticles (21). Nanoparticles are also presented with sizes between 1 and 1,000 nm (22) or 10 to 1,000 nm in diameter (23).

Biostimulants are eco-friendly substances, their use enhances plant tolerance to stress, and increased crop production has been widely reported in the literature. The utilisation of amino acids is a good example of a practice that reduces water consumption in the field. However, weekly treatments are needed to be effective, which makes this practice expensive for bigger farms. This could be because of the easy degradation of the biostimulant by soil microorganisms, which shortens their half-life period of activity. An interesting strategy to solve this problem is the use of encapsulation techniques, in which active substances are enclosed within an inert material (coat) that protects them against environmental degradation and provides controlled release. This process is mainly used for fertilisers and pesticides and has not yet been widely explored for biostimulants. Biostimulants and coats should be nontoxic (24).

Prolonging the half-life of an active substance in the soil is one of the most important qualities of a biostimulant commercial formulation. Nanoencapsulation permits continuous controlled release or protection of the active compound inside the particle to prevent natural degradation. One of the most important advantages of using biostimulants is the increase in production yield to build a more profitable farm business based on an optimal cost/productivity ratio. At the same time, easy degradation or uptake of biostimulants in the field is an indispensable characteristic to prevent its bioaccumulation. The disadvantage of biostimulants is its regular administration which decreases farmer profitability (24).

1.6.1. Encapsulation using ionotropic gelation technique

Ionotropic gelation is a technique that allows the formation of nano- and microparticles via electrostatic interactions between two ionic species (at least one of them must be a polymer) under certain conditions. When added to the reaction, a drug or bioactive molecule becomes trapped between the polymer chains and is contained within the micro- or nanoparticle structures. A drug-loaded polymer solution is dropped into an aqueous solution of multivalent cations which creates hydrogel beads. Cations or anions diffuse into drug-loaded polymer droplets, creating a three-dimensional network of ionically cross-linked units (Figure 5) (15).

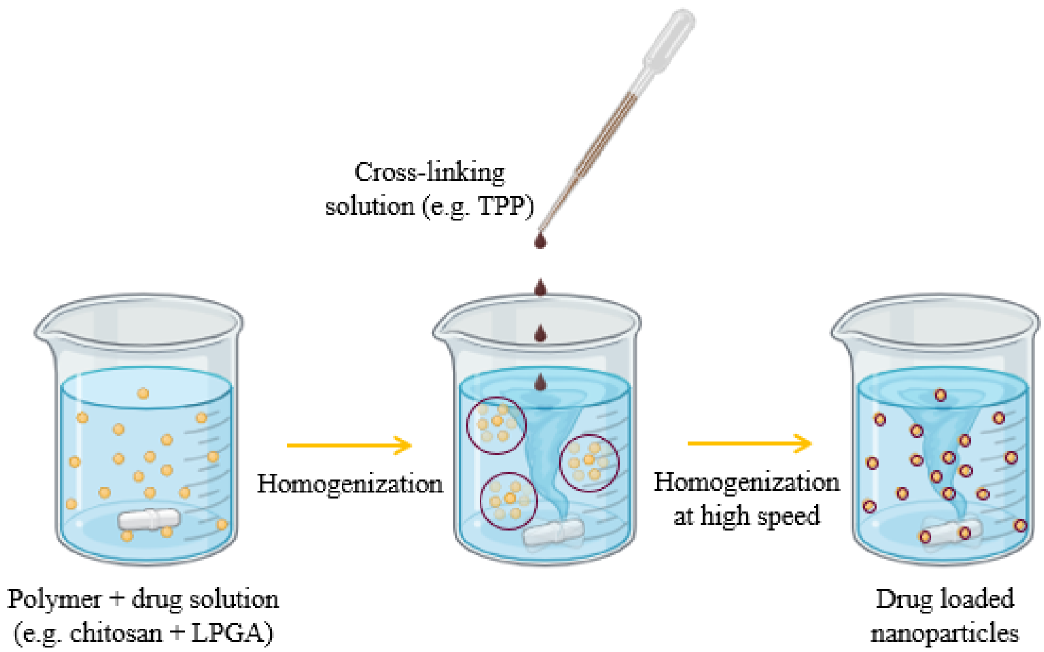


Figure 5. Encapsulation using ionotropic gelation technique. Based on Jiménez-Arias *et al.* (15)

The most important factors determining particle size are the polymer and drug concentrations, composition and pH of the cross-linking solution, solution flow velocity, and temperature (15). The capsules produced using this method are used for hydrophobic materials. Because the shell is made of a hydrophilic material, such as alginate or chitosan, this technique is generally applied to hydrophobic materials or those with very low solubility. Hydrophilic actives are difficult to encapsulate. Hydrocolloids are miscible with hydrophilic cores, making it very difficult to obtain good phase separation between the core and shell. Because a hydrogel bead is porous, encapsulation of hydrophilic compounds leads to lower encapsulation efficiency and poorly controlled release properties (25).

This technique has numerous advantages: it is fast, relatively cheap, and does not require expensive equipment or reagents (15). In addition, there is no need to use organic solvents because the mechanism is based on electrostatic interactions, not chemical interactions, avoiding the potential toxicity of chemicals or reagents. The disadvantage of this method is the difficulty to produce nanoparticles of uniform size, and limited research on polymers (other than chitosan) (26). When the polymer and drug concentrations are optimal, ionotropic gelation has a high encapsulation efficiency. Ionotropic gelation techniques have been used in agriculture to encapsulate biostimulants, such as silicon and gibberellic acid, with significant improvements in their behaviour (15).

1.6.1.1.Formation of chitosan nanoparticles through ionotropic gelation with TPP

Chitosan nanoparticles have been prepared, using different approaches, such as emulsification solvent diffusion, ionotropic gelation, microemulsion, polyelectrolyte complex and reverse micellar method. These nanoparticles share the features of chitosan and the important characteristics of nanoparticles, such as increased surface area and small size (13). They are characterised by sizes between 200 and 1,000 nm and zeta potentials between 20 and 60 mV, depending on the mass ratio of chitosan to TPP or the molecular weight of chitosan (26).

Chitosan nanoparticles' stability can be in part attributed to the ionic cross-linking of positively charged chitosan oligomers using polyanions. The advantages of ionic cross-linking related to chitosan nanoparticles prepared using this technique may be transferable to other approaches, regardless of whether ionic cross-linking is applicable (27).

The most commonly used polyanion for ionic cross-linking is TPP, which is non-toxic. Under constant stirring, the cation of chitosan crosslinks with the polyanion of TPP (phosphoric ion), leading to the formation of a hydrogel (26). The chitosan and TPP molar ratio and the evolved interactions are essential for the formation of the required nanoparticle diameter, because these parameters can affect the drug release properties (27).

1.7. Plants defence mechanisms

During their life cycle, plants are subjected to different environmental stresses, such as salinity, temperature, metal oxidative, and drought stress. They limit plant growth and productivity at different levels depending on the severity of the stress which can be reversible or irreversible (28,29). Plant responses to biotic and abiotic stresses comprise a complex network of reactions that involve different metabolic and physiological pathways of primary and secondary metabolism and oxidative stress (9). As a reaction to secondary oxidative stress, plants produce ROS, which causes damage through direct interaction with various macromolecules and peroxidation of membrane lipid components (28,29). ROS are a group of reactive oxygen species produced during the metabolism. These species are generated during oxidative processes, such as photosynthesis and respiration, and under normal conditions at low concentrations without negative effects on the plants. Under biotic or abiotic stresses, ROS levels increase as consequence of oxidative burst induced by a stress agent (9). The formation of free radicals exceeds the overall cellular antioxidative potential, leading to oxidative stress, and therefore has adverse effects on plant growth (28). High ROS concentrations can damage the lipid membranes, nucleic acids, and proteins (9).

Plants accumulate large amounts of different types of compatible solutes in response to various stressors. These solutes have low molecular weights and are highly soluble organic compounds that are usually non-toxic at high cellular concentrations. Under stressful conditions, they protect plants by contributing to cellular osmotic adjustment, enzyme/protein stabilisation, protection of membrane integrity, antioxidant responses, and ROS detoxification. These compatible solutes include polyols, proline, sucrose, trehalose, and quaternary ammonium compounds (QACs) (e.g. glycine betaine, pipecolate betaine, and proline betaine) (28). Osmotic adjustment is affected by the accumulation of free amino acids, proline, and sugars in roots and shoots (30).

1.8. Proline

1.8.1. Biosynthesis

The name 'proline' was proposed by Emil Fischer in 1904, and derivates from 'pyrrolidine' (31). The amino acid L-proline, or L-pyrrolidine 2-carboxy acid, is a proteinogenic amino acid, with remarkable conformational rigidity, essential for metabolism (31,32). Its accumulation has been reported under conditions of drought, heavy metals, high light and UV irradiation, high salinity, oxidative stress, and in response to biotic stress (32).

In plants, this amino acid is synthesised by the glutamate and ornithine pathways (28). Proline is mainly synthesised from glutamate, which is reduced to glutamate-semialdehyde (GSA) by Δ'-pyrroline-5-carboxylate synthetase (P5CS) and converted to Δ'-pyrroline-5-carboxylate (P5C) (28,32). The P5C intermediate is reduced to proline by Δ'-pyrroline-5-carboxylate reductase (P5CR) (28,32). Proline catabolism occurs in the mitochondria through the sequential action of proline dehydrogenase (ProDH) or proline oxidase (POX), which produces P5C from proline, and P5C dehydrogenase (P5CDH), which converts P5C to glutamate (28,32,33).

As an alternative pathway, proline can be synthesised from ornithine, which is first transaminated by ornithine-δ-aminotransferase (OAT), producing GSA and P5C, and then converted to proline (28,32). This pathway has been proposed to be important during seedling development and for stress-induced proline accumulation in some plants (28,32).

Biosynthesis, catabolism, and transport between cells and different metabolic surfaces (compartments) can determine the intracellular proline levels (28,32). Biosynthetic enzymes, such as P5CS1, P5CS2, and P5CR, are predicted to be localised in the cytosol, whereas enzymes involved in proline catabolism, such as ProDH2, P5CDH, and OAT, are predicted to be localised in the mitochondria (28,33).

ProDH and P5CDH are mitochondrial enzymes that use FAD and NAD^+ as electron acceptors, generating FADH2 and NADH, respectively, and delivering electrons for mitochondrial respiration (32,33). The P5C-proline cycle showed that P5C, which is produced from proline in the mitochondria, can be transported into the cytosol and reduced to proline by cytosolic P5CR. When the activity of P5CDH is limited, the P5C-proline cycle can transfer more electrons to the mitochondrial electron transport chain and then produce ROS (32,33).

Proline biosynthesis is upregulated by light and osmotic stresses, whereas its catabolism is activated in the dark and during stress relief and is controlled by ProDH and

P5CDH (32,33). ProDH transcription is activated by rehydration and proline but repressed by dehydration, which prevents proline degradation during abiotic stress (28,32,33). ProDH1 transcription is repressed during daylight and induced in darkness, indicating that illumination has opposing effects on P5CS1 and ProDH1 transcription (32,33).

1.8.2. Functions

Proline accumulation can influence the stress tolerance in several ways. It has been shown that this amino acid acts as a molecular chaperone that protects protein integrity and enhances different enzyme activities (e.g. prevention of protein aggregation and stabilisation of M4 lactate dehydrogenase during extreme temperatures and protection of nitrate reductase during heavy metal and osmotic stress) (32).

Proline has been attributed an antioxidant activity by various studies, which suggests ROS scavenging activity, and this amino acid acts as a singlet oxygen quencher (28,32). Proline treatment can decrease ROS levels in yeast and fungi (preventing programmed cell death), protect human cells against carcinogenic oxidative stress, and diminish lipid peroxidation in algal cells exposed to heavy metals. Proline can reduce the damaging effects of hydroxyl radicals and singlet oxygen on Photosystem II (PSII) in isolated thylakoid membranes (32).

However, compromised proline accumulation in *p5cs1* insertion mutants induces the accumulation of ROS and enhances oxidative damage. Proline protects and stabilises ROS-scavenging enzymes and activates alternative detoxification pathways (32).

Proline metabolism is likely to stabilise cellular homeostasis during stress conditions in a manner that is not completely understood. The accumulation of P5CS1 and P5CR in chloroplasts during salt stress suggests that, under certain conditions, glutamate-derived proline biosynthesis increases in plastids where photosynthesis occurs. Proline biosynthesis is a reductive pathway that requires NADPH for the reduction of glutamate to P5C, and this one to proline, and generates $NADP^+$ that can be used as an electron acceptor (32).

Proline has other protective functions in the mitochondria. After stress, proline pools supply reducing potential for mitochondria through the oxidation of proline by PDH and P5CDH, providing electrons for the respiratory chain and contributing to energy supply for resumed growth. Proline catabolism is an important regulator of cellular ROS balance and can influence various additional regulatory pathways (32).

1.9. Carbohydrates

1.9.1. Classification

Carbohydrates are an important source of energy for living organisms and serve as a mean by which chemical energy can be stored (34). These are usually represented by the formula $C_x(H_2O)_y$, where x and y represent numerical values between 3 and 12. Their chemistry is similar to that of alcohols and carbonyls (aldehydes and ketones) (35). Some function as structural components of the cell. They can be divided into two groups: soluble carbohydrates (monosaccharides, disaccharides, and oligosaccharides) and polysaccharides (e.g. starch and cellulose) (34).

Monosaccharides have only one sugar residue and are classified as aldoses (aldehyde functional groups) or ketoses (ketone functional groups). They are also classified according to the number of carbon atoms in the backbone, with prefixes, such as tri-(3), tetr-(4), pent-(5), hex-(6), and hepta-(7) (35). The three most commonly found monosaccharides are glucose, galactose, and fructose (34). Oligosaccharides contain 2 to 10 monosaccharide units. The most abundant possess two monosaccharide residues, referred to as disaccharides that include sucrose, maltose, lactose, cellobiose, and trehalose (Figure 6) (34,35).

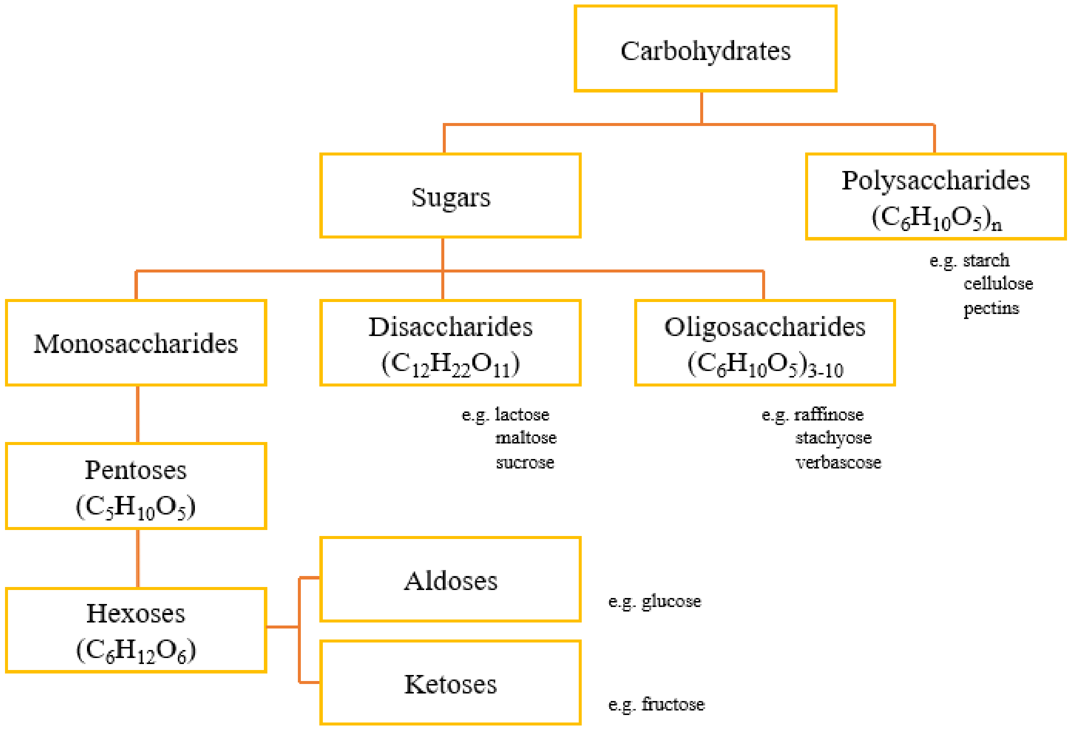


Figure 6. Classification of carbohydrates. Based on Kadler *et al.* (34)

1.9.2. Functions

The fixation of carbon and light energy by photosynthesis, which results in the formation of carbohydrate molecules rich in energy and production of oxygen, is essential for life on Earth. Soluble carbohydrates, such as sucrose, glucose, and fructose, maintain the structure and growth of plants. They also act as nutrients and regulators of metabolism, growth, stress responses and development from embryogenesis to senescence. Carbohydrates play a role in the regulation of growth, photosynthesis, carbon partitioning, carbohydrate and lipid metabolism, osmotic homeostasis, protein synthesis and gene expression during different abiotic stresses, and membrane stabilisation. An increase in the concentration of soluble carbohydrates enhances plant tolerance to abiotic stresses, such as cold, drought, and salinity (36).

1.9.3. Impact of drought stress

Plants are autotrophic, using light energy to convert CO_2 into carbohydrates which are involved in most metabolic and signalling pathways that control growth, development, and stress tolerance (37). They also function as storage compounds (38). The transport and partitioning of sugars from phototrophic leaves (source) to heterotrophic organs (sink) through the phloem are important parameters controlling crop productivity (37).

From the germination of seeds, roots will only depend on energy in the form of photosynthates obtained from the aerial parts of the plant. Roots can fix plants to the soil, are responsible for the uptake of water and ions, and are important for the flux of carbon from the atmosphere into the soil (37).

Environmental factors are also able to affect the allocation of sugars to the roots and microorganisms in the rhizosphere (37). Drought stress affects physiological and biochemical aspects associated with ion uptake, photosynthesis, phytohormone balance, respiration, sugar and nutrient metabolism, and translocation (38). This stress is one of the most important factors that significantly inhibits photosynthesis (36). Drought stress leads to sugar accumulation in plants and resets the source-sink relationship. Phloem transport is affected before stress affects photosynthesis, highlighting the importance of sugar transport and its sensitivity to different environmental conditions. It also causes the plant root system to take up the available water more efficiently, causing an increase in the root-shoot ratio, which is invariably facilitated by the transportation and accumulation of soluble sugars in roots (38).

The accumulation of sugar prevents oxidation of cell membranes in the case of water deficiency. Their accumulation decreases the rate of photosynthesis. Soluble sugars maintain the turgidity of leaves, prevent dehydration of membranes and proteins, and maintain leaf water content and osmotic adjustment in plants under drought stress (36).

Drought stress also facilitates the breakdown of storage sugars such as starch into soluble sugars, such as fructose, glucose, and sucrose, which leads to a decrease in the water potential of the cell. This takes up limited moisture present in the soil (38).

An increase in soluble sugar content is facilitated by the breakdown of stored starch in source tissues. These sugars are transported to sink tissues to address their needs and assist other stress-responsive adaptive mechanisms. The maintenance of the transport of sugars is also important for the plant's survival during stress, which secures their distribution throughout the plant system, facilitating their use as osmoprotectants and energy sources (38).

1.10. Objectives of the dissertation

This work aims to evaluate the pyroglutamic acid action as a biostimulant and to confirm if its chitosan encapsulation increased plant defence against drought during assays performed under controlled experimental conditions.

To test this hypothesis, this dissertation has the following objectives:

i. Verify the effect of different irrigations in maize plants and assess whether it is possible to maintain drought stress during a fifteen-day assay with daily half-strength Hoagland's solution application.

ii. Prepare chitosan nanoparticles with or without pyroglutamic acid entrapped, using an ionotropic gelation technique.

iii. Assess the influence of stress on the maize plant's development (50 % C), and the protective action of biostimulant (50 % Bs) and entrapped biostimulant (50 % EBs), through the measurement of functional characteristics (root and aerial part lengths) and markers (proline and sugar content).

CHAPTER II.

MATERIAL AND METHODS

2.1. Materials, reagents, and general equipment

2.1.1. Materials and general equipment

General laboratory equipment, such as precision balance (Precisa, Series 360 ES 225SM- DR, Switzerland), magnetic stirrer (Heidolph, MR 3001), refrigerator (Bosch) and a freezer (Liebherr ProfiLine), were used.

Peat (Siro) and a climatic chamber (Aralab, Model FITOCLIMA 12.000 PLH, Serial No. 2450) were used during the assays. A pH/ISE metre (Unicam, type 9455), vacuum controller (Vacuubrand), paper filter with a pore size of 8 µm (Whatman™) and 0.45 µm (Whatman®) and a multi-position magnetic stirrer with heater (Velp Scientifica, AM4) were used to prepare the nanoparticles.

A lab oven (Thermo Scientific, type Heratherm OMS180, serial number 41348079, Germany), moisture meter (Kern, MRS 120-03) and desiccator (Totech EU, Super Dry, ESDA-402-21) were used to prepare and preserve the maize plants from both the assays and samples for sugar content determination.

Liquid nitrogen was used to preserve the plants collected for the proline determination. Quartz (Fluka Chemika, Switzerland), a multi-position magnetic stirrer with heater (Velp Scientifica, AM4) and 90 mm diameter filter paper (Whatman™) were also used in proline measurement.

A centrifuge (Eppendorf™ Centrifuge 5430 R, Germany), vortex (Fisher Scientific, TopMix FB15024), water bath (Julabo SW22) and UV/VIS recording spectrophotometer (Shimadzu, UV-2401 PC) were used for the determination of proline and sugar content.

A microwave (Panasonic), Uniclave 99 vertical steam steriliser (A. J. Costa, Lda.), flow chamber (Thermo Scientific) and two lab ovens (Memmert and P Selecta) were used to analyse the peat.

Two mills (Taurus Aromatic; Tristar, Netherlands) were used to grind the material for sugar content determination.

2.1.2. Reagents

For the Hoagland solution, solution A was prepared with calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$) (Sigma-Aldrich, Germany, 99.0 – 103 %), ammonium nitrate (NH_4NO_3) (Merck, Darmstadt, Germany, ≥ 99.0 %), potassium nitrate (KNO_3) (Fluka Chemika, Switzerland, ≥ 99.0 %) and Rexolin® Q48 (YaraTera™, Netherlands). Solution B was prepared with potassium dihydrogen phosphate (KH_2PO_4) (Merck, Darmstadt, Germany, ≥ 99.5 %), potassium nitrate (Fluka Chemika, Switzerland, ≥ 99.0 %), boric acid (H_3BO_3) (Honeywell Fluka, USA, ≥ 99.8 %), copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) (Merck, Darmstadt, Germany, 99.0 – 100.5 %), zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Fluka Chemika, Switzerland, 99.5 – 103 %), manganese(II) sulphate tetrahydrate ($MnSO_4 \cdot 4H_2O$) (Merck, Darmstadt, Germany, ≥ 98.5 %), ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) (BDH, England, ≥ 99.0 %) and magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Fisher Scientific, Japan, 98.0 – 102.0 %).

Two chitosans were used to prepare the nanoparticles: one with 100 to 300 kDa and degree of deacetylation ≥ 75 % (Acros Organics, China) and the other with 310 to 375 kDa and degree of deacetylation ≥ 85 % (Biosynth, Bratislava, Slovakia). Aqueous glacial acetic acid (0.6 % v/v) (Panreac Quimica SA, Barcelona, Spain, 99.7 %), LPGA (Sigma-Aldrich, ≥ 99.0 %) and TPP (Thermo Scientific, Netherlands) were also used.

Sodium hydroxide (NaOH) (Honeywell Fluka™, Sweden, ≥ 98 %) was used in pH adjustment, during the preparation of the Hoagland solution and nanoparticles.

Ninhydrin (Sigma-Aldrich, India), sulfosalicylic acid (Fisher BioReagents, South Korea, 99.0 - 101.0 %), glacial acetic acid (Panreac Quimica SA, Barcelona, Spain, 99.7 %) and L-proline (Sigma Aldrich, Japan) were used during the proline measurement.

Culture media were prepared with nutrient agar (NA) (Liofilchem, Italy) and potato dextrose agar (PDA) (Liofilchem, Italy), for peat analysis. Saline solution was prepared with sodium chloride (NaCl) (Honeywell Fluka™, Germany, ≥ 99.5 %), potassium dihydrogen phosphate (Merck, Germany, ≥ 99.5 %) and potassium phosphate dibasic (HK_2O_4P) (Sigma-Aldrich, Japan, ≥ 98 %).

Anthrone (Thermo Scientific, Germany) and sulphuric acid (H_2SO_4) (Honeywell Fluka, Germany, 95.0 – 97.0 %) were used in sugar measurements.

2.2. Assays

2.2.1. Sowing and assay preparation

Maize seed germination was performed in specialised germination trays bought from an agricultural shop. Seeds from the local variety 'Milho de Santana', accession ISOP 1951, were obtained from the ISOPlexis germbank.

The assay methodology was based on that described by Jiménez-Arias *et al.* (3). The trays were filled with peat. The peat used is composed by blonde peat of *Sphagnum*, Siro Agro 1 (Pine bark hummus), coco peat and animal organic fertiliser. One maize seed was manually placed per tray cell and covered with peat. All trays were placed in larger containers filled with water for approximately 30 minutes to ensure complete hydration of the peat. The seedling trays were then transferred to a climatic chamber under controlled conditions: temperature 20 – 27 °C, humidity 60 – 75 %, and photoperiod 8 – 16 hours. For ten days, which was the time required to reach the two true-leaf stages, plant growth was evaluated, and any underdeveloped or anomalous plants were removed from the trays.

2.2.2. Layout of the different assays

During the practical part of this study, five different tests were conducted: the first three to establish the experimental design and the last two to assess biostimulant action.

In the first assay (Appendix I, Figure A.1), four 28-cell seedling trays (6.3 x 6.3 x 6.3 cm cells) were used to check for maize seed germination. Two of these were control seedlings receiving 100 % water, and the other two were water-deficit seedlings treated with 50 % water.

In the second assay (Appendix I, Figure A.2), six 28-cell seedling trays and one 40-cell seedling tray (5.3 x 5.3 x 6 cm cells) were used, the last of which was used only to assess maize development in smaller seedling cells. Three trays were used for seedlings receiving full irrigation (control), and the other three for seedlings subjected to water deficit conditions.

A third assay (Appendix I, Figure A.3) was conducted to determine the optimal concentration of LPGA. Three different concentrations were tested: 3, 5 and 10 mL. Five 28-cell seedling trays and one 40-cell seedling tray were used. Two trays represented water control seedlings (a 28-cell seedling tray and a 40-cell seedling tray). The remaining four were conducted under water-deficit conditions.

In the fourth assay (Appendix I, Figure A.4), five trays of 40 seedlings were used, two for the watered control seedlings and three for the water-deficit seedlings.

The fifth assay (Appendix I, Figure A.5) included ten 40-cell seedling trays, five with watered control seedlings and five under water-deficit conditions.

2.2.3. Treatments applied to the seedlings

The methodology used was based on Jiménez-Arias *et al.* (3). As previously mentioned, ten-day-old plants under healthy conditions were chosen, and two different experiments were conducted.

In the first experiment, the aim was to evaluate whether the expected effects of water deficit would appear in fully developed plants (Figure 7). The field water capacity per seedling cell tray was calculated daily, through the use of half-strength Hoagland's solution, and this value was used to define the irrigation of well-watered (WW) (100 %) and water-deficit (WD) (50 %) seedlings, that is, if it was used 10 mL of Hoagland's solution in well-watered seedlings, 5 mL were used for water-deficit ones.

In the second experiment, the influence of different treatments on maize seedlings was assessed according to the design shown in Appendix I. Four experimental treatments were applied in the WW and WD trials: **control (C) (no biostimulant applied)**, **empty particle (EP) (chitosan as the coating of the particle)**, **entrapped biostimulant (EBs) (chitosan as the coating and pyroglutamic acid as the core material of the particle)**, and **biostimulant (not entrapped pyroglutamic acid) (Bs)**. The experiment lasted for 15 days. On day 0, the first day of each assay, all plants were subjected to full irrigation, and on day 1, 3 mL of each treatment was applied to the soil in the fourth and fifth assays.

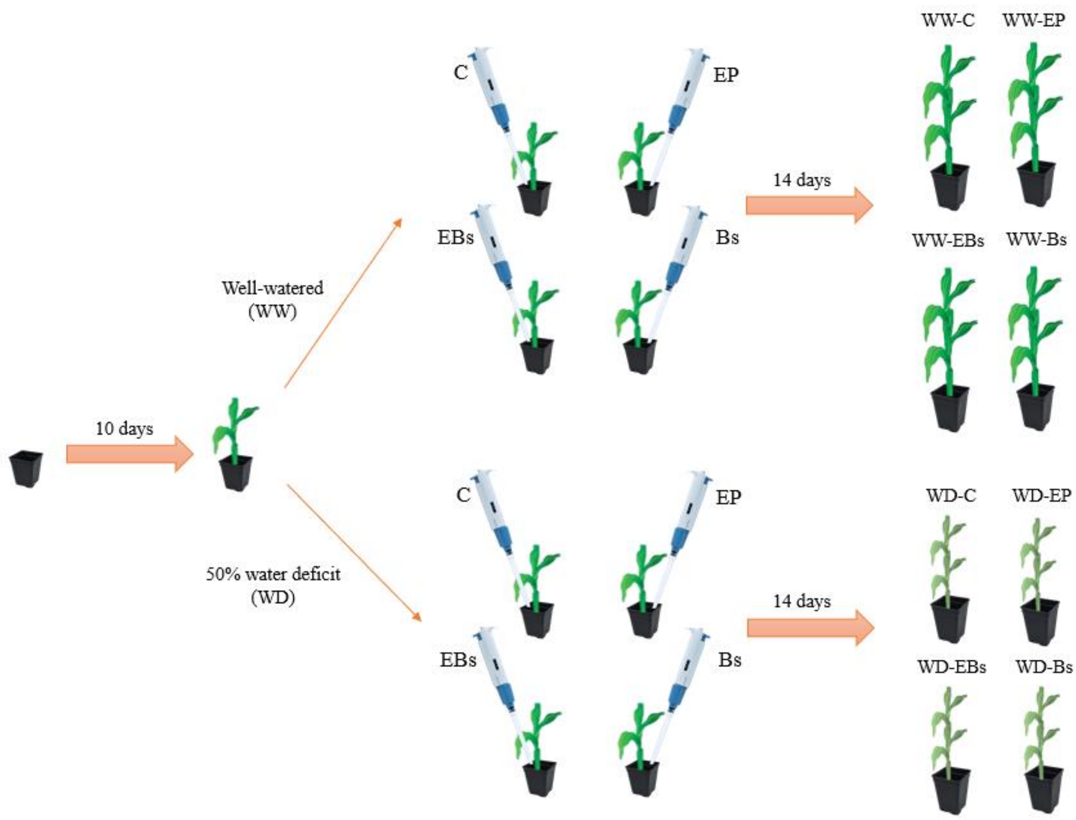


Figure 7. Representation of the treatments applied to the seedlings. Abbreviations: WW, well-watered; WD, water-deficit; C, control; EP, empty particle; EBs, entrapped biostimulant; Bs, biostimulant; WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

2.2.4. Hoagland's nutrient solution

The methodology was based on Jiménez-Arias *et al.* (3). All plants were irrigated with a half-strength Hoagland nutrient solution (Table I). According to Hoagland & Arnon (39), Hoagland's nutrient solution consists of a mixture of two stock solutions: A and B. Solution A was prepared with 70.0 g calcium nitrate tetrahydrate, 5.0 g ammonium nitrate, 5.0 g potassium nitrate and 7.2 g Rexolin® Q48. Solution B was prepared with 10.0 g potassium dihydrogen phosphate, 35.0 g potassium nitrate, 0.21 g boric acid, 0.02 g copper(II) sulphate pentahydrate, 0.02 g zinc sulphate heptahydrate, 0.165 g manganese(II) sulphate tetrahydrate, 0.015 g ammonium heptamolybdate and 40.0 g magnesium sulphate heptahydrate. These solutions are mixed, and the pH is adjusted to 6.0 with 0.5 M NaOH solution. During the assays, a half-strength Hoagland's nutrient solution was prepared as required. Appendix II, Tables A.1 and A.2, shows the quantities of Hoagland's nutrient solution applied during the fourth and fifth assays.

Table I. List of reagents used in the half-strength Hoagland's nutrient solution.

Stock solution A		Stock solution B	
Reagent	Hoagland's nutrient solution (g/L)	Reagent	Hoagland's nutrient solution (g/L)
$Ca(NO_3)_2 \cdot 4H_2O$	140	KH_2PO_4	20.0
NH_4NO_3	10.0	KNO_3	70.0
KNO_3	10.0	H_3BO_3	0.42
Rexolin® Q48	14.4	$CuSO_4 \cdot 5H_2O$	0.04
		$ZnSO_4 \cdot 7H_2O$	0.04
		$MnSO_4 \cdot 4H_2O$	0.33
		$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.03
		$MgSO_4 \cdot 7H_2O$	80.0

2.3. Chitosan nanoparticles

Two different chitosans with different molecular weights were used to prepare the nanoparticles: one with 100 to 300 kDa and degree of deacetylation ≥ 75 %, and the other with 310 to 375 kDa and degree of deacetylation ≥ 85 %. The first one was used for the first, second and third assays, and the last one for the fourth and fifth assays. Only the results of the fourth and fifth assay were analysed.

The procedure for nanoparticle preparation followed methods described by Fan *et al.* (40) and Jiménez-Arias *et al.* (41), using the ionotropic gelation technique. The procedure started with the preparation of a 0.2 % chitosan solution using aqueous glacial acetic acid solution (0.6 % v/v) as the solvent. The solution was placed in a magnetic stirrer at room temperature until it was completely dissolved. The pH, which was 3.6, was adjusted to a value of 4.7 using 2 M NaOH solution in the pH/ISE meter. After adjustment, the chitosan solution was passed through two filters: first through one with a pore size of 8 µm and then through one with a pore size of 0.45 µm, with a vacuum controller assisting the process. The TPP solution was prepared in distilled water at a concentration of 0.5 mg/mL in a magnetic stirrer. It was stored at 4 °C before being used. 1 mM of LPGA, used as the 'main' biostimulant (core material of the particle), was dissolved in the chitosan solution, and stirred in a magnetic stirrer heated several times until 50 °C was reached. The TPP solution was then added dropwise, and the mixture was transferred to an ice-water bath for 30 minutes with stirring. An opalescent solution was obtained and stored at 4 °C. The same procedure was used to prepare empty nanoparticles (without LPGA).

2.4. Measurements and weighting

The assays lasted for 15 days, after which all treated plants were collected. The roots of the maize plants were washed to remove the peat. For the first four assays, after cleaned, the plants were put in paper bags and dried in a lab oven at 65 °C for two days. Each dried plant was weighed using the precision balance and stored in a desiccator. For the last assay, after being collected, the maize plants were cleaned. Some were measured in roots and aerial parts (stem and leaves) and stored in vacuum bags at -30 ºC, and others were put in paper bags, dried as previously explained, and stored.

2.4.1. Root and aerial part stress index (SI)

Based on Robinson *et al.* (42) and Gouveia *et al.* (43), the root and aerial part stress index was calculated with the following equation:

$$SI = \frac{L_{unstressed} - L_{stressed}}{L_{unstressed}}$$

Where ‘L’ represents the mean length of the root and aerial part. The SI ranges from 0 to 1, representing the effect of the environment on plant growth. These values tend toward 0 when the plant is less sensitive to stress (SI → 0), and to 1 with the increase of the plant’s stress sensitivity (SI → 1).

2.5. Proline (Pro)

2.5.1. Harvest of plant material for determination of Proline

On the last day of the fourth assay, leaf samples were randomly taken from ten maize plants. They were placed in a box with liquid nitrogen and stored at -30 ºC. In the fifth assay, leaves were collected randomly at 0, 1, 4, 8, 12, and 14 days of the assay. On day 0, three leaf samples were collected and treated as described for the fourth assay. On the remaining sampling days, leaf samples of sixteen plants were collected, representing a duplicate of each experimental variant under WW and WD conditions. All samples were treated as described for the fourth assay.

2.5.2. Construction of proline’s calibration curve

According to Shabnam *et al.* (44) and Lee *et al.* (45), a 1 % sulfosalicylic acid solution was prepared by weighing 1.165 g of sulfosalicylic acid and dissolving it in distilled water to obtain 100 mL. This solution is stable at room temperature and protected from light, for one week. A 1.25 % ninhydrin solution was prepared. 1.25 g of ninhydrin was weighed, dissolved in 100 mL of glacial acetic acid and stored. This solution was kept at 4 ºC and protected from light for two days. A standard solution of 0.6 mM L-proline was prepared by dissolving 0.006918 g of proline in 100 mL of 1 % sulfosalicylic acid solution. This solution was prepared daily.

To obtain the calibration curve of proline, different volumes of proline were pipetted into 5 mL amber volumetric flasks, with sulfosalicylic acid (1 %) added as described in Table II.

Table II. Standards and respective concentrations of L-proline used in the making of the calibration curve.

Proline's standard curve points	Concentration of L-proline on each point (mM)	Concentration of L-proline on each point (µg/mL)	Volume to pipette of the standard solution of L-proline (mL)	Final volume with sulfosalicylic acid 1 % (mL)
0	0.00	0.00	0.00	5.00
1	0.01	1.15	0.08	4.92
2	0.02	2.30	0.17	4.83
3	0.03	3.45	0.25	4.75
4	0.04	4.61	0.33	4.67
5	0.06	6.91	0.50	4.50
6	0.09	10.36	0.75	4.25
7	0.12	13.82	1.00	4.00
8	0.15	17.27	1.25	3.75

Then, 1 mL of each standard curve point and 2 mL of the acidic ninhydrin reagent were added to the test tubes and vortexed. The 'blank' consisted of the same solution that forms 'point 0', that is, sulfosalicylic acid and acidic ninhydrin reagent (1:2). The test tubes were covered with aluminium foil and placed in a water bath at ± 100 ºC for 30 minutes. The reaction was then stopped in an ice-water bath for 10 minutes. A UV/VIS spectrophotometer was used to measure absorbance at 508 nm after 10 minutes. The equation of the obtained standard curve was $y = 6.02629\ x - 0.00436191$, with a correlation coefficient (R^2) of 0.99908 (Appendix III, Figure A.6).

2.5.3. Preparation of plant material and sample dosing

Following Shabnam *et al.* (44) and modified Lee *et al.* (45), each leaf sample collected during the fourth and fifth assays was weighed. Sulfosalicylic acid in a ratio of 1:10 (p/v) and quartz sand were added to the samples in a mortar, and the material was macerated (46,47). The extract was collected and centrifuged at 7,830 rpm for 15 minutes at 4 ºC (48). The supernatant was collected. An additional 3 mL of sulfosalicylic acid was added to the sample pellet, vortexed, and centrifuged. This procedure was repeated twice (49). The obtained supernatants were added to a tube, and the final volume was filtered. The final supernatant volume of each sample was measured and recorded. 1 mL of sample supernatant and 2 mL of ninhydrin solution were added to the test tubes and vortexed. The test tubes were sealed with aluminium foil and placed in a water bath at 100 ºC for 30 minutes. Then the test tubes were placed in an ice water bath for 10 minutes. After this period, their absorbance was measured at 508 nm, using as 'blank' the 'point 0' of the standard curve.

2.5.4. Calculation for proline's concentration

The concentration of proline in fresh weight (FW) was calculated according to Lee *et al.* (45), using the following equation:

$$\mu mol\frac{proline}{g}FW = \frac{\left(\mu g\frac{proline}{mL}\right) \times mL\ extraction\ buffer/115.5\ \mu g}{(g\ sample)}$$

Where 'µg proline/mL' represents the concentration obtained by the standard curve and the absorbance of the FW sample; 'mL extraction buffer' represents the volume (in millilitres) obtained in the extraction; '115.5 µg/µmol' represents the molar mass of proline and 'g sample' represents the fresh weight of sample used for proline dosage.

2.6. Peat microbiology analysis

2.6.1. Harvest of peat for evaluation of microorganisms

In the fourth assay, peat was collected from sixteen plant samples from each treatment in duplicate. Water capacity of the samples was measured using a moisture meter. The peat was then placed in tubes, previously disinfected with 70 % ethanol, and stored at -30 ºC. A similar set of sixteen peat samples was also taken in the fifth assay and treated as described for the fourth assay. A sample of untreated peat was collected directly from the purchased peat bag to assess the potential formation and quantify the number of microorganisms present.

2.6.2. Preparation of the media and saline solution

The NA and PDA media were prepared following the manufacturer's instructions with some adjustments depending on the quantity of samples. The media were poured into 500 mL glass flasks. To dissolve the media components was used distilled water, and the flasks were placed in a microwave. Once the components were completely dissolved, the flasks were placed in a steriliser. After sterilization, they were cooled at room temperature and then stored in a refrigerator until further use. To pour into plates, the flasks were placed in the microwave to re-dissolve the media and then used. This procedure was carried out in a flow chamber, and all materials used were sterilised.

The saline solution was adapted from Dionísio *et al.* (50), and was composed of sodium chloride, potassium dihydrogen phosphate, and potassium phosphate dibasic. The preparation of this solution involved weighing all reagents, combining them, and suspending the mixture in a magnetic stirrer at room temperature until complete dissolution. Subsequently, the saline solution was placed on a steriliser, cooled to room temperature, and stored in a refrigerator for later use. Smaller glass flasks were preferred since it is easier to grab them to pour the media into the plates. Also, is important not to fill them completely because the medium can overflow while being heated in the microwave.

2.6.3. Successive dilution method

Following Dionísio *et al.* (50), 1 g of soil from the selected samples was weighed and mixed with 100 mL of saline solution. 9 mL of the saline solution was poured into two plastic tubes corresponding to two dilutions (1:10 and 1:100) (Figure 8). An earlier test was performed to determine how many dilutions were required, how many plates were necessary, and the concentration of the cells of interest. 1 mL of a mixture of saline solution and soil was poured into the first tube and gently shaken. Then, 1 mL of the previous mixture was poured into the next tube and shaken. After the dilutions were made, 100 µL of each tube was poured into plates in triplicate. Glass beads used to spread the sample onto the plate were placed before the sample was applied. At the end of each application, the NA plates were placed in a lab oven at 28 ºC to 30 ºC and the PDA plates were kept at room temperature in a closed environment (in this case, a lab oven). The incubation period for bacteria was 2 days and that for fungi was 7 days.

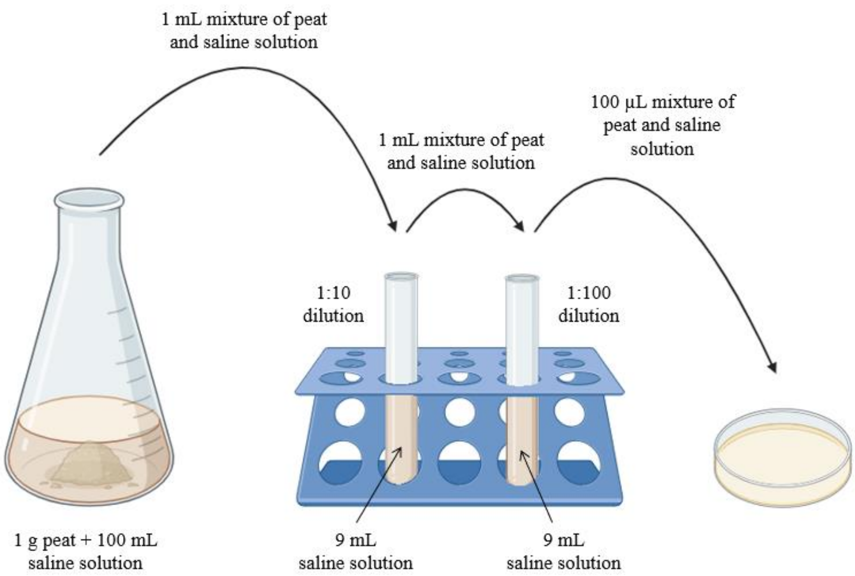


Figure 8. Successive dilutions method of an initial peat sample to obtain 1:10 and 1:100 dilutions.

2.6.4. Calculation of colony-forming units

According to Dionísio *et al.* (50), the number of colony-forming units (CFUs) per gram was calculated as following:

$$CFU\ g^{-1} = (average\ count \times selected\ dilution \times 10)g^{-1}$$

Where the 'average count' represents the average of each sample, and 'selected dilution' represents 1:10 or 1:100 dilutions.

2.7. Total soluble sugars (TSS)

2.7.1. Harvest of plant material for determination of TSS

In the fifth assay, on days 0, 1, 4, 8, 12 and 14, the entire maize plants (roots, stems and leaves) were randomly collected for TSS determination. On day 0, five samples were collected, washed, carefully dried with tissue paper, and their roots, stems, and leaves were measured, placed in a vacuum-sealed plastic bag, and stored at -30 ºC. On each mentioned day, four plants per treatment were collected, in a total of thirty-two samples per day. All samples were treated in the same way as described for day 0.

2.7.2. Construction of glucose's calibration curve

According to McCready (51) and following Bailey (52), 100 mg of glucose was weighed and the value registered. The dry residue of the glucose was measured using a moisture meter. The glucose was then dissolved in 100 mL of distilled water, in a volumetric flask.

To obtain the calibration curve of glucose, different volumes of glucose were pipetted into 50 mL volumetric flasks, as described in Table III.

Table III. Standards and respective concentrations of glucose used in the making of the calibration curve.

Glucose's standard curve points	Concentration of glucose on each point (µg/mL)	Volume to pipette of the standard solution of glucose (mL)	Final volume (mL)
0	0	0	50
1	20	1	50
2	40	2	50
3	60	3	50
4	80	4	50
5	100	5	50

0.5 mL of glucose was added to the test tubes from each point of the glucose calibration curve, and 5 mL of the anthrone reagent was also added. Test tubes were prepared in triplicate and maintained in water with ice. All tubes were covered with aluminium foil, carefully vortexed, and placed in a water bath at 100 ºC for 7 minutes. After that, the tubes were cooled for 30 minutes at room temperature in the dark and the absorbance was measured at 620 nm. The 'blank' was the 'point 0' of the standard curve of glucose. The equation of the obtained standard curve was $y = 0.00297816\ x + 0.0176375$, with an R^2 value of 0.99668 (Appendix III, Figure A.7).

2.7.3. Preparation of anthrone solution

According to McCready (51) and following Bailey (52), the 0.02 % anthrone solution (in sulphuric acid 70 % v/v) was prepared daily and kept for 12 hours at 4 ºC, protected from light.

2.7.4. Preparation of plant material and sample dosing

To prepare the samples for the determination of carbohydrate content, the maize plants from each sample were divided into roots and aerial parts (stems and leaves), dried in a lab oven at 65 ºC, and grinded. The material was stored in a desiccator until use.

According to McCready (51) and following Bailey (52), 50 mg of sample material was weighed in triplicate and the dry residue was measured using a moisture meter. 1 mL of 80 % heated ethanol was added to each sample and homogenised in a vortex. This ethanol was added immediately before centrifugation, at 5,000 rpm for 10 minutes at 23 ºC. The supernatant was collected. The sample pellet was resuspended in 1 mL of 80 % heated ethanol, homogenised with a vortex, and centrifuged. The supernatant obtained was added to the previous tube. To the total supernatant obtained, 5 mL of aqueous ethanol (a mixture of 4 mL of 80 % ethanol and 1 mL of distilled water) was added. The final extract was centrifuged and filtered under the same conditions as described previously, and the filtered volume was adjusted to 25 mL with distilled water.

For each sample, 0.5 mL of the obtained extract was added to test tubes in triplicate, placed in water with ice, and 5 mL of the anthrone reagent was added. All tubes were covered with aluminium foil, carefully vortexed, and subjected to the same procedure as for the construction of the glucose calibration curve. The content of TSS was measured at 620 nm against a 'blank' made with 0.5 mL of distilled water and 5 mL of the anthrone reagent.

2.7.5. Calculation of TSS

According to McCready *et al.* (51) and following Bailey (52), the number of soluble carbohydrates in the maize samples was determined using the glucose standard curve equation and expressed in g/100 g DW.

2.8. Statistical analysis

All samples were measured in triplicate. For the analysis of the data, the IBM SPSS statistical software was used to perform One-Way ANOVA, followed by Tukey's HSD test, to detect significant differences between the samples and parameters analysed. Pearson correlation was used to detect significant correlations between the different parameters studied.

CHAPTER III.

RESULTS AND DISCUSSION

3.1. Soil water content

As mentioned previously, peat was collected from different samples in the fourth and fifth assays on the final day of each experiment. In the fourth assay (Figure 9A), differences in peat moisture between WW and WD conditions were observed, with higher values recorded for the former and lower values for the latter.

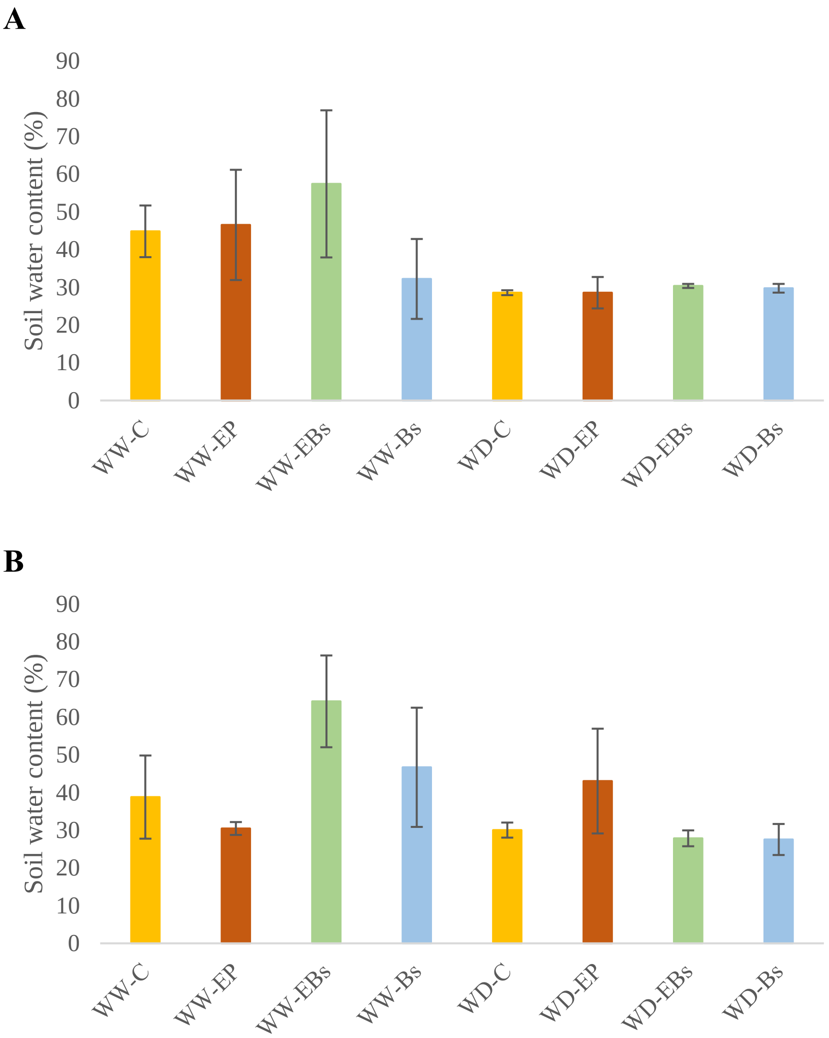


Figure 9. Soil water content (%) obtained from the fourth (A) and fifth (B) assays. The results are expressed as the mean of soil water content per sample ± standard error. No significant differences were detected by One-Way ANOVA between the different treatments in either assay. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

These results serve as a validation test to assess whether the calculated daily quantities of Hoagland's nutrient solution supplied to the maize correctly maintained the stress conditions. In the fifth assay (Figure 9B), stress conditions were maintained in most treatments (WD: C, EBs, and Bs) applied to the maize. However, in the WD-EP experiment, stress conditions were not reliably maintained, even when compared to WW-EP. This discrepancy might be attributed to undetected water retention or accumulation behaviours. Despite consistently supplying 100 % of the solution to some plants (WW plants) and 50 % to others (WD plants), the appropriate water quantity for the cell-tray size might not have been provided. Nevertheless, stress conditions were observed in both assays and in the majority of the experimental variants.

Peat is an organic substrate whose properties depend on the composition and uniformity of the mixture. It exhibits various types based on different plant species and climatic conditions of origin. Peat is highly porous, with a significant water-holding capacity. It is used alongside other substrates and is formed from the partial decomposition of plants, such as *Carex* and *Sphagnum*, commonly found in poorly drained areas (peat bogs). It thrives under anaerobic conditions and low temperatures, with low nutrients and pH. Its advantages include high water retention, porosity, low bulk density, slow degradation, and relatively high cation exchange capacity. However, disadvantages include contributing to soil-borne diseases and creating a biological vacuum during sterilization, which can be filled by pathogenic fungi (53). Despite originating from the same lot, variations in its mixture can impact the behaviour of the peat tray.

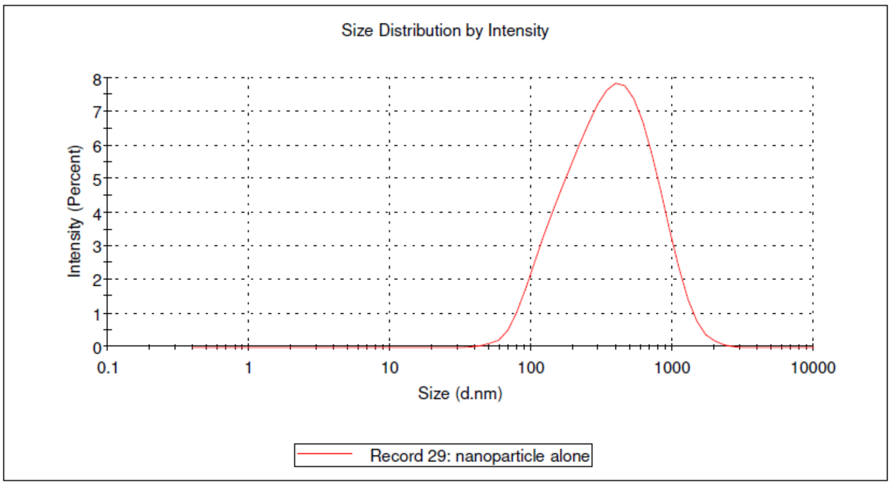
Plant available water can be defined as the difference between field capacity and permanent wilting point. Field capacity represents water retained in the soil after excess water drains but might not be accessible to the plant, while the permanent wilting point signifies the water content unextractable by plants from the soil (54–56). Soils vary in texture from coarse-textured sand to fine-textured clay, with intermediate-textured soils demonstrating the highest available water capacity (54). Peat's strength classification remains somewhat unclear, as it's debated whether peat should be treated as a frictional material akin to sand or cohesive-like clay. Surficial peats, found as submerged deposits, exhibit low unit weight and submergence, leading to low vertical stress for consolidation. Consequently, peat exhibits high porosities and hydraulic conductivities similar to fine sand or silty sand. As it consolidates, porosity decreases, and hydraulic conductivity becomes comparable to clay (57). Therefore, categorising peat as more similar to sand or clay proves challenging due to the absence of specific parameters in this study.

3.2. Chitosan nanoparticles

Chitosan nanoparticles were prepared before the start of the assays to modulate biostimulant activity in stressed maize plants, to verify the effectiveness of the methodology and to confirm the successful production of chitosan particles for use in the assays. The characterisation of these particles involved observation via dynamic light scattering (DLS) analysis[1]. DLS analysis was used to determine the sizes of EP and EBs. EP was 280 nm in size, whereas EBs was 400 nm in size (Figure 10). These dimensions align with the typical range of sizes exhibited by chitosan nanoparticles, that is, a size between 200 and 1,000 nm (26). The ionic gelation technique employed for chitosan nanoparticles formation is known to produce nanoparticles with irregular shapes and sizes (26,58,59). The size distribution of the resulting nanoparticles might be affected by the concentration of acetic acid used to dissolve the chitosan and the temperature during cross-linking. Additionally, the stirring speed might impacted the reaction yield (59).

[1] This analysis was conducted by the Institute of Natural Products and Agrobiology (IPNA – CSIC) in La Laguna, Tenerife.

			Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	279,6	Peak 1:	439,1	100,0	309,3
PdI:	0,326	Peak 2:	0,000	0,0	0,000
Intercept:	0,952	Peak 3:	0,000	0,0	0,000
Result quality	Good				



			Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	399,5	Peak 1:	644,1	89,2	300,1
PdI:	0,371	Peak 2:	101,3	10,2	28,67
Intercept:	0,943	Peak 3:	5092	0,6	554,8
Result quality	Refer to quality report				

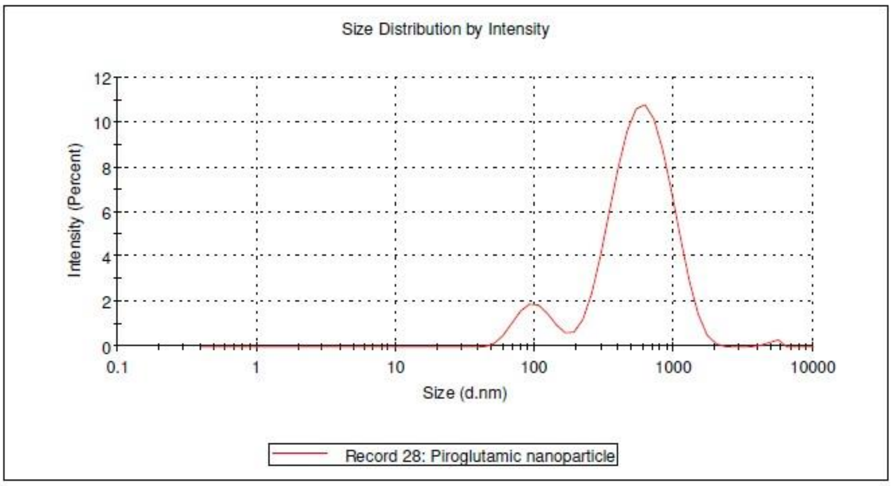


Figure 10. Dynamic Light Scattering (DLS) analysis was used to measure the diameter of the empty particle (EP) and the entrapped biostimulant (EBs) in the nanometre range. The size of the EP at the selected concentration was 280 nm, and the size of the EBs at the selected concentration was 400 nm.

3.3. Peat analysis

Through the method of successive dilution, explained in section 2.6.3., the bacterial and fungal colonies and CFUs in the samples were isolated and counted.

Table IV. Sum of the bacterial colony numbers detected in the initial (collected directly from the bag) and final peat soil samples used in the fourth and fifth assays at dilutions of 1:10 and 1:100. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

	Peat (colonies)		Bacteria (colonies)			
			Fourth assay		Fifth assay	
Dilutions	**1:10**	**1:100**	**1:10**	**1:100**	**1:10**	**1:100**
Peat	744	268				
WW-C			2687	892	2348	1292
WW-EP			1059	95	2514	480
WW-EBs			2449	931	3128	550
WW-Bs			2562	444	2484	508
WD-C			2361	270	1332	125
WD-EP			1915	230	1178	65
WD-EBs			1759	846	2162	135
WD-Bs			1281	171	884	184

In the peat collected directly from the bag, bacterial counts showed 744 colonies at a 1:10 dilution and 268 at a 1:100 dilution (Table IV). In the fourth assay, under WW conditions and a 1:10 dilution, the highest bacterial colony count was observed in WW-C (2687 colonies), whereas the lowest count was in WW-EP (1059 colonies). At the same assay but with a 1:100 dilution, the highest count was WW-EBs (931 colonies), and the lowest count in WW-EP (95 colonies). Under WD conditions and a 1:10 dilution, bacterial colonies ranged from WD-C (2361 colonies) to WD-Bs (1281 colonies). Meanwhile, at 1:100 dilution, counts varied from WD-EBs (846 colonies) to WD-Bs (171 colonies).

In the fifth assay, under WW conditions and at a 1:10 dilution, the highest count was observed in WW-EBs (3128 colonies), and the lowest was in WW-C (2348 colonies). At a 1:100 dilution, the treatment with the highest count was WW-C (1292 colonies), and the lowest was WW-EP (480 colonies). Under WD conditions and a 1:10 dilution, counts ranged from WD-EBs (2162 colonies) to WD-Bs (884 colonies), while at a 1:100 dilution, counts varied from WD-Bs (184 colonies) to WD-EP (65 colonies).

Despite variations observed in the fourth (Figures 11A and 11B) and fifth (Figures 11C and 11D) assays, a consistent decrease in bacterial colonies was evident under WD conditions compared to WW conditions for both 1:10 and 1:100 dilutions. In the fifth assay, WD-EP showed an anomalous variation, with an increase in colony count in the 1:10 dilution compared to WW-EP. The variation in bacterial colonies exhibited a significant difference ($p < 0.05$) between WW and WD conditions in both the fourth and fifth assays. However, no significant difference was detected among the experimental conditions, except for the variation between WD-EP and WD-EBs in the fourth assay (1:100 dilution), where Tukey's HSD test detected a significant difference ($p < 0.05$) under stress conditions. Overall, it can be concluded that bacterial communities did not interfere with the results of the fifth assay, in terms of uptake of biostimulants by the plant, under stress or non-stress conditions. When exposed to low soil water content, microbial activities can be widely affected due to reduced diffusion or an increased risk of cell dehydration (60). Microorganisms in soil have different strategies to cope with stress, including tolerance mechanisms, such as accumulating compatible solutes, spore production, or avoidance strategies like dormancy (60,61). The variation in these strategies varies across different microbial groups, influencing their activity and growth under stress conditions (60). Metze *et al.* (60) identified Actinobacteria as the most drought-tolerant phylum, primarily driven by some families including Intrasporangiaceae, Nocardiaceae, Nocardioidaceae, and Streptomycetaceae, and particularly by specific genera within them, such as *Marmoricola*, *Oryzihumus*, *Rhodococcus*, and *Streptomyces*. Among these, *Streptomyces* accounted for the majority of community growth in their study.

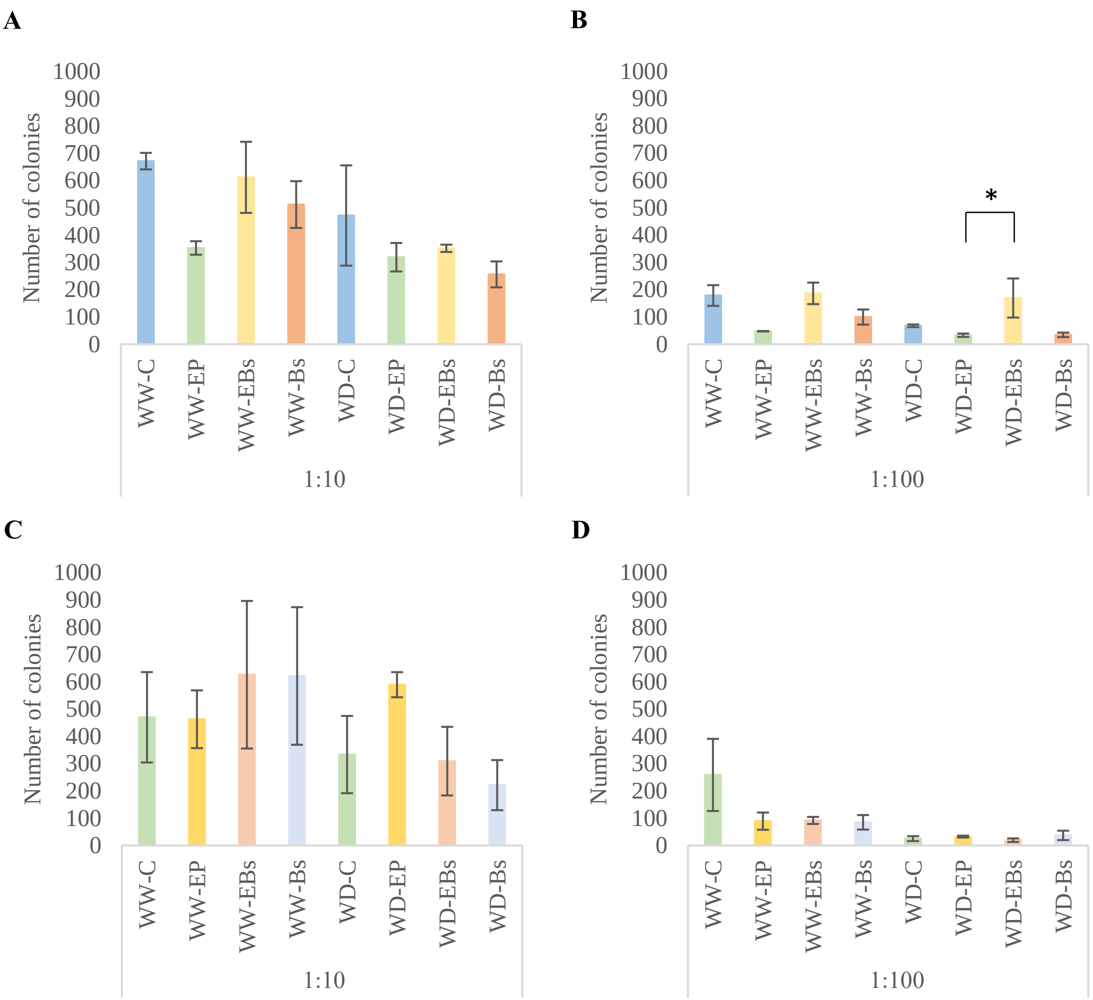


Figure 11. Number of bacterial colonies detected in the fourth (A and B) and fifth (C and D) assays under WW and WD conditions for all the experimental variants at dilutions of 1:10 and 1:100. The results are expressed as the mean of the number of bacterial colonies per sample ± standard error. Asterisks represent statistical significance ($p < 0.05$) in Tukey's HSD test. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Table V. Sum of the fungal colony numbers detected in the initial (collected directly from the bag) and final peat soil samples used in the fourth and fifth assays at dilutions of 1:10 and 1:100. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; EBs, water-deficit entrapped biostimulant; Bs, water-deficit biostimulant.

	Peat (colonies)		**Fungi (colonies)**			
			Fourth assay		**Fifth assay**	
Dilutions	**1:10**	**1:100**	**1:10**	**1:100**	**1:10**	**1:100**
Peat	961	107				
WW-C			1601	547	1279	217
WW-EP			704	85	1249	300
WW-EBs			1857	322	3222	323
WW-Bs			1782	269	2711	522
WD-C			1360	126	672	107
WD-EP			1024	184	1306	36
WD-EBs			783	120	752	97
WD-Bs			753	121	595	111

For fungi, peat collected directly from the bag showed 961 colonies at a 1:10 dilution and 107 colonies at a 1:100 dilution (Table V). In the fourth assay, under WW conditions and a 1:10 dilution, the treatment with the highest fungal colony count was WW-EB (1857 colonies), while WW-EP (704 colonies) exhibited the lowest count. In the same assay and at a 1:100 dilution, the treatment with the highest count was WW-C (547 colonies) and the treatment with the lowest count was WW-EP (85 colonies). Under WD conditions and a 1:10 dilution, counts ranged from WD-C (1360 colonies) to WD-Bs (753 colonies). Meanwhile, at a 1:100 dilution, counts varied from WD-EP (184 colonies) to WD-EBs (120 colonies).

In the fifth assay, under WW conditions and at a 1:10 dilution, the treatment with the highest number of fungal colonies was WW-EBs (3222 colonies), while the lowest was WW-EP (1249 colonies). In the same assay and a 1:100 dilution, WW-Bs (522 colonies) showed the highest count, and WW-C (217 colonies) the lowest. Under WD conditions at a 1:10 dilution, counts varied from WD-EP (1306 colonies) to WD-Bs (595 colonies), and at a 1:100 dilution, counts ranged from WD-Bs (111 colonies) to WD-EP (36 colonies).

Despite variations observed in the fourth (Figures 12A and 12B) and fifth (Figures 12C and 12D) assays, fungal colony counts exhibited a decrease under WD conditions compared to WW conditions. However, in the fifth assay at a 1:10 dilution, an anomalous increase in the number of colonies was noted in WD-EP relative to WW-EP. The variation in fungal colonies showed a significant difference ($p < 0.05$) between WW and WD conditions, in both the fourth and fifth assays. Nevertheless, no significant difference was detected among the experimental conditions overall. Exceptions were observed in the variation of fungal colonies between WD-C and WD-EP in the fourth assay at a 1:10 dilution, where Tukey's HSD test detected a significant difference ($p < 0.01$). Additionally, in the fifth assay, significant differences were noted between WW-EP and WW-Bs at a 1:100 dilution ($p < 0.05$), and between WD-EP and other experimental variants at a 1:10 dilution ($p < 0.01$). Overall, it can be concluded that fungal communities did not interfere with the results of the fifth assay regarding the uptake of biostimulants by plants under stress conditions because the number of fungal colonies decreased under WD conditions. Furthermore, there was no significant difference between the experimental conditions at a 1:100 dilution. The exception of WD-EP at the 1:10 dilution might be linked to the higher soil moisture content detected in Figure 9.

The fungi present in the peat might have utilised the introduced chitosan particles within their metabolic processes, potentially contributing to the observed increase in colony numbers. Chitin, an important component of fungal cell walls and insect exoskeletons, serves as the basis for chitosan production (62). Generally, fungi are more susceptible to the effects of chitosan compared to bacteria. Chitosan directly interferes with the growth of fungi through interactions with phospholipid components with a negative charge on their membranes, which leads to the leakage of cellular components and binding to their DNA (63). Nevertheless, certain fungi belonging to the Ascomycota, Basidiomycota, and Zygomycota phyla have the capability to produce chitosan. Notably, entomopathogenic and nematophagous fungi exhibit resistance to this polymer (64).

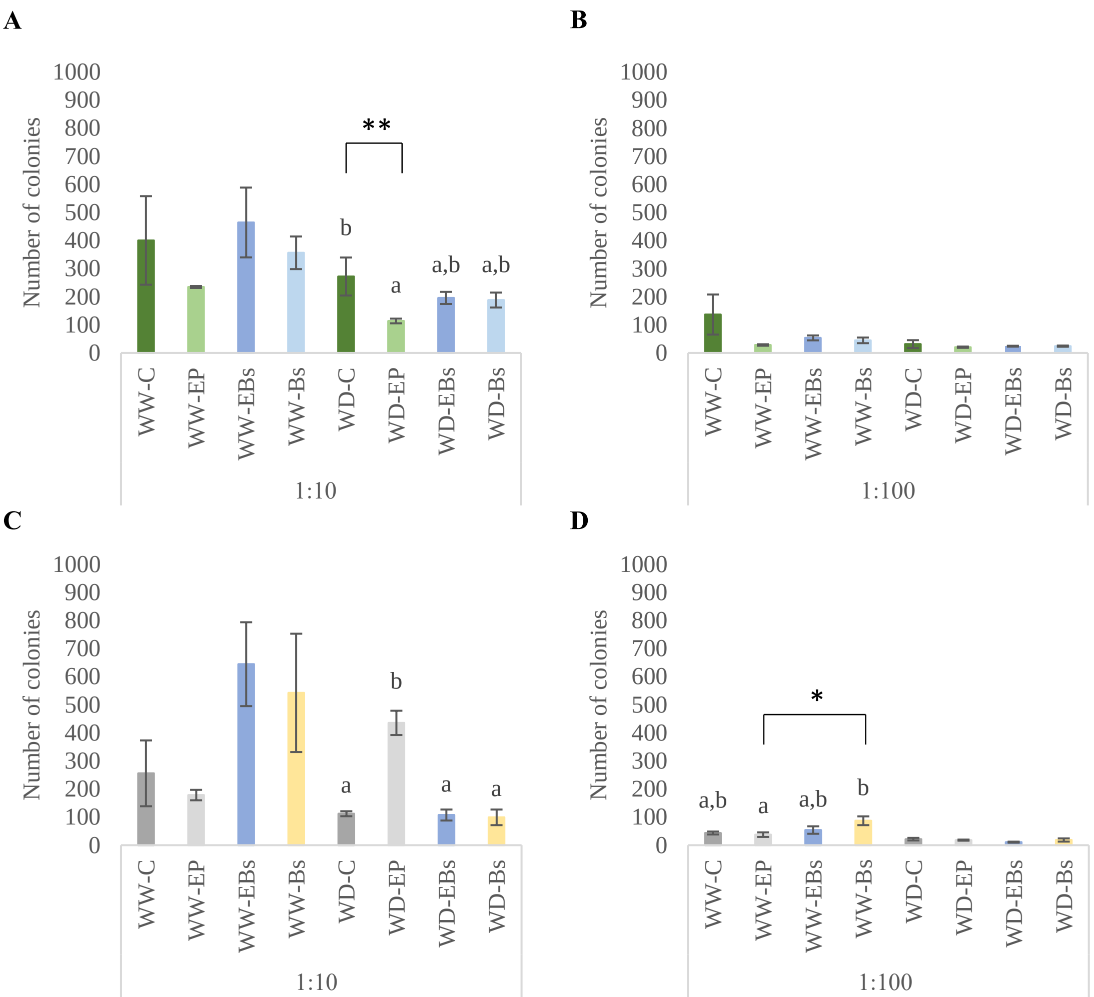


Figure 12. Number of fungal colonies detected in the fourth (A and B) and fifth (C and D) assays under WW and WD conditions for all the experimental variants at dilutions of 1:10 and 1:100. The results are expressed as the mean of the number of fungal colonies per sample ± standard error. Different letters represent statistical significance by One-Way ANOVA ($p < 0.05$). Asterisks represent statistical significance (* $p < 0.05$; ** $p < 0.01$) in Tukey's HSD test. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Table VI. Bacterial and fungal CFUs per gram of peat, in initial (collected directly from the soil) and final peat soils in the fourth and fifth assays were calculated. The results show the number of CFUs expected under WW and WD conditions and experimental variants (treatments) applied to *Zea mays*. The results are expressed as the mean of the number of CFUs per sample ± standard error. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

	Peat	Bacteria		Peat	Fungi	
		4th assay	5th assay		4th assay	5th assay
Peat	4.52×10^6 ± 27.27			3.24×10^6 ± 23.18		
WW-C		15.9×10^6 ± 89.80	15.4×10^6 ± 105.89		9.76×10^6 ± 94.33	6.31×10^6 ± 65.64
WW-EP		3.61×10^6 ± 76.05	16.0×10^6 ± 78.08		2.39×10^6 ± 46.18	5.86×10^6 ± 20.97
WW-EBs		15.0×10^6 ± 94.18	15.0×10^6 ± 142.86		9.47×10^6 ± 81.10	11.6×10^6 ± 112.92
WW-Bs		12.0×10^6 ± 80.92	13.0×10^6 ± 128.00		6.89×10^6 ± 55.20	13.1×10^6 ± 115.19
WD-C		11.0×10^6 ± 120.28	6.39×10^6 ± 79.32		4.99×10^6 ± 55.58	2.40×10^6 ± 15.13
WD-EP		9.62×10^6 ± 47.26	5.65×10^6 ± 161.75		3.66×10^6 ± 12.07	4.19×10^6 ± 104.93
WD-EBs		9.93×10^6 ± 45.89	10.0×10^6 ± 72.60		4.00×10^6 ± 31.44	3.35×10^6 ± 14.87
WD-Bs		4.97×10^6 ± 43.44	4.82×10^6 ± 50.33		3.90×10^6 ± 30.82	2.14×10^6 ± 18.21

The total number of CFUs per gram of soil sample was determined (Table VI) for the initial (collected directly from the bag) and final soil samples in the fourth and fifth assays. The initial peat samples presented 4.52×10^6 and 3.24×10^6 CFUs of bacteria and fungi, respectively.

In the fourth assay, under WW conditions, the experimental variant with the highest number of bacterial (15.9×10^6) and fungi CFUs (9.76×10^6) was WW-C. The treatment with the lowest number of bacterial (3.61×10^6) and fungal CFUs (2.39×10^6) was WW-EP. Under WD conditions, the experimental variant WD-C also showed the highest number of bacterial CFUs (11.0×10^6) and fungal CFUs (4.99×10^6). The treatment with the lowest number of bacterial CFUs (4.97×10^6) was WD-Bs, and the one with the lowest fungal CFUs (3.66×10^6) was WD-EP.

In the fifth assay, under WW conditions, the experimental variant with the highest number of bacterial CFUs (16.0×10^6) was WW-EP, while in terms of fungal CFUs (13.1

x 10^6), WW-Bs exhibited the highest count. Conversely, the treatment with the lowest number of bacterial CFUs (13.0 x 10^6) was WW-Bs, and for fungal CFUs (5.86 x 10^6), it was WW-EP. Under WD conditions, the experimental variant with the highest number of bacterial CFUs (10.0 x 10^6) was WD-EBs, while for fungal CFUs (4.19 x 10^6), WD-EP showed the highest count. On the other hand, the treatment with the lowest number of bacterial (4.82 x 10^6) and fungal CFUs (2.14 x 10^6) was WD-Bs.

The growth and behaviour of microbial populations depend on the interactions between plant species and soil. The number of microorganisms varies in and between the different soil types and conditions. Among them, bacteria are the most prevalent, with counts ranging from 4 x 10^6 to 2 x 10^9 CFUs/g of dry soil (65). Even though there was one exception, that is, WW-EP in the fourth assay (3.61 x 10^6), the majority of CFUs counts were consistent within this established range. In natural ecosystems, peat can be classified into two categories: acrotelm, which is the upper layer receiving oxygen from the atmosphere, and typically inhabited by aerobic microorganisms, and catotelm, which is beneath the acrotelm in which general microorganisms are anaerobic (66). Similar to bacteria, fungi are present in the acrotelm, with an average count of approximately 3.7 x 10^6 CFUs/g of dry peat (66,67). Although the assays were not conducted in "open air", the number of fungal CFUs obtained was around the value estimated for dry peat, except for the counts observed in WW-C (9.76 x 10^6) and WW-EBs (9.47 x 10^6) in the fourth assay, and in WW-EBs (11.6 x 10^6) and WW-Bs (13.1 x 10^6) in the fifth assay.

In Figure 13, bacterial colonies obtained from both the fourth (Figure 13A) and fifth (Figure 13C) assays are shown, in their majority represented by regular and irregular shapes with different shades of beige, as well as filamentous ones. Fungal colonies observed in the fourth (Figure 13B) and fifth (Figure 13D) assays showed greater diversity, including regular yellowish-beige shapes with a reddish-pink centre, filamentous structures, as well as both regular and irregular shapes in different shades of beige. Additionally, some colonies appeared greenish, while others were white in colour.

The most well-studied microbial communities associated with plants include mycorrhizal fungi, nitrogen-fixing bacteria, and plant growth-promoting rhizobacteria (61). Investigations showed that although bacteria have been subdivided into more than 100 phyla, fewer than 10 are abundant in the soil. The estimated relative abundance of the main phyla varies between different soils or samples. Among these, the phyla Acidobacteria, Actinobacteria, and Proteobacteria are frequently abundant, whereas members of the Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, and

Planctomycetes are less abundant. Despite the relatively low number of bacterial phyla present in soil, the diversity of species within these phyla appears to be considerably high when compared to other environments (68). The biology, distribution, and ecology of fungi have not been studied sufficiently due, for example, to their morphological characteristics. Some fungal species exhibit variations in their forms throughout their life cycles, creating challenges in their study. Additionally, their relationships with other organisms contribute to the complexity of studying and culturing them under laboratory conditions. This also applies to soil fungi. While certain fungal species exhibit visible and distinct structures, others, like arbuscular mycorrhizal or endophytic fungi, require microscopic observation for their detection and study (69).

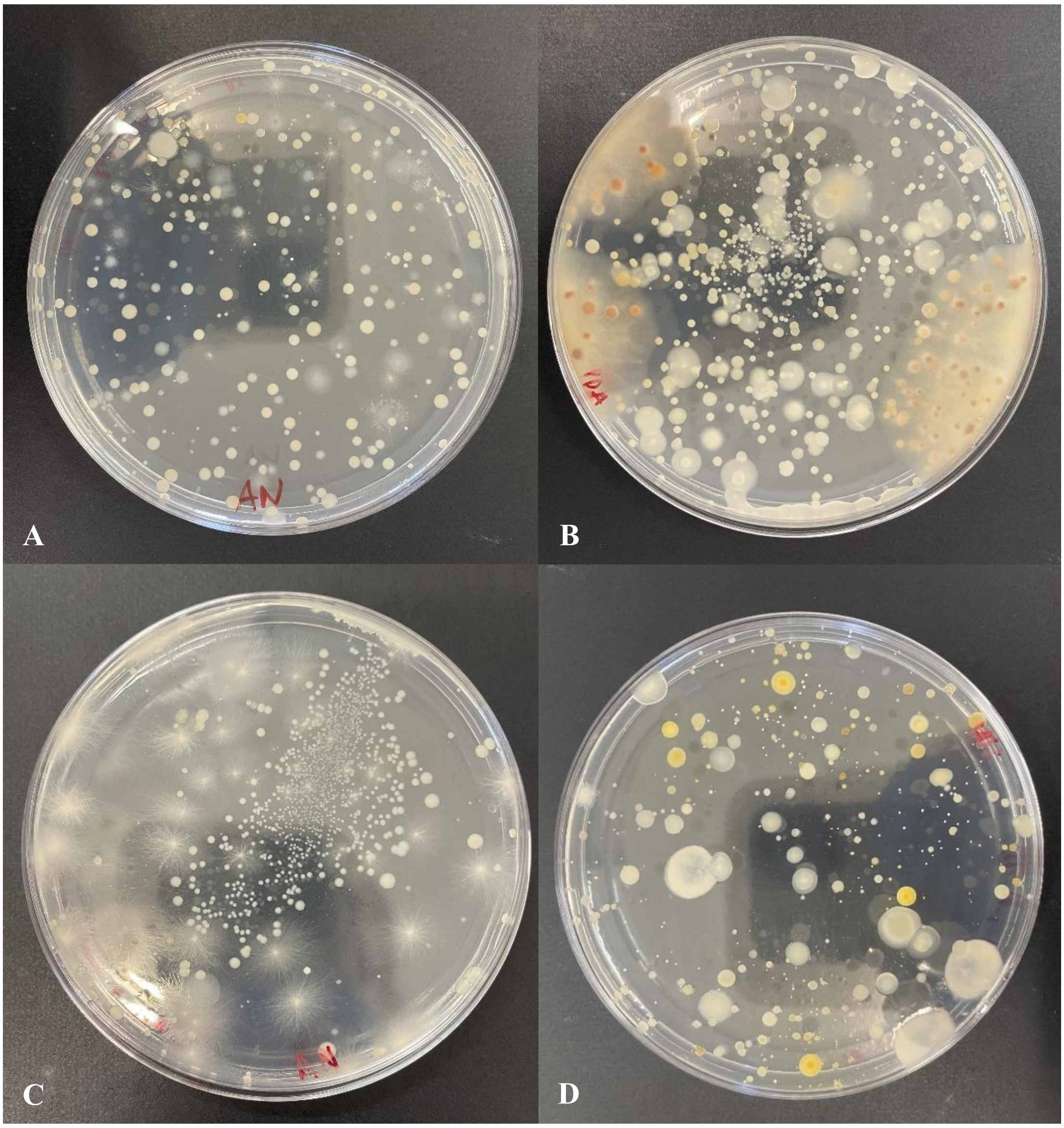


Figure 13. Bacterial and fungal colonies obtained in the fourth (A and B) and fifth (C and D) assays, using the successive dilution method. Bacterial colonies are represented by A and C, and fungal colonies are represented by B and D.

Comparisons were made between the total bacteria and fungi communities in the initial peat (collected directly from the bag) and the WW peats from the fourth and fifth assays (Figure 14). The aim was to assess potential differences between untreated peat and peat that remained under non-stress conditions, receiving 100 % irrigation until the last day of the assays. A significant difference in the number of bacterial colonies was observed between the initial peat and the WW peat from both the fourth ($p < 0.01$) and fifth assays ($p < 0.05$). For fungal colonies, no significant difference was observed between the initial peat and the WW peat in both assays. This suggests that the number of fungal colonies in WW peats was similar to that of the initial peat fungal flora after 15 days.

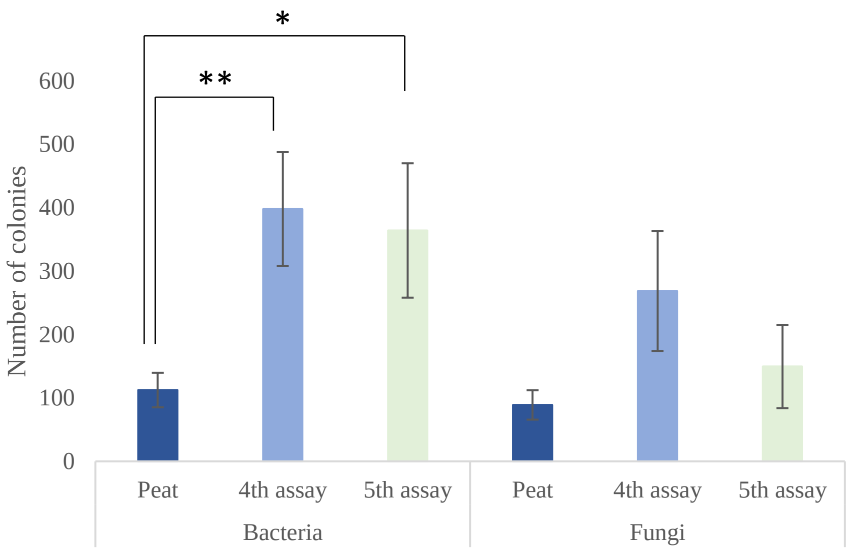


Figure 14. Number of bacterial and fungal colonies observed in the initial peat (collected directly from the bag) and the WW peats from the fourth and fifth assays, after 15 days. The results are expressed as the mean of the number of bacterial and fungal colonies per sample ± standard error. Asterisks represent statistical significance (* $p < 0.05$; ** $p < 0.01$) in Tukey's HSD test.

3.4. Testing drought stress in maize and evaluating the action of entrapped biostimulants

3.4.1. Whole-plant biomass dry weight

The dry biomass weights of maize plants in the fourth assay for each treatment are shown in Figure 15. Although there wasn't a significant difference observed between WW and WD conditions, a decrease in the dry biomass of the experimental variants, particularly in C and EP, was noticeable. In the entrapped pyroglutamic acid (EBs) and free pyroglutamic acid (Bs) treatments, the decrease in plant dry biomass of the plant was low. This suggests a potential success in delivering the biostimulant and coping with drought stress. In the molecular structure of glutamic acid, the presence of two carboxylate groups (-COOH) enhances its ability to bind with the amine group (-NH_2) of chitosan, strengthening its hydrophilic properties (70). In the case of pyroglutamic acid, the number of carboxylate groups decreases to one. Nevertheless, it retains the capability to bind, even with less strength, to the amine group of chitosan. This property facilitates its penetration into the root structure.

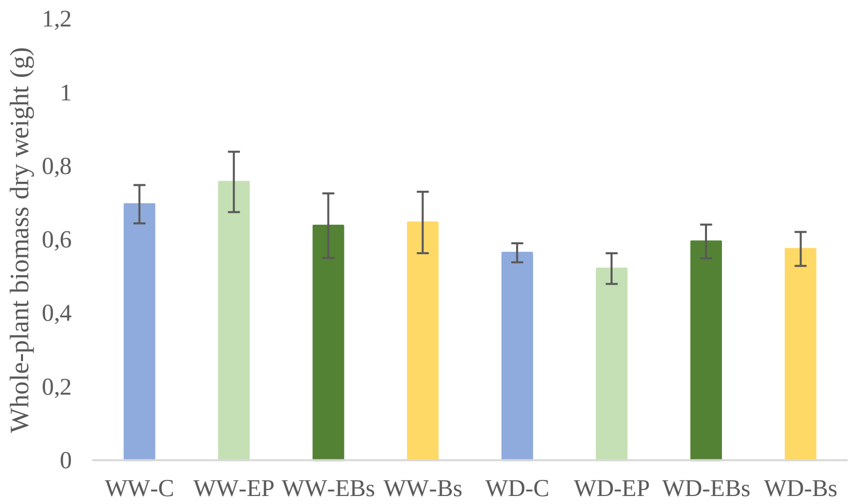


Figure 15. Whole-plant biomass dry weight (g) under WW and WD conditions for all the experimental variants in the fourth assay. The results are expressed as the mean of dry weight per sample ± standard error. No significant differences were detected by One-Way ANOVA for the different treatments. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

In Efeoğlu *et al.* study (71), three maize cultivars were examined, subjecting them to drought stress by withholding irrigation for 12 days after sowing, followed by re-watering for the subsequent 6 days. Their findings indicated a significant decrease in dry biomass under both stress and recovery conditions. In this study, a reduction in the dry biomass of maize plants was detected between WW and WD conditions, although this decrease did not reach statistical significance.

3.4.2. Proline

The plant's response to drought stress often involves an increase in proline (Pro) content. It was hypothesised that the biostimulant might function as a plant defence elicitor, either directly or by enhancing proline synthesis. To assess Pro levels, maize leaves were harvested on day 14 of the fourth assay (Figure 16).

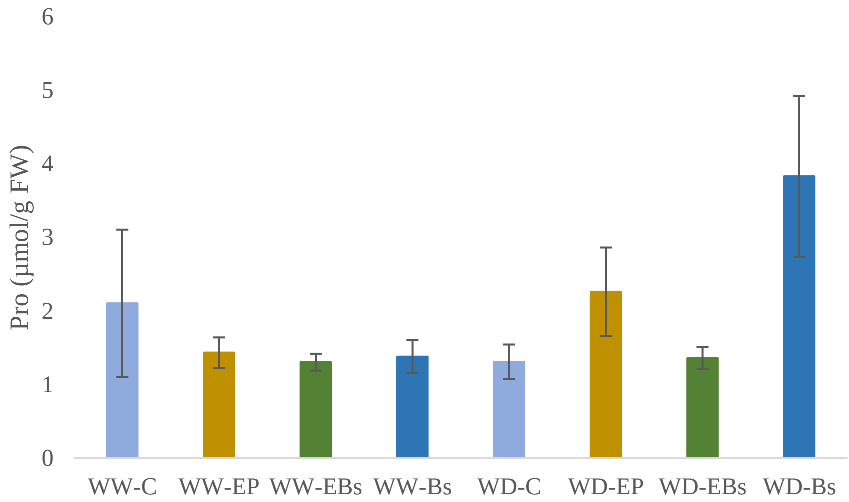


Figure 16. Proline (µmol/g FW) content under WW and WD conditions for all the experimental variants during the fourth assay. The results are expressed as the mean of Pro content per sample ± standard error. No significant differences were detected by One-Way ANOVA for the different treatments. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Under WW conditions, no significant difference ($p < 0.05$) was observed in the Pro content among the experimental variants. Similarly, under WD conditions, there was no significant difference in Pro content among the experimental variants. Pro levels increased in WD-EP and WD-Bs compared to WD-C, but there were no evident differences in WD-EBs. This observation suggests that the free biostimulant (WD-Bs) might have acted at the beginning of the plant response to drought stress. By day 14, the plant might have elicited its defence mechanism, evident through Pro synthesis in the leaves. Meanwhile, the entrapped biostimulant (WD-EBs) potentially continued to alleviate plant stress at the root level and might not have been required for reinforcement through leaf Pro synthesis. This conclusion finds support in the sustained whole-plant dry biomass observed in WD-EBs and WD-Bs (Figure 15). At the same time, a moderate increase in Pro content was observed in leaves treated with WD-EP. Behboudi *et al.* (72) investigated the application of chitosan nanoparticles in *Hordeum vulgare* L. under WW and WD conditions administered via foliar or soil application. They found that drought stress significantly increased Pro content compared to well-watered plants. For both applications, in well-watered plants, the use of chitosan nanoparticles showed no significant effect on Pro content compared to the control (no nanoparticle application). However, in plants subjected to drought stress, the application of these nanoparticles significantly increased Pro levels compared to the control. These findings suggest that chitosan could potentially trigger Pro synthesis mechanisms, particularly under stress conditions. Nevertheless, further validation is necessary. Proline serves as an osmoprotectant that accumulates in response to various stresses. This accumulation may be due to an increase in Pro synthesis (activation of P5CS1) or a reduction in Pro degradation (suppression of ProDH) in response to drought stress, leading to elevated levels of proline. However, while an increase in this amino acid is anticipated, only a few plant species possess the ability to produce sufficient Pro to mitigate the effects of abiotic stress (73).

Other explanations for these results could be related to maize variability, considering the utilisation of a Madeiran variety known for its substantial genetic and phenotypic diversity. The number of plants assessed per assay was limited since the beginning of the assays depended on the plants that reached the two true-leaf stages. The fact that the harvest was made only on the last day of the assay does not allow us to deeply discuss what led to the present results for the proline content.

3.5. Validating the action of the entrapped biostimulant in the cope of drought stress

3.5.1. Root length

In the fifth assay, the influence of drought stress on the development of maize plants was investigated, and the plants were collected periodically. Root lengths were measured on days 1, 4, 8, 12, and 14 (Figure 17).

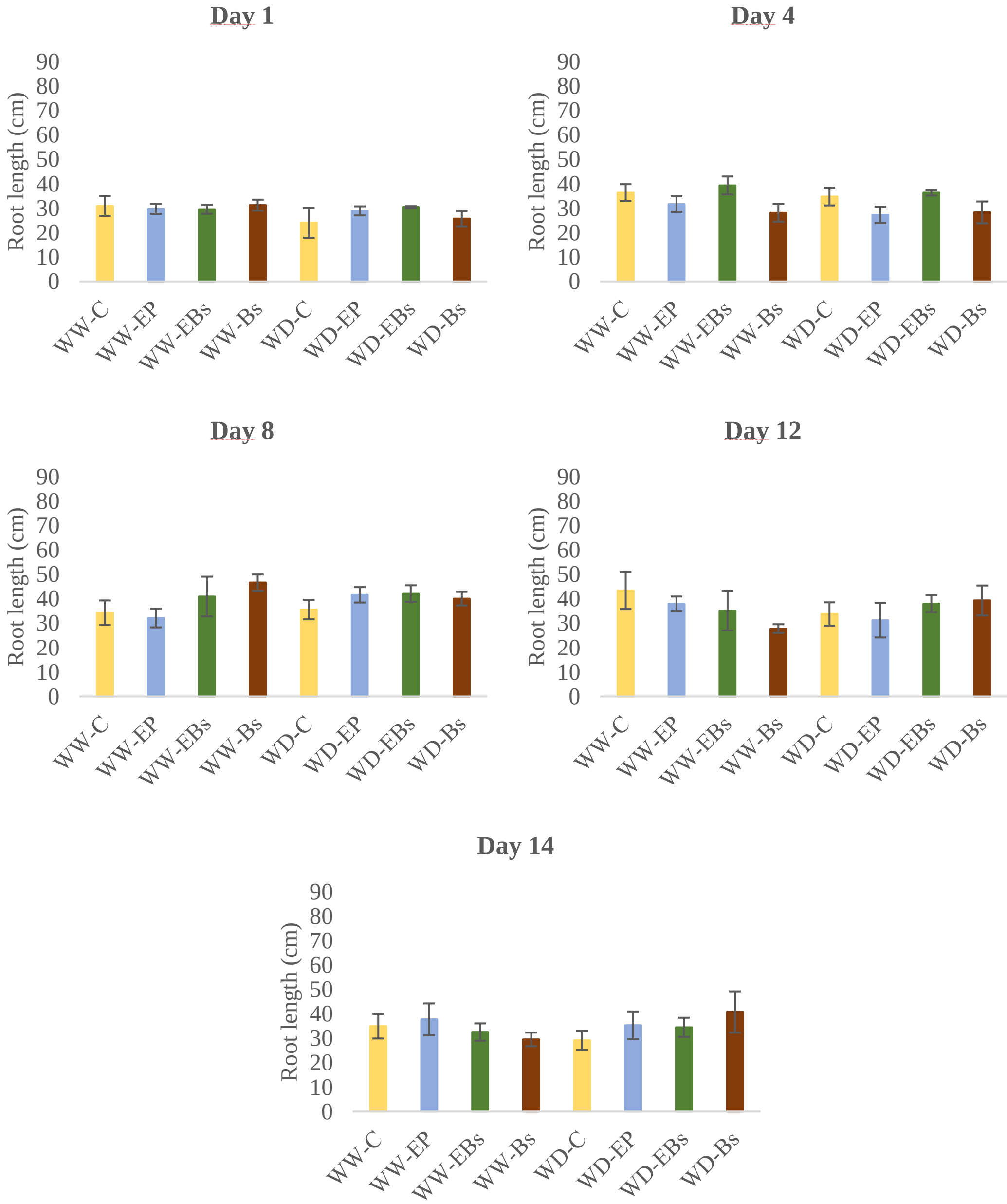


Figure 17. Root length (cm) under WW and WD conditions for all the experimental variants during the fifth assay. The results are expressed as the mean of root length per sample ± standard error. No significant differences were detected by One-Way ANOVA between the different treatments. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Under WW conditions, root length showed considerable variability, and therefore, a clear trend could not be observed. These results might come from the fact that the roots were picked up at each sampling day from different individual plants. To analyse these data, lengths from days 1, 8, and 14 were used, representing the beginning, middle, and end of the experiment, respectively. Comparing the data from the first day with day 8, WW-EBs and WW-Bs treatments demonstrated an enhancement in root length. However, the trend was not sustained by day 14, as there was a decline in root length. This suggests that during the experimental duration of biostimulant action, whether entrapped or not, there might not have been sufficient efficacy to consistently influence root growth across most of the experimental variants. Under WD conditions, there was also a variation in the root length of the measured plants, but a clear trend seemed to exist, where the experimental variants compared to control plants seemed to promote or maintain root growth. When plants were subjected to stress, it was observed that on day 8, WD-EBs exhibited greater efficiency than WD-Bs, whereas on day 14, the opposite trend occurred. Tukey's HSD test analysis did not detect significant differences ($p < 0.05$) in root length among the experimental variants under WW and WD conditions. The observed variability and the absence of a statistically significant trend might be attributed to several factors, including the uneven number of samples and the inability to measure roots on the same plants consistently throughout the 14-day assay.

The utilisation of the regional variety (landraces) 'Milho de Santana' might account for the substantial variability observed in root length results, as the high genetic variability of landraces can be reflected in root morphological traits. This diversity can be exhibited at different levels and patterns in different species, as well as in distinct cultivars within species or even within the populations of a regional variety (74).

Under drought stress, the plant's root response is vital for maintaining crop productivity. The architecture, distribution, and size of roots determine how the plant will access water for proper physiological functioning of the shoots. To cope with this stress, plants often allocate a substantial amount of assimilates to maintain growth and access water from deeper soil layers, decreasing the growth of the shoot (aerial part) (75). Consequently, roots continue to grow within the soil, while shoot growth is deprived (76). Roots exhibit morphological adaptability in response to soil physical conditions, which makes plants adapt better to the chemical and physical properties of the soil, particularly during stress conditions such as drought (61). Studies have shown that a higher root-to-shoot ratio is one of the main parameters associated with better adaptation to drought stress in maize, rice, and wheat. Long and few lateral roots are associated with deeper rooting, greater water uptake, and higher grain yield under drought stress in maize (75). The dimensions of the cell trays used have constrained root growth, potentially limiting space for expansion. This limitation was detected when the plants were removed from the cell trays, where some roots were damaged during the process.

It is important to mention that although maize plants were collected on day 0, the data were not used in any of the results of the fifth assay (lengths, Pro, and TSS) because the treatments were not applied.

3.5.2. Aerial part length

The length of the aerial part of maize plants was measured during the fifth assay on days 1, 4, 8, 12, and 14 (Figure 18).

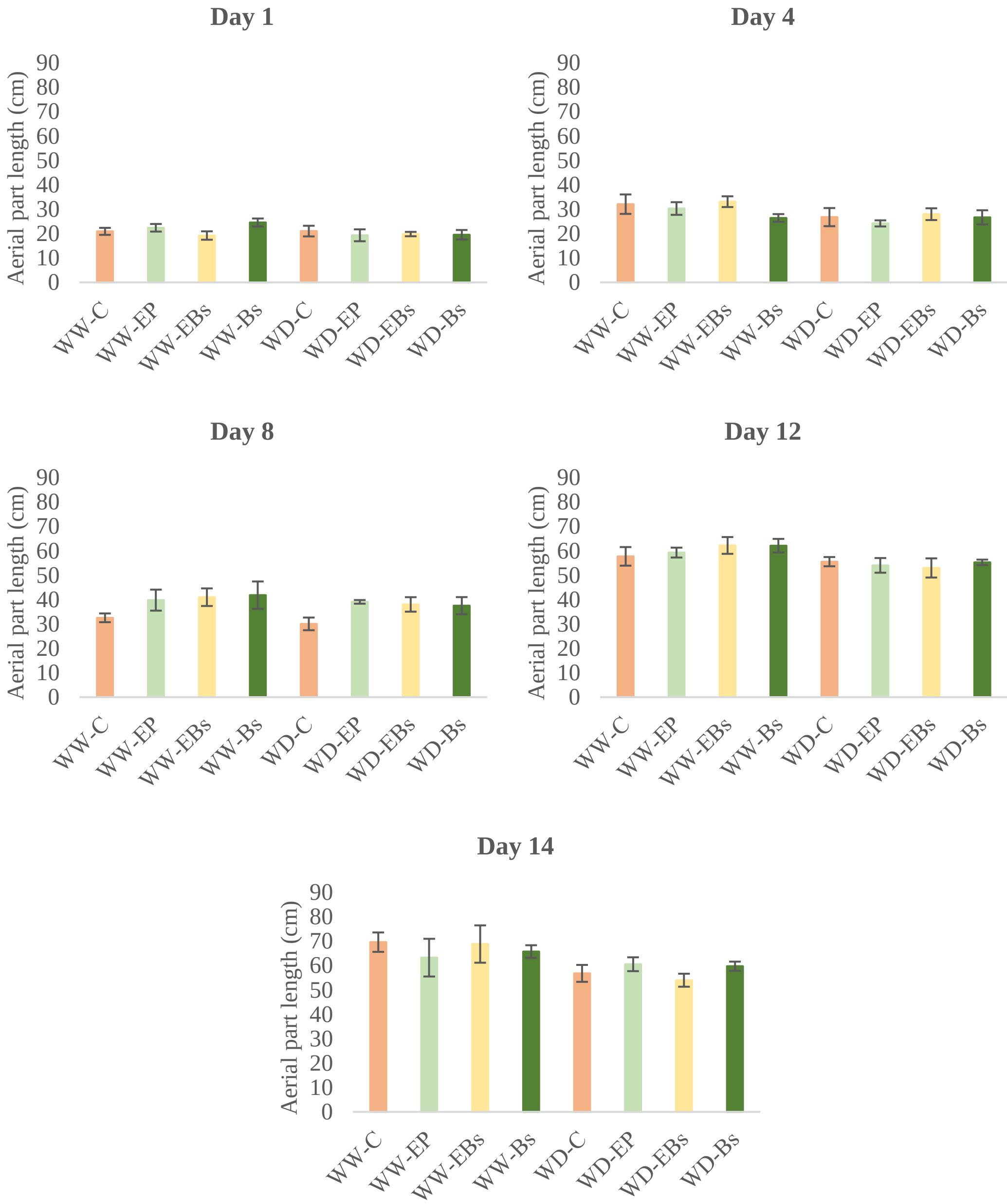


Figure 18. Aerial part length (cm) under WW and WD conditions for all the experimental variants during the fifth assay. The results are expressed as the mean of aerial part length per sample ± standard error. No significant differences were detected by One-Way ANOVA between the different treatments. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Under WW conditions, an increase in both stem and leaf lengths was observed during the fifth assay. Data from days 1, 8, and 14 were analysed to represent the beginning, middle, and end of the experiment, respectively. A comparison between the first day and days 8 and 14 revealed that on day 8, WW-EBs and WW-Bs promoted aerial part growth, which was confirmed on the last day of the assay. This indicates that the application of the biostimulant, regardless of entrapment, had a significant effect on the growth of the aerial parts across most experimental variants. Under WD conditions, an increase in both stem and leaf growth was noted throughout the assay, where the experimental variants appeared to facilitate or sustain aerial part growth compared to control plants. As in the root lengths under WD conditions, it was evident that on day 8, WD-EBs exhibited more efficacy than WD-Bs, but on day 14, the trend reversed. Despite these observations, Tukey's HSD test analysis comparing the aerial part lengths across different assessed days under both WW and WD conditions, and among various experimental variants, did not detect any significant differences ($p < 0.05$) between the application or non-application of the entrapped biostimulant and the lengths of the aerial parts.

In maize, the number of leaves ranges from 8 to 20 and are alternatively present on the nodes. Their growth consists of increased leaf size and number of leaves, which are structural components, whereas light interception, photosynthesis, and transpiration are functional traits (77). When subjected to drought, the aerial part of the plant can adapt to it, reducing the leaf area and number of leaves to decrease water loss (76,77). Flux assimilation, light interception, and turgor pressure are important factors for leaf elongation. The turgor of leaves is reduced, and leaves become curled or folded, which consequently reduces the leaf area, light interception, and photosynthetic activity. With reduced leaf area, plant water requirements are reduced, which increases the probability of plant survival under limited water availability conditions. However, chloroplast and chlorophyll contents and photosynthetic activity are reduced, decreasing grain yield (77). This study showed that the entrapped biostimulant and the free biostimulant affected aerial part growth, increasing it even under WD conditions.

3.5.3. Root and aerial part stress index

The stress index (SI) was calculated to determine the possible increase in plant tolerance to drought stress as determined by the experimental variants. In Figure 19, a comparison between root (R) and aerial part (AP) lengths in the fifth assay was performed to determine whether EBs and Bs influenced growth of these plant parts. The biostimulant, entrapped or not, showed a protective effect, which could be verified by the elongation of the root. However, the aerial part was not affected, or its development was slower. This means that the biostimulant, entrapped or not, acted at root level, not being necessary in the aerial parts. The maize plant showed some sensitivity to stress, although not very severe.

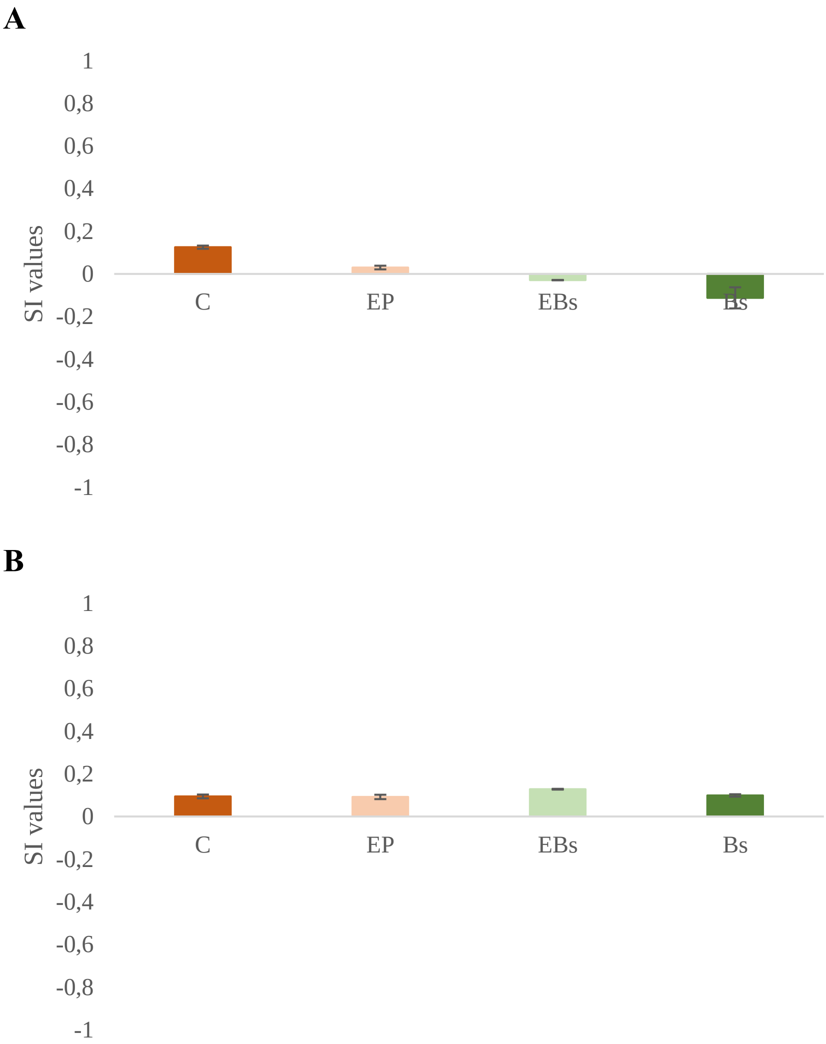


Figure 19. Stress index (SI) values calculated, comparing root (R) (A) and aerial part (AP) (B) lengths to evaluate the tolerance to drought in experimental variants in the fifth assay. The results are expressed as the mean value calculated to the plants analysed per sample ± standard error. No significant differences were detected by One-Way ANOVA for the different treatments. Abbreviations: C, control; EP, empty particle; EBs, entrapped biostimulant; Bs, biostimulant.

3.5.4. Whole-plant length

With the root and aerial part lengths, it was possible to determine the length of the whole-plant in the fifth assay on days 1, 4, 8, 12, and 14 (Figure 20).

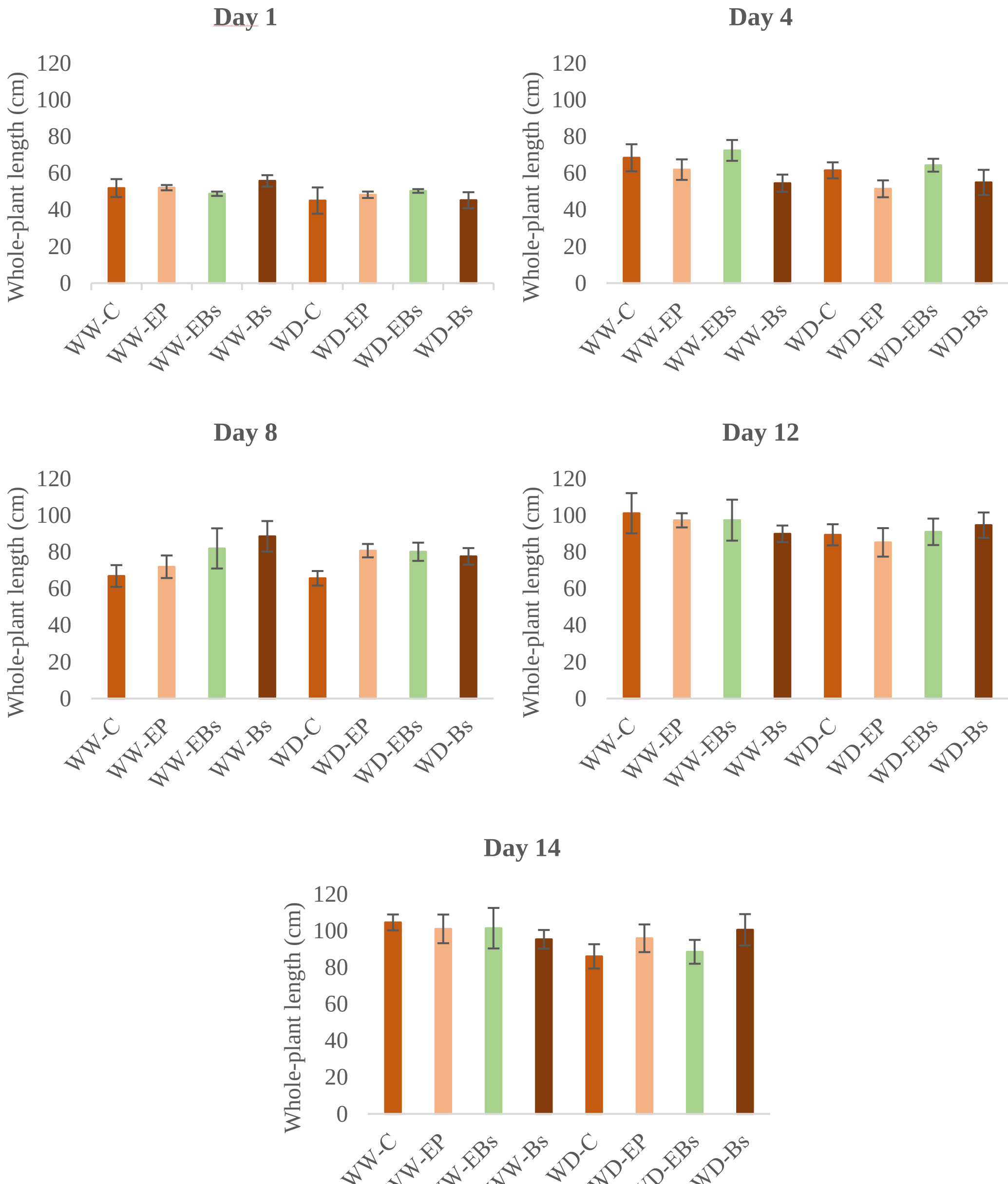


Figure 20. Whole-plant length (cm) under WW and WD conditions for all the experimental variants during the fifth assay. The results are expressed as the mean of whole-plant length per sample ± standard error. No significant differences were detected by One-Way ANOVA between the different treatments. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Under WW conditions, there was an increasing trend observed. Similar to the analysis of root and aerial parts, only the data on days 1, 8, and 14 were considered. On day 1,WW-Bs showed a greater value for whole-plant length than WW-C and WW-EBs, which presented similar values. However, this difference was not statistically significant. The values of all experimental variants increased from day 1 to day 8, with an abnormal decrease in WW-Bs on day 4. In comparison to day 1, WW-EBs presented a higher value than WW-C, and, as previously mentioned, WW-Bs also presented the highest whole-plant measurement, but again not statistically different from the other variants. On day 14, WW-EBs presented a higher value than WW-Bs and similar to WW-C. This indicates that the entrapped biostimulant influenced plant growth. Under WD conditions, on day 1, all experimental variants had similar values. From days 1 to 8, all the variants showed an increase in plant length. WD-EBs and WD-Bs presented higher values than WD-C. From days 12 to 14, there was a slight decrease in WD-EBs. On day 14, WD-C presented a length value closer to that of WD-EBs, and WD-Bs showed the highest value for the plant, but no statistical difference was determined. EBs protected the plant from drought, allowing its growth, but further applications might have been needed to ensure a constant protection.

3.5.5. Proline

In the fifth assay, Pro content was measured in plant leaves harvested on days 1, 4, 8, 12, and 14, and the results are presented in Figure 21. Under WW conditions, Pro content in leaves showed wide variation, without any clear trend. Analysing the data from days 1 and 14, Pro content varied from around 1.0 to 1.90 and 1.0 to 2.0 μmoles.g^{-1} FW in WW-C and WW-EBs, respectively. This variation suggested that Pro synthesis in the leaves occurred randomly and might not be directly linked to drought stress. At the same time, under WD conditions (Figure 21B) and on days 1, 8, and 14, Pro content was more or less constant in the control (WD-C) and experimental variants. This confirms that the elicitation of stress tolerance occurred in plant roots and was not linked with the mobilisation of plant defence mechanisms against drought stress at the leaf level. This might be linked to the protective role played by the biostimulants. An abnormal peak of variation in Pro content in WD-Bs on the first day was observed, as well as on day 12, where Pro content nearly doubled in all experimental variants. This latter variation in Pro content was challenging to explain and could potentially be a random occurrence resulting from an experimental error. These findings require further validation, perhaps through analysis of root metabolism under stress conditions. Overall, the statistical analysis did not show a significant difference ($p < 0.05$) in the variation in Pro content in the maize leaves between WW and WD conditions or among the experimental variants.

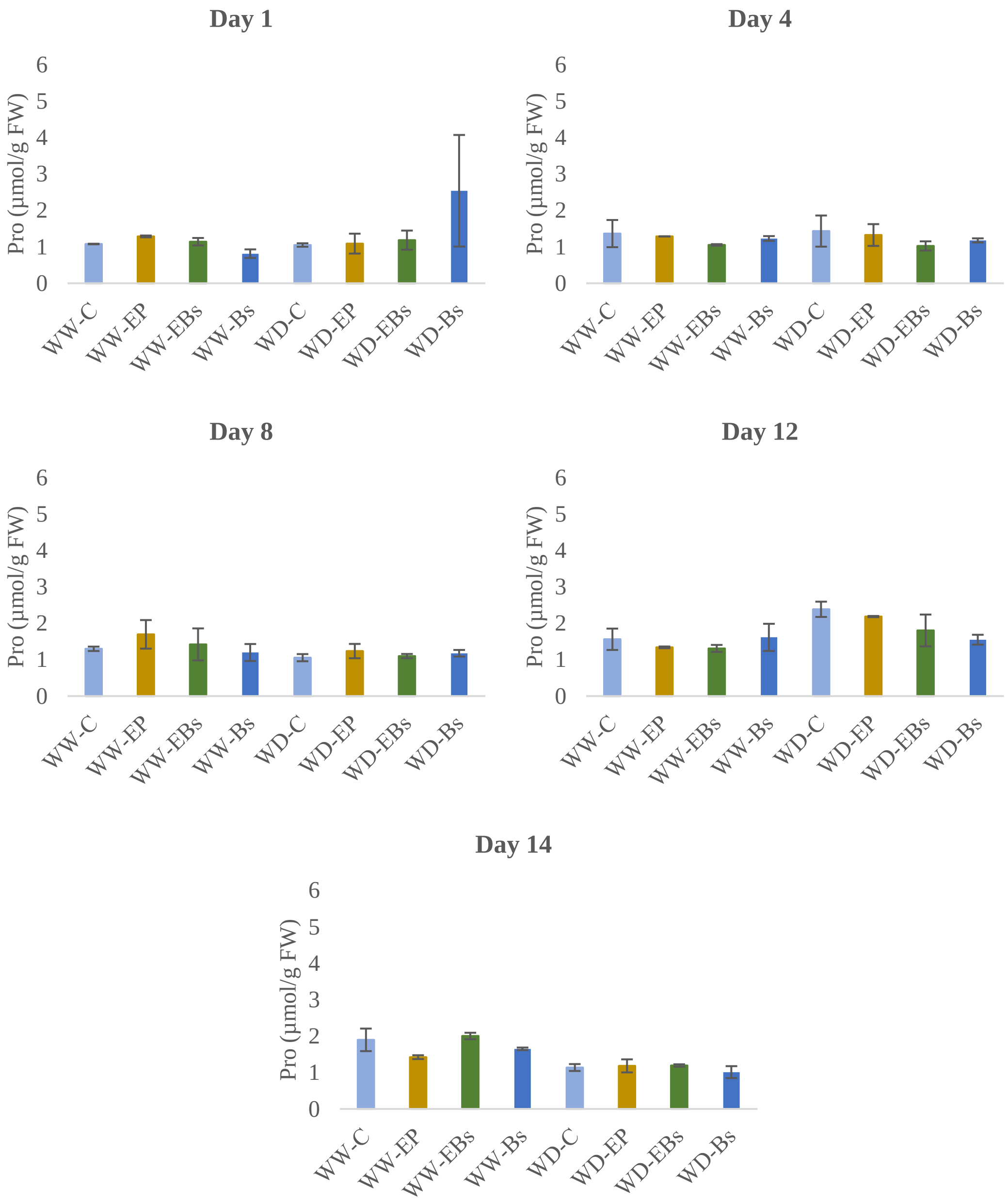


Figure 21. Proline (µmol/g FW) content under WW and WD conditions for all the experimental variants during the fifth assay. The results are expressed as the mean of Pro content per sample ± standard error. No significant differences were detected by One-Way ANOVA between the different treatments. Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

Behboudi *et al.* (78) showed that in *Triticum aestivum* cv. Pishtaz, Pro content remains low without the application of nanoparticles (control) in soil and in the absence of stress. Meanwhile, Bakhoum *et al.* (79) demonstrated that drought stress caused a significant increase in Pro content in lupine plants when chitosan was applied to the leaves.

3.5.6. Total soluble sugars

To understand if the drought stress targeted carbon fixation, and if the experimental variants increased this metabolic process, the total soluble sugars (TSS) were measured on days 1, 4, 8, 12, and 14, in maize roots and aerial parts (stems and leaves), in the fifth assay (Figures 22 and 23).

3.5.6.1. Root

Under WW conditions, TSS in the roots showed an intensive variation of the variants in the first 24 hours of the experience, with a following decrease in sugar content and its variation in the remaining days. On day 1, the value of WW-EBs was higher than in WW-C, and statistical different from the other variants. This result could have been determined by regular metabolic processes in the root and individual features of the plants, not determined by the application of the treatments.

Under WD conditions, a similar variation was detected in the first day of the experience, in which sugar content values are higher than in WW conditions, as well as in the remaining days. This variation might be attributed to the stress conditions triggering an active mobilisation of sugars in the experimental variants WD-EP, WD-EBs, and WD-Bs. However, this variation could also be influenced by the variability present in the maize samples analysed. On the first day, WD-EBs presented higher TSS content compared to WD-C, being the difference not statistically significant, but inferior to WD-EP. WD-Bs presented the lowest value of TSS content, being statistically different from the others experimental variants. WD-EP presented the highest value of sugar content, potentially due to the absence of protective effect and the active metabolization of sugars in response to stress conditions.

On day 8, TSS content showed a decrease in all the experimental variants, with a probable origin in the individual variability of the sampled plants. Although the sugar content is higher in WD-EBs (in relation to WD-C and slightly to WD-EP) and in WD-Bs (in relation to WD-C), and these differences are statistically significant, it is still lower than on day 4. Considering the variation of TSS on day 4, this indicates that EBs was protecting the roots from drought, and somehow affecting in a more moderate way sugar metabolism until the last day of the assay, maintaining the variation of sugars moderate in relation to WD-C, whereas these compounds should be actively metabolised to avoid the negative effects of stress. On day 14, the effect of Bs is greater than that of the other variants (except for C). Nevertheless, these findings need a deeper study and further validation.

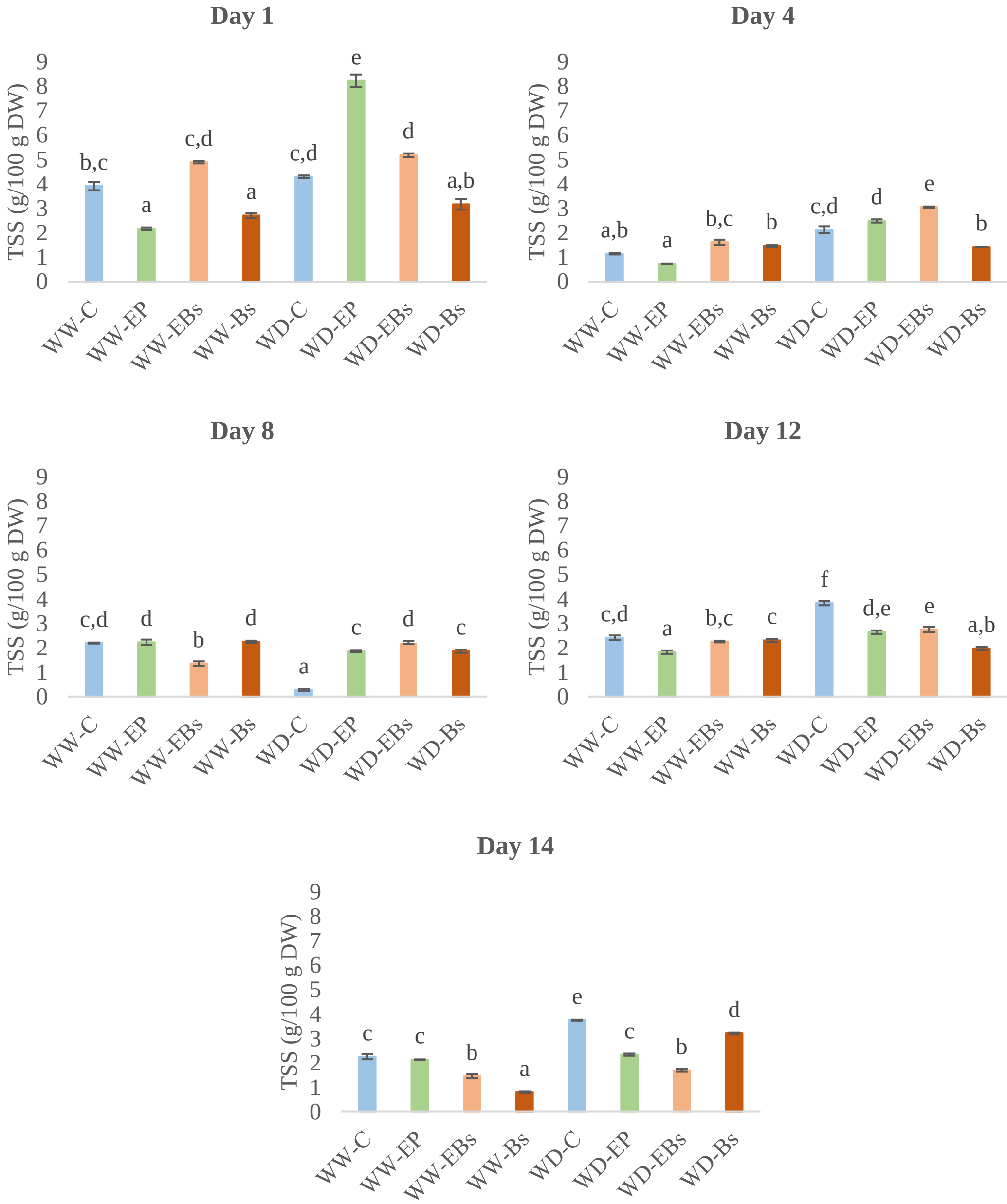


Figure 22. Total soluble sugars (TSS) (g/100 g DW) present at the root under WW and WD conditions for the experimental variants during the fifth assay. The results are expressed as the mean of TSS content per sample ± standard error. Different letters represent statistical significance by One-Way ANOVA ($p < 0.05$). Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

In the presence of drought stress, maize plants needed to actively use the carbohydrates reserved in the roots, increasing the flow of the soluble sugars to the root. It is important to mention that the relative strength of the source and sink regulates the rate of photosynthetic assimilation (80). Briefly, the plant vascular system is composed by the xylem, which is responsible for transporting water and nutrients from the roots to the shoots, and the phloem, which is responsible for transporting nutrients produced in the shoots to the other parts of the plant (81). Fully developed leaves are the source organs of sugars, responsible for assimilating carbon, which assimilates CO_2 from the atmosphere, converts it into sugars, and exports it to sink organs, such as roots and other vegetative organs, where they are stored or used (80,81). It was reported the role of nanoparticles in increasing soluble sugar content to reduce drought stress, in several plant species. However, their participation in sugar signalling during stress requires further study. Some nanoparticles are known to decrease plant growth by affecting cellular macromolecules, photosystems, and ROS accumulation (38).

An increased demand in sink organs leads to photosynthesis activation in source organs, whereas reduced sink strength causes sugars to accumulate in source organs. This causes downregulation of photosynthesis-related genes and photosynthetic rate, which shows that the carbon requirements from the sink organs also influence the activity of the source leaves. Therefore, a high positive correlation exists between the source strength (carbon assimilation and export) and sink strength (sugar import and usage). During drought, reduced soil water availability results in decreased water movement through the xylem, which leads to less water reaching the different organs, decreasing cell turgor pressure (influences stomatal behaviour and cell expansion). The decrease in water flow increases phloem viscosity, which reduces sugar transport (81). In this situation, the accumulation of osmolytes in the root can improve water absorption and uptake, and that can be achieved using metabolites to produce energy and sugar soluble accumulation.

3.5.6.2. Aerial part

The soluble sugar content in the aerial part of *Zea mays*, in the absence and presence of stress, was determined. Under WW conditions, the variation of TSS content in maize leaves between the first and last days occurred in a narrow range, despite some variability observed in some variants. The maximal value of WW-EBs was observed on day 12 of the assay. Although some variability was observed, these findings indicate that the experimental conditions do not promote or increase the concentration of soluble sugars in the source organ. Under WD conditions, the variation of TSS followed the same pattern of WW conditions, with slight differences across all experimental variants between days 1 and 8. On day 1, WD-C and WD-EBs presented sugar values around 1.59 and 2.07 g/100 g DW, respectively. From days 12 to 14, WD-C sugar content increased, ranging from 3.16 to 5.23 g/100 g DW. Sugar content remained below these values in WD-EBs, and the maximal value of TSS in WD-Bs reached around 4.0 g/100 g DW on day 14. These variations suggest that between days 1 and 8, drought stress did not notably influence sugar content. However, between days 12 and 14, there was an increase in sugar content in the leaves, likely in response to stress. A similar trend occurred in roots under stress conditions. Additionally, EBs and Bs appeared to have a moderate effect, maintaining the release of TSS below WD-C.

Exposure to drought stress often triggers plant defence responses, and the sugar reserved in the roots can be used to adapt to this situation. Many studies have demonstrated that water deficit induces an increase in the vegetative root-to-shoot ratio, a response that may be interpreted as the reallocation of sugars to roots. Hummel *et al.* suggested that water deficit may increase the assimilation of carbon in the leaves, which could be exported to the roots where is delipidated (37).

Other possible explanation for the increase in leaf TSS content is associated with plant growth, under moderate drought, which decreases before photosynthesis and leads to an excess of carbon skeletons that can be related to osmolytes production (82).

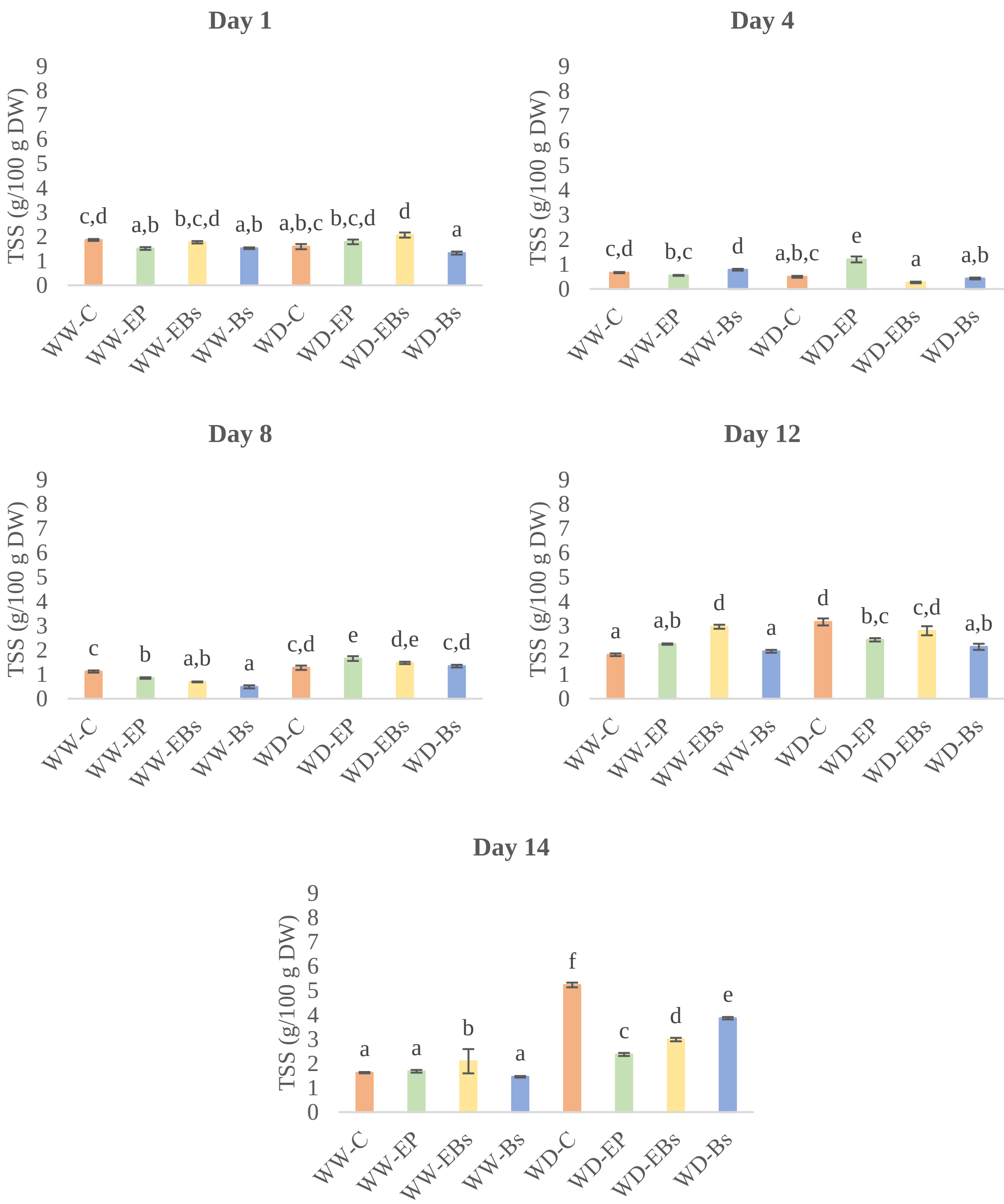


Figure 23. Total soluble sugars (TSS) (g/100 g DW) present at the aerial parts under WW and WD conditions for all the experimental variants during the fifth assay. In the absence of stress, more specifically, on day 4, EBs had negative absorbance values; therefore, it was not used for comparison. The results are expressed as the mean of TSS content per sample ± standard error. Different letters represent statistical significance by One-Way ANOVA ($p < 0.05$). Abbreviations: WW-C, well-watered control; WW-EP, well-watered empty particle; WW-EBs, well-watered entrapped biostimulant; WW-Bs, well-watered biostimulant; WD-C, water-deficit control; WD-EP, water-deficit empty particle; WD-EBs, water-deficit entrapped biostimulant; WD-Bs, water-deficit biostimulant.

3.6. Correlation between parameters

The existence of Pearson correlations between the different assay's parameters were analysed to understand their behaviour and to detect a possible understanding of the biostimulant action to cope with stress. The correlation varies between -1 and +1, where 0 indicates no correlation and 1 indicates a complete or perfect correlation, and the correlation strength increases from 0 to +1 and from 0 to -1 (83). According to De Winter (84), it will be considered a correlation of 0.2, moderate; 0.4, strong; and 0.8, very strong. Correlations with significant differences are described. These were present only in the fifth assay.

A significantly strong negative correlation, $r = -0.608$, $p < 0.05$, between Pro and length was detected in the aerial parts of the plant on day 4, independent of the absence or presence of stress (Appendix IV, Table A.3). Under drought conditions, Pro is synthesised in the shoots and roots through independent synthesis pathways. This amino acid can also be transported to the root through the phloem by Pro porters as there are high levels of its development in the phloem (33). The increase in Pro in the roots determines its decrease in the aerial parts, which can explain this negative correlation. In addition, before significant changes in photosynthesis occur, there is a frequent reduction in the growth of the aerial part. For example, in grapevines, shoot length is significantly reduced even before significant changes in leaf water potential are observed, which accentuates growth sensitivity to water deficit. In multiple species, shoot growth is more affected than root growth, as observed by increased root-to-shoot ratios during drought stress, whereas in other species, root growth is more affected than shoot growth (81). In addition, a significantly very strong negative correlation, $r = -0.810$, $p < 0.05$, was detected between Pro and length in the aerial parts, on day 4, in the absence of stress (Appendix IV, Table A.4). Under WW conditions, plants do not need to decrease their aerial part length, and Pro does not need to accumulate in plant tissues.

A significantly strong positive correlation, $r = 0.521$, $p < 0.05$, was observed between Pro and TSS in the roots and on day 12 in the absence or presence of stress (Appendix IV, Table A.5). Accumulation of proline and soluble sugars is related to plant response to drought and interactions between their signalling pathways have been suggested. A lower concentration of hexoses could slow carbon flow through glycolysis, decreasing the supply of pyruvate to the tricarboxylic acid cycle with a reduction in glutamate production and, consequently, proline biosynthesis. Another possible alternative is that reduced hexose availability can reduce NADPH production in the cytosol via the oxidative pentose phosphate pathway (induced earlier by osmotic stress), which in turn affects proline production. Regarding the catalytic function of P5CS1, the reduced capacity to accumulate soluble sugars may depend on the reduced ability of the *p5cs1* mutant to generate proline and regenerate $NADP^+$. A reduction in $NADP^+$ regeneration could overload the photosynthetic electron transport chain and affect the soluble sugars production (82). Another effect is the increase in ROS production needed to activate plant defence mechanisms against drought. Thus, a significantly strong negative correlation, $r = -0.722$, $p < 0.05$, between Pro and TSS was detected on day 12 in the aerial parts and in the presence of stress (Appendix IV, Table A.6).

In the roots, a significantly strong negative correlation, $r = -0.414$, $p < 0.05$, was detected between dry biomass weight and length, as well as a significantly strong negative correlation, $r = -0.677$, $p < 0.05$, between TSS and Pro, on day 14, independent of the absence or presence of stress (Appendix IV, Table A.7). Eziz *et al.* (85) determined that drought increased, on average, root mass fraction, but decreased stem mass fraction, leaf mass fraction, and reproductive mass fraction. When subjected to stress, plants tend to elongate their roots to obtain more water, which leads to a decrease in shoot length and consequently, plant biomass. In addition, sugars are stored in roots, under stress conditions, to increase their length. More specifically, in the presence of stress on day 14, a significantly strong negative correlation, $r = -0.584$, $p < 0.05$, between dry biomass weight and length was detected (Appendix IV, Table A.8).

In the aerial parts, a significantly strong negative correlation, $r = -0.502$, $p < 0.05$, between Pro and TSS was detected on day 14, independent of the absence or presence of stress, as well as a significantly moderate negative correlation, $r = -0.350$, $p < 0.05$, between length and Pro (Appendix IV, Table A.9). In the absence of stress, on day 14, a significantly strong positive correlation, $r = 0.834$, $p < 0.05$, between Pro and TSS was detected (Appendix IV, Table A.10). In the presence of stress, on day 14 (Appendix IV, Table A.11), a significantly moderate positive correlation, $r = 0.329$, $p < 0.05$, was detected between dry biomass weight and Pro. According to the literature, under stress conditions, Pro accumulates in the roots to protect them from drought and allow their growth, which consequently decreases shoot biomass. Lum *et al*. (86) determined a positive correlation between Pro and dry matter.

In the whole-plant, a significantly moderate negative correlation, $r = -0.359$, $p < 0.05$, between dry biomass and length was detected on day 14, independent of the absence or presence of stress (Appendix IV, Table A.12). In the case of drought, even though there is a decrease in shoot length, the same does not happen for root length, which leads to a consequent decrease in biomass. However, Lum *et al*. (86) reported a positive correlation between dry matter and shoot length, as well as between dry matter and root length. In the presence of stress, on the same day (Appendix IV, Table A.13), a significantly strong negative correlation, $r = -0.547$, $p < 0.05$, was observed between length and Pro. However, Lum *et al*. (86) showed a positive correlation between Pro, and root and shoot lengths. It was also suggested by the authors that, under drought conditions, Pro accumulation suggests that it might act as a compatible solute that regulates and decreases water loss and supplies energy for the survival and growth of plants.

CHAPTER IV.

CONCLUSION AND FUTURE PERSPECTIVES

This study began with the modulation of the suitable conditions for the assays. A total of five assays were conducted to evaluate the efficacy of biostimulants and entrapped biostimulants in mitigating drought stress in plants. The fourth and fifth assays were designed to assess the impact of biostimulants under drought stress conditions, where parameters such as whole-plant dry biomass weight, root, aerial part and whole-plant lengths, root and aerial part stress index, Pro, and TSS in roots and aerial parts were analysed.

Soil water content measurements were conducted to verify whether the daily quantities of the nutrient solution calculated and applied to the plants were the most appropriate. Stress conditions were verified for the fourth and fifth assays and in most of the experimental variants. The preparation of chitosan nanoparticles aimed to verify the efficiency of the methodology and ensure the successful acquisition of the nanoparticles. DLS analysis confirmed that the particles used in the assays were within the size range of chitosan nanoparticles. Peat analysis revealed a reduction in the number of bacterial and fungal colonies at dilutions of 1:10 and 1:100, without interference in the results of the fifth assay regarding biostimulant uptake. Bacterial CFUs were in accordance with the expected range, except for WW-EP in the fourth assay, and fungal CFUs were also in accordance with the expected range, except for WW-C and WW-EBs in the fourth assay, and WW-EBs and WW-Bs in the fifth assay. Bacterial colonies exhibited regular and irregular shapes with different shades of beige, along with filamentous structures. Fungi colonies presented greater diversity, with regular yellowish-beige shapes with reddish-pink centres, filamentous growth, as well as regular or irregular shapes in various shades of beige, greenish, and white. The initial peat (collected directly from the bag) and WW controls of the fourth and fifth assays were also compared. Fungal community was similar to the initial peat, which did not happen with the bacterial community.

For the whole-plant biomass dry weight in the fourth assay, EBs and Bs presented a minimum decrease at the end of the experiment, indicating that the nanoparticles succeeded in delivering the biostimulant and also coping with stress. Pro levels were assessed to verify its increase in the presence of stress. However, in the fourth assay, no significant differences in Pro content were observed among the experimental variants in the absence or presence of stress. In the presence of stress, experimental variants showed higher root length compared to the control, and the biostimulant was more efficient than EBs. The same happened for the aerial parts under WD conditions. Comparing the root and aerial part lengths, it was possible to conclude that EBs and Bs presented a protective effect, visible through root elongation, and that the aerial part was not affected by them, or its development was slower. In both organs, there was some sensitivity, which was visible in the control and empty particles. Regarding the whole-plant length, the biostimulant showed the highest values in the presence of stress. EBs protected the plant, but reapplication would be necessary. In the fifth assay, Pro content remained relatively stable across both the control and experimental variants under stress conditions. In TSS analysis, the entrapped biostimulant achieved the highest value on day 12. Generally, the experimental variants did not promote or increase sugar content in the aerial parts.

The main correlations detected were: between Pro and length, in which Pro is transported and accumulated in the roots under stress conditions, which leads to a decrease of shoot length; Pro and TSS, in which both sugars and Pro, though produced in the leaves, are transported and accumulated in the roots during stress conditions; biomass and length, in which stress leads to an elongation of the roots, leading to a decrease in shoot length and consequently decreases the biomass; and biomass and Pro, in which Pro is, under stress, accumulated in the roots and participates in metabolic processes, leading to root growth and a subsequent decrease in biomass, as previously described.

One solution could be to increase the stress applied from 50 % to 25 % in water-deficit irrigation. The number of samples was restricted by the size of the climatic chamber used. This affected the results expressed in this dissertation, in the fact that the number was different for the different treatments, which affected the deviations. The genetic variability of the regional variety also affected the deviations. After stabilisation of the results obtained for future assays in the climatic chamber, it would be important to start with greenhouse studies and then field studies to observe how maize behaves in the environment. Regarding chitosan nanoparticles, it would have been important to observe their shape, as it might have affected the delivery of pyroglutamic acid. For this reason, the term “entrapped” was used instead of “encapsulated”. An encapsulation efficiency test could have been done as well. More studies are needed on the utilisation of chitosan as a nanoparticle with pyroglutamic acid entrapped. At last, the regional variety used, ‘Milho de Santana’, showed how much variable it was, and is important to investigate more about it, as well as the other varieties found in Madeira, to increase our knowledge about them and explain such variability. A commercial variety could have been used for comparison to the regional variety because of its low genetic variability.

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APPENDIX

Appendix I: Assay's schematization

100 %
B

	Bs5	Bs4	Bs3		Bs2	Bs1
EBs7	EBs6	EBs5	EBs4	EBs3	EBs2	EBs1
EP7	EP6	EP5	EP4	EP3	EP2	EP1
	C4	C3		C2	C1	

100 %
A

C1			C2	C3		C4
EP1	EP2			EP3	EP4	EP5
EBs1	EBs2	EBs3	EBs4	EBs5	EBs6	EBs7
Bs1	Bs2	Bs3		Bs4	Bs5	Bs6

50 %
B

Bs4	Bs3				Bs2	Bs1
EBs4		EBs3			EBs2	EBs1
EP6	EP5	EP4	EP3	EP2		EP1
C7	C6	C5	C4	C3	C2	C1

50 %
A

	C1		C2	C3	C4	C5
EP1	EP2	EP3	EP4	EP5	EP6	EP7
EBs1	EBs2	EBs3		EBs4	EBs5	
	Bs1	Bs2	Bs3	Bs4	Bs5	Bs6

Figure A.1. Layout of the first assay, with four 28-cell seedling trays and the application of treatments (C, EP, EBs, and Bs) under WW (100 % irrigation) and WD (50% irrigation). Legend: Cx, control; EPx, empty particle; EBsx, entrapped biostimulant; Bsx, biostimulant; x is a number given to a plant; "black" means that there was no germination; "grey" means an outlier.

50 %
C

Bs7	Bs6	Bs5	Bs4	Bs3	Bs2	Bs1
EBs6	EBs5	EBs4	EBs3		EBs2	EBs1
EP6	EP5		EP4	EP3	EP2	EP1
C6	C5	C4	C3	C2	C1	

100 %
C

C1	C2	C3				
EP1	EP2		EP3	EP4	EP5	EP6
EBs1		EBs2	EBs3	EBs4		EBs5
Bs1	Bs2	Bs3	Bs4	Bs5	Bs6	

100 %
B

Bs7	Bs6	Bs5	Bs4	Bs3	Bs2	Bs1
EBs7	EBs6	EBs5	EBs4	EBs3	EBs2	EBs1
EP6		EP5	EP4	EP3	EP2	EP1
C6	C5	C4	C3		C2	C1

50 %
B

C1	C2		C3	C4	C5	C6
EP1	EP2	EP3	EP4		EP5	EP6
EBs1	EBs2	EBs3		EBs4	EBs5	
Bs1	Bs2	Bs3	Bs4			Bs5

100 %
A

Bs7	Bs6	Bs5	Bs4	Bs3	Bs2	Bs1
EBs5		EBs4		EBs3	EBs2	EBs1
EP7	EP6	EP5	EP4	EP3	EP2	EP1
C7	C6	C5	C4	C3	C2	C1

50 %
A

	C1	C2	C3	C4	C5	C6
EP1	EP2	EP3	EP4	EP5	EP6	EP7
EBs1	EBs2	EBs3	EBs4	EBs5	EBs6	EBs7
Bs1	Bs2	Bs3	Bs4	Bs5	Bs6	Bs7

Figure A.2. Layout of the second assay, with six 28-cell seedling trays and one 40-cell seedling tray, and the application of treatments (C, EP, EBs, and Bs) under WW (100 % irrigation) and WD (50% irrigation). The 40-cell seedling tray was used only to assess maize development in smaller seedling cells. Legend: Cx, control; EPx, empty particle; EBsx, entrapped biostimulant; Bsx, biostimulant; x is a number given to a plant; "black" means that there was no germination; "grey" means an outlier.

50 %

				Bs10U	Bs10T	
Bs5Y	Bs5X	Bs5W	Bs5V	Bs5U	Bs5T	Bs5S
	Bs3W		Bs3V	Bs3U	Bs3T	Bs3S
C27	C26	C25	C24	C23	C22	C21

50 %

C8	C9	C10	C11	C12		C14
Bs3G	Bs3H	Bs3I	Bs3J	Bs3K		Bs3M
Bs5G	Bs5H	Bs5I	Bs5J	Bs5K	Bs5L	
Bs10H	Bs10I	Bs10J	Bs10K	Bs10L	Bs10M	Bs10N

50 %

Bs10S	Bs10R		Bs10Q	Bs10P	Bs10O	Bs10N
Bs5R	Bs5Q	Bs5P		Bs5O	Bs5N	Bs5M
Bs3R	Bs3Q		Bs3P	Bs3O	Bs3N	
C20	C19	C18		C17	C16	C15

50 %

C1	C2	C3	C4	C5	C6	C7
Bs3A	Bs3B	Bs3C		Bs3D	Bs3E	Bs3F
Bs5A		Bs5B	Bs5C	Bs5D	Bs5E	Bs5F
Bs10A	Bs10B	Bs10C	Bs10D	Bs10E	Bs10F	Bs10G

100 %

C39	C38	C37	C36	C35	C34	C33	C32
	C31	C30	C29	C28	C27	C26	C25
C24	C23	C22	C21	C20	C19	C18	C17
C16	C15	C14	C13	C12	C11	C10	C9
C8	C7	C6	C5	C4	C3	C2	C1

100 %

C1		C2		C3	C4	
C5	C6	C7	C8	C9	C10	C11
C12		C13	C14	C15	C16	C17
C18	C19	C20			C21	C22

Figure A.3. Layout of the third assay, with five 28-cell seedling trays and one 40-cell seedling tray, and the application of treatments (C and Bs) under WW (100 % irrigation) and WD (50% irrigation). Three different concentrations of Bs were evaluated: 3, 5, and 10 mL. Legend: Cx, control; Bsx, biostimulant; x is a number given to a plant; “black” means that there was no germination; “grey” means an outlier.

50 %
B

C13	C12	C11	C10		C9	C8	
Bs8	Bs7	Bs6	Bs5	Bs4	Bs3	Bs2	Bs1
EBs8	EBs7	EBs6	EBs5	EBs4		EBs2	EBs1
EP8	EP7	EP6	EP5	EP4	EP3	EP2	EP1
C7	C6	C5	C4	C3	C2		C1

100 %
A

C1		C2	C3	C4	C5	C6	C7
EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8
EBs1		EBs2	EBs3	EBs4	EBs5	EBs6	EBs7
Bs1	Bs2	Bs3	Bs4		Bs5	Bs6	Bs7
C8	C9	C10	C11		C12	C13	C14

100 %
B

	C14		C13	C12	C11	C10	C9
		Bs6	Bs5	Bs4	Bs3	Bs2	Bs1
	EBs7	EBs6	EBs5	EBs4	EBs3	EBs2	EBs1
EP7	EP6	EP5	EP4	EP3	EP2		EP1
C8	C7	C6	C5	C4	C3	C2	C1

50 %
C

C1	C2	C3		C4	C5	C6	C7
EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8
	EBs1	EBs2	EBs3	EBs4	EBs5	EBs6	EBs7
Bs1	Bs2	Bs3	Bs4	Bs5		Bs6	Bs7
C8	C9	C10	C11	C12	C13		C14

50 %
A

C16	C15	C14	C13	C12	C11	C10	C9
Bs7	Bs6	Bs5		Bs4	Bs3	Bs2	Bs1
	EBs5	EBs4	EBs3	EBs2			EBs1
EP7	EP6	EP5	EP4	EP3	EP2		EP1
C8	C7	C6	C5	C4	C3	C2	C1

Figure A.4. Layout of the fourth assay, with five 40-cell seedling trays, and the application of treatments (C, EP, EBs, and Bs) under WW (100 % irrigation) and WD (50% irrigation). Legend: Cx, control; EPx, empty particle; EBsx, entrapped biostimulant; Bsx, biostimulant; x is a number given to a plant; "black" means that there was no germination; "grey" means an outlier.

100 %
C

					Bs3		
	EBs7	EBs6					
				EP4			
					C3		

50 %
C

		C3					
		EP3					
			EBs4				
		Bs3					
/	/	/	/	/	/		/

50 %
B

		Bs14	Bs13	Bs12	Bs11	Bs10	
	Bs7						
			EBs5				
			EP5				
		C6					

100 %
D

				C5	C6	C7	
						EP7	
				EBs5			
				Bs5			
			Bs12				

100 %
B

				EBs12	EBs11	EBs10	EBs9
			Bs5				
	EBs7						
				EP4			
			C5				

50 %
D

					C6		
						EP7	
				EBs5			
				Bs5	Bs6		
	EBs10	EBs11	EBs12	EBs13	EBs14	EBs15	

50 %
A

	EP15	EP14	EP13	EP12	EP11	EP10	EP9
		Bs6					
			EBs5				
		EP6					
			C5				

100 %
E

				C5	C6		
				EBs5		EBs7	
					Bs6		
EP9	EP10	EP11	EP12				

100 %
A

	C15	C14	C13	C12	C11		
			Bs5				
		EBs6		EBs4			
				EP4			
	C7						

50 %
E

					C6		
							EP8
				Bs5		Bs7	
	C10		C12	C13	C14		

Figure A.5. Layout of the fifth assay, with ten 40-cell seedling trays, and the application of treatments (C, EP, EBs, and Bs) under WW (100 % irrigation) and WD (50% irrigation). This layout shows the number of samples obtained in the last day of the fifth assay. Legend: Cx, control; EPx, empty particle; EBsx, entrapped biostimulant; Bsx, biostimulant; x is a number given to a plant; "black" means that there was no germination; "grey" means an outlier.

Appendix II: Application of treatments and Hoagland's nutrient solution

Table A.1. Quantities applied of the treatments and Hoagland's nutrient solution in the fourth assay. Abbreviations: C, control; EP, empty particle; EBs, entrapped biostimulant; Bs, biostimulant; NS, Hoagland's nutrient solution.

Day	Date	Treatments								Notes
		C		EP		EBs		Bs		
		100 %	50 %	100 %	50 %	100 %	50 %	100 %	50 %	
1	01/03/2023	10 mL NS	5 mL NS	3 mL EP + 7 mL NS	3 mL EP + 2 mL NS	3 mL EBs + 7 mL NS	3 mL EBs + 2 mL NS	3 mL Bs + 7 mL NS	3 mL Bs + 2 mL NS	-
2	02/03/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	-
3	03/03/2023	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	-
4	04/03/2023	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	Double irrigated.
5	05/03/2023	-	-	-	-	-	-	-	-	-
6	06/03/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	-
7	07/03/2023	7 mL NS	3,5 mL NS	7 mL NS	3,5 mL NS	7 mL NS	3,5 mL NS	7 mL NS	3,5 mL NS	-
8	08/03/2023	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	-
9	09/03/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	-
10	10/03/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	-
11	11/03/2023	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	Double irrigated.
12	12/03/2023	-	-	-	-	-	-	-	-	-
13	13/03/2023	15 mL NS	7,5 mL NS	15 mL NS	7,5 mL NS	15 mL NS	7,5 mL NS	15 mL NS	7,5 mL NS	-
14	14/03/2023	-	-	-	-	-	-	-	-	-

Table A.2. Quantities applied of the treatments and Hoagland's nutrient solution in the fifth assay. Abbreviations: C, control; EP, empty particle; EBs, entrapped biostimulant; Bs, biostimulant; NS, Hoagland's nutrient solution; Pro, proline.

Day	**Date**	**Treatments**								**Notes**
		C		**EP**		**EBs**		**Bs**		
		100 %	**50 %**	**100 %**	**50 %**	**100 %**	**50 %**	**100 %**	**50 %**	
1	13/04/2023	10 mL NS	5 mL NS	3 mL EP + 7 mL NS	3 mL EP + 2 mL NS	3 mL EBs + 7 mL NS	3 mL EBs + 2 mL NS	3 mL Bs + 7 mL NS	3 mL Bs + 2 mL NS	Material collected: 8 plants for Pro, and 16 for other parameters.
2	14/04/2023	-	-	-	-	-	-	-	-	-
3	15/04/2023	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	Double irrigated.
4	16/04/2023	-	-	-	-	-	-	-	-	-
5	17/04/2023	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	Material collected: 16 plants for Pro, and 32 for other parameters.
6	18/04/2023	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	5 mL NS	2,5 mL NS	-
7	19/04/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	-
8	20/04/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	Material collected: 16 plants for Pro, and 32 for other parameters.
9	21/04/2023	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	10 mL NS	5 mL NS	-
10	22/04/2023	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	20 mL NS	10 mL NS	Double irrigated.
11	23/04/2023	-	-	-	-	-	-	-	-	-
12	24/04/2023	30 mL NS	15 mL NS	30 mL NS	15 mL NS	30 mL NS	15 mL NS	30 mL NS	15 mL NS	Material collected: 16 plants for Pro, and 32 for other parameters; double irrigated.
13	25/04/2023	-	-	-	-	-	-	-	-	-
14	26/04/2023	-	-	-	-	-	-	-	-	Material collected: 16 plants for Pro, and 32 for other parameters.

Appendix III: Standard curves

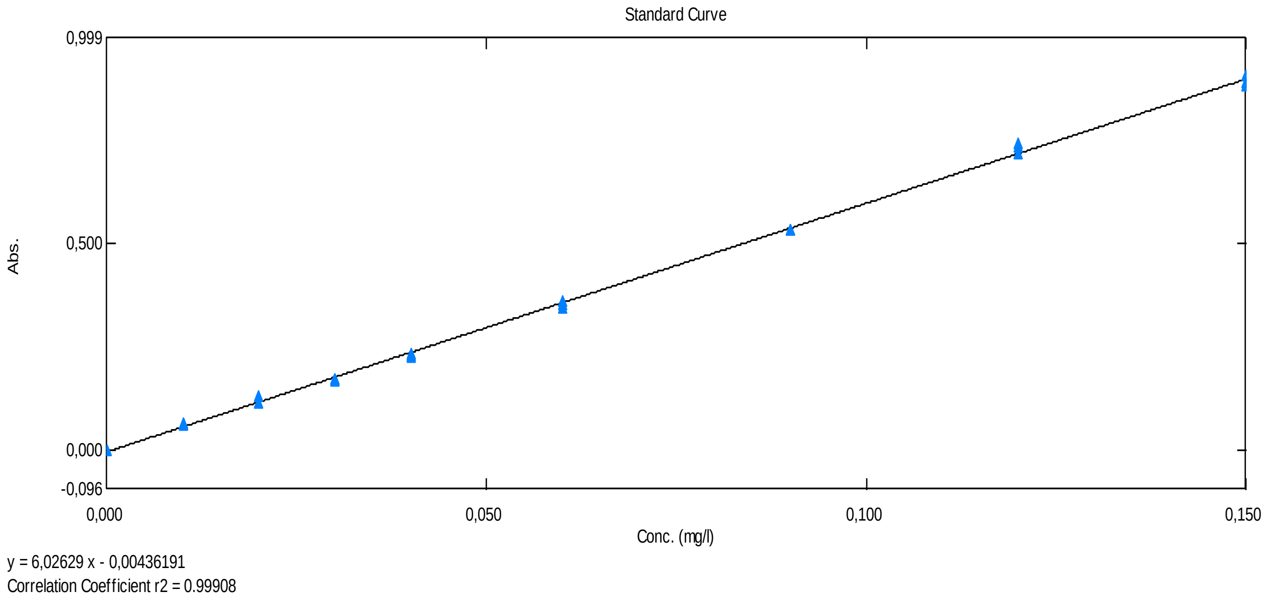

Figure A.6. Standard curve of proline with the equation y = 6.02629 x – 0.00436191 and a correlation coefficient (R^2) of 0.99908.

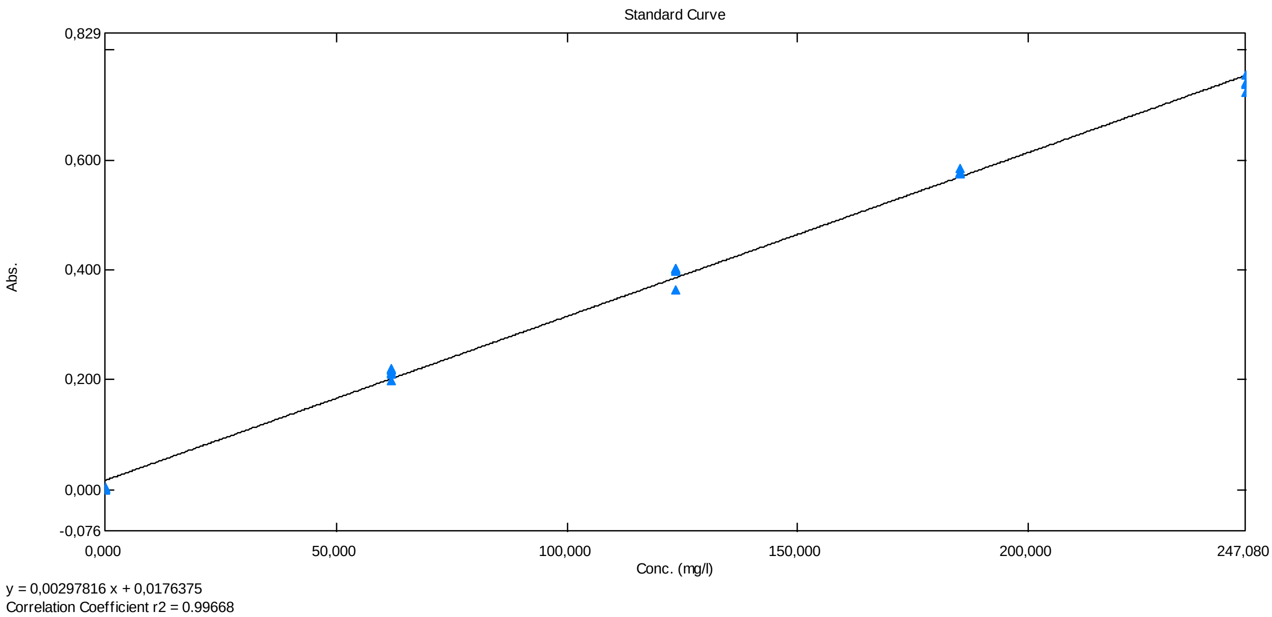

Figure A.7. Standard curve of total soluble sugars with the equation y = 0.00297816 x + 0.0176375 and a correlation coefficient (R^2) of 0.99668.

Appendix IV: Parameters correlation

Table A.3. Pearson correlation between Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts, independent of the presence or absence of stress, on day 4. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Aerial parts Day 4 (general)		Pro (µmol/g FW)	TSS (g/100 g DW)	Length (cm)
Pro (µmol/g FW)	Pearson correlation Sig.		0.093 0.752	-0.608* 0.012
TSS (g/100 g DW)	Pearson correlation Sig.	0.093 0.752		-0.110 0.576
Length (cm)	Pearson correlation Sig.	-0.608* 0.012	-0.110 0.576	

Table A.4. Pearson correlation between Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts in the absence of stress on day 4. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Aerial parts Day 4 (no stress)		Pro (µmol/g FW)	TSS (g/100 g DW)	Length (cm)
Pro (µmol/g FW)	Pearson correlation Sig.		-0.183 0.729	-0.810* 0.015
TSS (g/100 g DW)	Pearson correlation Sig.	-0.183 0.729		-0.320 0.311
Length (cm)	Pearson correlation Sig.	-0.810* 0.015	-0.320 0.311	

Table A.5. Pearson correlation between Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the roots, independent of the presence or absence of stress, on day 12. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Roots Day 12 (General)		Pro (µmol/g FW)	TSS (g/100 g DW)	Length (cm)
Pro (µmol/g FW)	Pearson correlation Sig.		0.521* 0.038	0.123 0.649
TSS (g/100 g DW)	Pearson correlation Sig.	0.521* 0.038		0.025 0.894
Length (cm)	Pearson correlation Sig.	0.123 0.649	0.025 0.894	

Table A.6. Pearson correlation between Pro (μmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts in the presence of stress on day 12. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Aerial parts **Day 12 (stress)**		Pro (μmol/g FW)	TSS (g/100 g DW)	Length (cm)
Pro (μmol/g FW)	Pearson correlation Sig.		-0.722* 0.0.43	0.411 0.312
TSS (g/100 g DW)	Pearson correlation Sig.	-0.722* 0.0.43		0.075 0.783
Length (cm)	Pearson correlation Sig.	0.411 0.312	0.075 0.783	

Table A.7. Pearson correlation between biomass (g), Pro (μmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the roots, independent of the presence or absence of stress, on day 14. Asterisks represent statistical significance (* $p < 0.05$; ** $p < 0.01$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Roots **Day 14 (General)**		Biomass (g)	Pro (μmol/g FW)	TSS (g/100 g DW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		0.373 0.155	-0.045 0.708	-0.414* 0.018
Pro (μmol/g FW)	Pearson correlation Sig.	0.373 0.155		-0.677** 0.004	-0.200 0.458
TSS (g/100 g DW)	Pearson correlation Sig.	-0.045 0.708	-0.677** 0.004		0.088 0.633
Length (cm)	Pearson correlation Sig.	-0.414* 0.018	-0.200 0.458	0.088 0.633	

Table A.8. Pearson correlation between biomass (g), Pro (μmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the roots in the presence of stress on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Roots **Day 14 (Stress)**		Biomass (g)	Pro (μmol/g FW)	TSS (g/100 g DW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		-0.006 0.988	0.248 0.138	-0.584* 0.018
Pro (μmol/g FW)	Pearson correlation Sig.	-0.006 0.988		-0.243 0.562	0.335 0.417
TSS (g/100 g DW)	Pearson correlation Sig.	0.248 0.138	-0.243 0.562		-0.129 0.634
Length (cm)	Pearson correlation Sig.	-0.584* 0.018	0.335 0.417	-0.129 0.634	

Table A.9. Pearson correlation between biomass (g), Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts, independent of the presence or absence of stress, on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Aerial parts Day 14 (General)		Biomass (g)	Pro (µmol/g FW)	TSS (g/100 g DW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		-0.140 0.242	0.373 0.155	-0.111 0.546
Pro (µmol/g FW)	Pearson correlation Sig.	-0.140 0.242		-0.502* 0.047	-0.350* 0.049
TSS (g/100 g DW)	Pearson correlation Sig.	0.373 0.155	-0.502* 0.047		-0.258 0.335
Length (cm)	Pearson correlation Sig.	-0.111 0.546	-0.350* 0.049	-0.258 0.335	

Table A.10. Pearson correlation between biomass (g), Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts in the absence of stress on day 14. Asterisks represent statistical significance ($p < 0.01$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Aerial parts Day 14 (No stress)		Biomass (g)	Pro (µmol/g FW)	TSS (g/100 g DW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		-0.122 0.492	0.111 0.793	-0.313 0.239
Pro (µmol/g FW)	Pearson correlation Sig.	-0.122 0.492		0.834** 0.010	0.065 0.810
TSS (g/100 g DW)	Pearson correlation Sig.	0.111 0.793	0.834** 0.010		-0.456 0.257
Length (cm)	Pearson correlation Sig.	-0.313 0.239	0.065 0.810	-0.456 0.257	

Table A.11. Pearson correlation between biomass (g), Pro (µmol/g FW), TSS (g/100 g DW), and length (cm) in the fifth assay for the aerial parts in the presence of stress on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline; TSS, total soluble sugars.

Aerial parts Day 14 (Stress)		Biomass (g)	Pro (µmol/g FW)	TSS (g/100 g DW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		0.329* 0.044	-0.006 0.988	-0.242 0.366
Pro (µmol/g FW)	Pearson correlation Sig.	0.329* 0.044		-0.142 0.738	-0.107 0.692
TSS (g/100 g DW)	Pearson correlation Sig.	-0.006 0.988	-0.142 0.738		0.106 0.802
Length (cm)	Pearson correlation Sig.	-0.242 0.366	-0.107 0.692	0.106 0.802	

Table A.12. Pearson correlation between biomass (g), Pro (µmol/g FW), and length (cm) in the fifth assay for the whole-plant, independent of the presence or absence of stress, on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline.

Whole-plant Day 14 (General)		Biomass (g)	Pro (µmol/g FW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		0.373 0.155	-0.359* 0.043
Pro (µmol/g FW)	Pearson correlation Sig.	0.373 0.155		0.045 0.868
Length (cm)	Pearson correlation Sig.	-0.359* 0.043	0.045 0.868	

Table A.13. Pearson correlation between biomass (g), Pro (µmol/g FW), and length (cm) in the fifth assay for the whole-plant in the presence of stress on day 14. Asterisks represent statistical significance ($p < 0.05$). Abbreviations: Pro, proline.

Whole-plant Day 14 (Stress)		Biomass (g)	Pro (µmol/g FW)	Length (cm)
Biomass (g)	Pearson correlation Sig.		-0.006 0.988	-0.547* 0.028
Pro (µmol/g FW)	Pearson correlation Sig.	-0.006 0.988		0.268 0.522
Length (cm)	Pearson correlation Sig.	-0.547* 0.028	0.268 0.522	