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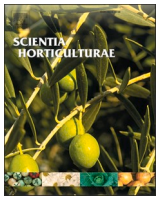

Research Paper

Field evaluation and characterization of a novel biostimulant for broccoli (*Brassica oleracea* var. *italica*) cultivation under drought and salt stress which increases antioxidant, glucosinolate and phytohormone content

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ABSTRACT

Anthropogenic global warming is affecting crop yield and thus compromising food security. Drought and salinization of the irrigation water or soil are increasingly frequent in most arable land, specifically in arid and semiarid areas such as the Mediterranean basin. Many crops have been displaced or their yield has plummeted in recent years, causing food shortages and price increases. In a context of global population growth and reduction in the availability of natural resources used in agriculture, finding novel tools that help farmers to maintain yield in an increasingly arid and saline scenario is of pivotal importance. This is particularly important in organic agriculture, where the number of available tools is very limited. Biostimulants are substances or microorganisms of natural origin whose function is to stimulate plant processes related to nutrient absorption, nutrient use efficiency, tolerance to abiotic stress or the quality of the agricultural products obtained. In the present work, we have evaluated, in field, the agronomic effectiveness of a novel biostimulant formulation in a cruciferous crop of great interest in the Mediterranean basin (broccoli) under control conditions, water and salt stress. Our research has shown that our product had a positive effect on broccoli production and in delaying flowering time. We have also found that the application of our biostimulant increased enzymatic and non-enzymatic antioxidant defense under drought conditions. Similarly, when exposed to salinity, the biostimulant increased the concentration of different phytohormones and glucosinolates. Taken together, our biostimulant increases the tolerance of broccoli to salt stress and water limitation by increasing the antioxidant response, the level of glucosinolates and eliciting the hormonal response.

1. Introduction

Climate change and the subsequent increase in aridity have become a major threat to natural and cultivated ecosystems. The increase in global temperatures and the alteration in the rainfall patterns is increasing soil desertification. Dry periods are longer, and droughts are becoming more frequent. In forests, this is driving the migration of species (Taïbi et al., 2014), while agriculture is facing a 50 % reduction in global food production and a shift towards crops that are better adapted to the current conditions. This aridification of agricultural soils is threatening the Mediterranean basin, particularly in the southeast region of the Iberian Peninsula, one of the main producers of horticultural crops in Europe. Considering altogether the negative effects of global warming on crop yield are expected to be dramatic (Millán 2014; Hussain et al., 2019).

Drought is one of the major constraints for crop yield. In response to this stress, plants activate different cellular mechanisms, which involve morphological and structural changes, expression of resistance genes, hormone synthesis or osmotic regulation to alleviate the effects of drought (Yang et al., 2021). Under drought conditions, plants experience a loss of turgor, which leads to a decrease in plant height due to reduced cell expansion, changes in the number and surface of leaves,

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Abbreviations

1-MeO-I3M	1-methoxyindole-3-methyl glucosinolate
3-PPG	3-phenylpropyl glucosinolate
4-MeO-I3M	4-methoxyindole-3-yl-methyl glucosinolate
4-OH-I3M	4-hydroxyindol-3-yl-methyl glucosinolate
8-MSO	8-methylsulfinyloctyl glucosinolate
ABA	Abscisic acid
APX	Ascorbate peroxidase
BS	Biostimulant
CAT	Catalase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CK	Cytokinins
DW	Dry weights
EU	European union
Fm	Maximum fluorescence
Fv	Variable fluorescence
FW	Fresh weight
GA	Gibberellins
GLU	glucosinolates
GR	Glutathione reductase
I3M	Indole-3-yl-methyl glucosinolate
IAA	Indole-3-acetic acid
IPR	Isopentenyladenosine
JA	Jasmonic acid
JA-Ile	Isoleucine JA conjugate
LC/ESI-QTOF-MS	Liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry
MDA	Malondialdehyde
OLD	Oxidative damage to lipids
OPDA	12-oxo-phytodienoic acid
PA	Phaseic acid
PK	Potassium phosphate
Pro	Proline
PSII	Photosystem II
PVPP	Polyvinylpolypyrrolidone
ROS	Reactive oxygen species
RWC	Relative water content
SA	Salicylic acid
SOD	Superoxide dismutase
Total Chl	Total chlorophyll
SPAD	Soil Plant Analysis Development
TFC	Total flavonoid content
TPC	Total phenolic compounds
TSS	Total soluble sugar
TW	Turgid weight
UPLC	Ultra Performance Liquid Chromatography
WUE	water use efficiency

and wilting (Gisbert et al., 2020). Under normal conditions, irrigation promotes root development (elongation, formation of secondary roots and increase in root hairs), but drought conditions block this developmental program. It also affects plants at the cellular, physiological and biochemical levels. The photosynthetic and transpiration rates decrease when the relative water content of the soil is reduced, leading to a subsequent decrease in biomass. As plant cells start to lose water, there is an increase in the biosynthesis of regulatory osmotic substances (osmolytes) to maintain the turgor pressure. Regulatory osmotic substances include organic molecules such as amino acids (proline), molecules derived from amino acids (glycine, betaine and polyamines), or sugars (mannitol, fructose or sucrose) or potassium (Cao et al., 2013). However, osmotic regulation also has limitations. Although it may improve stress resistance temporarily, it has a limited effect. If the stress is severe, the turgor pressure of the cell cannot be maintained, and the effects of drought will appear even when the correct range of osmotic adjustment of the water potential is reached (Szabados and Savouré, 2010; Osakabe et al., 2014).

Soil salinization is often aggravated by drought. Water shortage leads to overexploitation of water sources, and the lowering of the phreatic level next to coastal areas allows the entry of seawater. Additionally, extensive irrigation also causes the accumulation of salts in the soil. Salinity affects more than 1 billion hectares worldwide, of which 77 million hectares are used for agriculture. As a result of the effects of salinity, 1.5 million hectares are abandoned each year (Hussain et al., 2019). Salinity harm crops through various mechanisms: salt in the soil generates an osmotic effect, which is similar to drought, since high external ionic content increases osmotic potential and biophysically impedes water absorption through the roots. Once inside the plant, sodium becomes a toxic cation (Serrano et al., 1999) that competes with potassium, which is the major mineral nutrient (Mulet et al., 2023) . If the ratio Na^+/K^+ in the cytoplasm increases, it reduces the activity of enzymes participating in essential biochemical pathways, such as sulfate assimilation (Murguia et al., 1995), reduction of photosynthetic ratio, the elimination of antioxidants, and it also disrupts membranes. Reactive oxygen species (ROS) production is also higher under salinity conditions, especially oxygen (O^{2-}) (James et al., 2011), superoxide (OH^-) and hydrogen peroxide (H_2O_2) (Munns & Termaat, 1986) or free radicals (Taïbi et al., 2021) . These oxidants can disrupt the functions of various cellular compartments, in addition to causing damage to DNA, proteins and lipids and interfering with plant metabolism. In response to all these processes, the plant synthesizes phytohormones that govern the response against stress (Pedranzani et al., 2003; Yang et al., 2021) .

Phytohormones are small organic molecules that play a crucial role in the growth, development and reproduction of plants, as well as in tolerance to abiotic stress through the regulation of different cellular functions at the molecular level. Among the characterized hormones, abscisic acid (ABA), ethylene, salicylic acid (SA), jasmonic acid (JA), cytokinins (CK) and auxins improve tolerance to abiotic and biotic stresses (Verma et al., 2016). For example, JA and ABA participate in regulating stomatal closure in response to drought stress (Riemann et al., 2015), while auxins play a dynamic role in mediating and improving plant tolerance to non-infectious stresses (Kazan, 2013). On the other hand, SA also plays an essential role in signaling and defense responses against drought stress (Miura and Tada, 2014) .

As a result of this challenging panorama caused by climate change, there is a great demand for novel tools that help farmers to maintain yield under adverse environmental conditions. Biostimulants have been proposed as one of those tools. Biostimulants are substances or microorganisms whose function is to stimulate or enhance natural processes that improve the tolerance of plants to abiotic stress, generate an increase in nutrient absorption or improve some of the agronomic characteristics of the crop. Unlike fertilizers or pesticides, which aim to directly provide nutrients to plants or directly attack the pest, biostimulants stimulate plant nutrition or defense processes, regardless of the product's composition (du Jardin, 2015)

Currently, the use of biostimulants in agriculture is growing exponentially, especially since the approval of the EU regulation in July 2022 (Regulation (EU) n°2019/1009). In addition, farmers are demanding novel products suitable not only for conventional agriculture but for organic farming, since there is a very limited number of authorized inputs effective to maintain yield in organic agriculture, especially under the new conditions imposed by climate change. Biostimulants based on non-microbial formulations may play an important role in increasing the

tolerance of plants to abiotic stress by triggering numerous pathways and processes that increase the plant's ability to withstand unfavorable environmental conditions (du Jardin, 2015) .

Our laboratory has developed a new biostimulant (Calbio) using a methodology based on the evaluation in model organisms such as *Saccharomyces cerevisiae* and *Arabidopsis thaliana* under laboratory conditions Calbio is based on a combination of natural extracts (yeast extract, licorice root extract, willow root extract and Ascophyllum nodosum algae extract) and was selected from among more than 200 tested combinations (Benito et al., 2022). This biostimulant increased the growth and development of model organisms under conditions of salt or osmotic stress. In the present study, we chose to test this product in broccoli (*Brassica oleracea* var. *italica*) for several reasons. First, Spain is the main European broccoli producer, with a total cultivation area of 6392 ha (ESYRCE, 2022), almost all under artificial irrigation (Fig. 1). Second, the demand for broccoli is increasing due to the growing trend in vegetarianism and veganism, because of its low caloric content and its contribution to a balanced diet and because broccoli is a food rich in health promoting molecules, including some with a preventive action against cancer (Zaghdoud et al., 2016; Baladia et al., 2024). The third factor is the limited number of microbial inputs due to the biochemical particularities of broccoli's secondary metabolism. Broccoli is rich in glucosinolates, a family of secondary plant metabolites derived from amino acids and found almost exclusively in plants of the Brassicaceae family (Schreiner et al., 2011). Root exudates of brassicas are rich in glucosinolates, which become biologically active compounds upon hydrolyzation, such as isothiocyanates, which are very effective antimicrobial compounds. These molecules protect the plants from pathogens, but at the same time, inhibit the presence of beneficial microorganisms, such as arbuscular mycorrhizal fungi or plant growth-promoting bacteria. So, broccoli cultivation, especially under organic farming regimens, has fewer tools to maintain yield. The fourth reason to undertake this field study in broccoli is that the biostimulant used in this project was developed using *Arabidopsis thaliana* (Saporta et al., 2019; Benito et al., 2022), which is a standard model plant for molecular biology and, at the same time, is phylogenetically related to broccoli, as both are cruciferous plants. So, the main objective of the current work is to confirm whether the results observed under laboratory conditions could be translated into field applications, and once confirmed the effectiveness of our biostimulant in real field conditions, characterize its effect on broccoli under water and salt stress at the physiological and biochemical levels.

2. Material and methods

2.1. Plant material

The assays were conducted with broccoli (*Brassica oleracea* var. *italica*) cultivar Parthenon F1 (Sakata Ibérica S. L. U., Valencia, Spain) (Fernández-León et al., 2013). It is a vigorous variety of small size and few regrowth. It produces very uniform, compact, heavy and dark green heads. The florets are very short and small in size.

2.2. Biostimulant formulation

The biostimulant (BS) used in this study was a precommercial non-microbial biostimulant based on a combination of natural extracts (yeast extract, licorice root extract, willow root extract and *Ascophyllum nodosum* algae extract, each one at a concentration of 200 µg/mL) (Calbio; Caldic Ibérica S. L., Barcelona, Spain). The product is available for scientific purposes upon request to Caldic. This formulation was the result of a functional evaluation in *A. thaliana* and *S. cerevisiae* developed in our research group (Saporta et al., 2019; Benito et al., 2022).

2.3. Experimental design and growth conditions

The field trials were carried out at the Sinyent experimentation and technology transfer farm, owned by the Valencian Association of Farmers AVA-ASAJA, located in Polinyà del Xúquer, (Valencia, Spain) (39°11′46.4"N, 0°23′24.5"W) from December 2022 to April 2023. The meteorological register during the cultivation can be found in **Supplementary table 1**.

The seedlings were obtained from the commercial nursery CUCALA AGRICOLA S.L. (Benigànim, València, Spain) (38°56'42" N, 0°25'42" W), grown in coconut fiber alveoli. The transplant was carried out on December 27, 2022, when all the seedlings presented 3 true leaves unfolded and a homogeneous size between them. Before transplanting, a pre-emergent herbicide (Pendimethalin 33 % w/v. EC) was applied at a dose of 5 L/ha and a broth volume of 600 L/ha. After this, the cultivation sites and drip lines were prepared. For each crop row, a 16 mm pipe with

Fig. 1. Experimental setup, following the standard procedure for broccoli cultivation in the Mediterranean area.

1.5 L/h self-compensating drippers was installed at a distance of 0.5 m between drippers.

The experimental plot consisted of 30 plants per treatment arranged in consecutive blocks. The treatments were distributed in continuous blocks, with each lane of culture receiving a different treatment. The plants were arranged in a double row per lane, with a distance of 0.5 m x 0.5 m per plant (Fig. 1). Irrigation was provided between 2-3 times per week by drip, throughout the growth cycle (except for plants under drought stress). During the first stages of the crop cycle, 2 weekly waterings were carried out with an irrigation time of 15 min. During the growth period of the floral head, there was a rise in temperatures, so the irrigation frequency was increased to 3 times per week. During the crop cycle, drip fertilization was carried out with a nutrient solution appropriate for the requirements of the crop in each of the phases. A water-soluble NPK complex fertilizer of composition 19-6-6 (ammoniacal N 14 %, urea N 5 %, P_2O_5 6 %, K_2O 6 %, SO_3 46 %, MgO 2 % (w/v), chlorides < 2 %) was used.

The plant groups consisted of (1) control plants under standard irrigation conditions (no stress+control; grey bars), (2) plants under drought conditions (drought+control; grey bars), (3) plants under salinity conditions (salinity+control; grey bars), (4) plants treated with BS under normal irrigation conditions (no stress+BS treatment; blue bars), (5) plants treated with BS under drought conditions (drought+BS treatment; blue bars) and (6) plants treated with BS under salinity conditions (salinity+BS treatment; blue bars). BS application was performed at two different application times: 5 days after transplantation (Application A) and before flower head formation (growth stage BBCH 41; Meier, 2018; Application B). A total concentration of 800 µg/mL of biostimulant was used, applying 100 mL of the solution around the base of the stem of each plant for its assimilation by the root system.

The application of water and saline stress began three days after Application B (BBCH 41). Drought stress was induced by stopping the irrigation. The salt stress was applied by irrigating with 140 mM NaCl for 10 min once per week, replacing the usual irrigation. Stress conditions were maintained until harvest. The irrigation with salinity was applied using a wheelbarrow-type motor sprayer equipment model NOUKI 103-2R-YC82AS (Vila Grancha S.L., Albal, Valencia, Spain), connected directly to the drip line.

The harvest was carried out 39 days from the start of the stress treatment. The time of harvest was determined by the physiological maturity of the crop, when the broccoli heads reached a commercial diameter (BBCH 49; Meier, 2018). At the time of harvest, samples were taken from each tissue (leaf, root and head) to be evaluated, frozen in liquid nitrogen and stored at -80 °C until use.

2.4. Agronomic parameters

After harvesting, the weight, the floral head perimeter and the inflorescence height were measured for each of the collected heads. The height was measured from the cutting point of the head (two centimeters below the appearance of the first florets) to the highest point of the inflorescence. The flowering time of the heads was also determined, due to uneven flowering between treatments. For doing this we considered that a plant has entered flowering when it has reached BBCH 51 at the time of harvest (Meier 2018). All the plants in the study were evaluated, so it was possible to estimate the percentage of plants that had early flowering with respect to the total number of plants in the experiment by applying the equation:

% early flowering plants = (plants with BBCH 51 at harvest / total plants) x 100

For the measurement of the Soil Plant Analysis Development index (SPAD), the non-destructive optical method SPAD-502Plus (Minolta, Osaka, Japan) was used, which allowed for the estimation of the concentration of chlorophylls indirectly. For a given plant species, a higher SPAD value indicates a healthier plant. To perform the data collection, the reader was placed a few centimeters above the apex of the leaves located at an average height of the plant. 10 biological replicates were performed per treatment and 3 technical replicates were carried out for each of the measurements. The measurements were all made at 12 p.m. (at noon), to avoid the influence of solar radiation on the measurements.

2.5. Extraction and measurement of photosynthetic pigments

The extraction and measurement of photosynthetic pigments were performed following the method described by Lichtenthaler (1987). Samples from fresh leaves (100 mg) were macerated in 100 % (v/v) methanol, followed by shaking for 30 min at room temperature. After sample centrifugation, the supernatant was transferred to new tubes and diluted 1:25. Absorbance was measured in a TECAN Infinite® 200 PRO microplate reader (TECAN, Männensdorf, Switzerland) at wavelengths of 665.2 nm, 652.4 nm and 470 nm. The residual pellet of plant material was dried at 65 °C for 4 days (dry weight (DW)). Results were expressed in µg pigment/g dry weight.

2.6. Efficiency of Photosystem II

The efficiency of photosystem II was measured with a HandyPEA fluorometer (Hansatech, Pentney, England) according to the manufacturer's specifications. For measurement, 6 biological replicates per treatment and three technical replicates were used. This fluorometer recorded data relating to the Fv/Fm ratio, known as the maximum quantum yield of photosystem II (PSII). Before this measurement, the plant leaves were dark adapted for 45 min. Maximum fluorescence was measured after exposure of the leaves to an intense flash of light. The results were expressed as the ratio between variable fluorescence (Fv) and maximum fluorescence (Fm).

2.7. Relative water content (RWC) and water use efficiency (WUE)

Leaf disks from young leaves (1 cm diameter) from five plants per each treatment were weighed (fresh weight (FW)) immediately after harvesting, then placed in a water-saturated vial at 4 °C for 24 h and weighed (turgid weight (TW)). The samples were then oven-dried at 60 °C for a period of 48 h and DW were obtained. Relative water content (RWC) (Ruiz-Lozano and Azcon 1997) and water use efficiency (WUE) (Marulanda et al., 2007) were obtained according to the following formulas:

$$\text{RWC}\ (\ \%) = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgor weight} - \text{dry weight}}\ x\ 100$$

$$WUE = \frac{fresh\ weight - dry\ weight}{dry\ weight}$$

Water use efficiency was expressed in g H_2O/g dry weight.

2.8. Determination of osmolytes, phenols and flavonoids

We used a modification of the method described by Bligh and Dyer (1959). 100 mg of fresh weight of each of the analyzed tissues were crushed. The extraction was carried out with 375 µL of methanol (x2), 750 µL of chloroform and 0.88 % (w/v) NaCl solution, homogenizing the sample by vortexing after applying each solution. Finally, the samples were centrifuged at 12,300 g at 4 °C. The methanolic phase was transferred to a new tube and the samples were stored at -80 °C until use. For all the analyses mentioned, 3 biological replicates and 3 technical replicates were used.

2.8.1. Determination of total soluble sugars (TSS)

The total soluble sugar (TSS) determination in leaf, root and head

was carried out according to the procedure described by Irigoyen et al., (1992). For each sample, 100 µL of extract and 3 mL of anthrone reagent were added to a tube. The samples were boiled in a water bath at 100 ºC for 10 min and then cooled on ice. The absorbance reading was performed in 96-well plates using the TECAN Infinite® 200 PRO microplate reader at a wavelength of 620 nm. The anthrone reagent without extract was used as a blank. The standard curve was performed using glucose in a range of 20 to 400 µg/mL (w/v). The result of the analysis was expressed in mg TSS/g of FW.

2.8.2. Proline determination

To determine the proline (Pro) content in leaf, root and head, we followed the protocol described by Bates et al., (1973). For the reaction, 100 µL of methanol, 200 µL of acetic acid, 200 µL of ninhydrin reagent and 100 µL of extract were added consecutively. The tubes were incubated at 96 ºC for 1 h. The reaction was stopped by ice cooling and adding 1 mL of toluene, then samples were stirred with vortex and incubated for 5 min at room temperature. The absorbance reading was performed on the TECAN Infinite® 200 PRO microplate reader at a wavelength of 520 nm on 96-well plates. To carry out the standard curve, concentrations of 0 to 300 µM of Pro from a stock solution of 1 mM were used. The results obtained were expressed in nmol of Pro/g FW.

2.8.3. Determination of total phenols

To determine the total phenols in leaves, we followed the protocol proposed by Blainski et al., (2013), with minor adjustments to perform the reaction in microplates. 10 µL of the extract used for the TSS and Pro determination was diluted in 140 µL of Milli-Q water. Then, 10 µL of the Folin-Ciocalteu reagent was added and allowed to react at room temperature for 5 min. After this incubation, 35 µL of 1.42 M Na_2CO_3 was added and the samples were incubated in darkness for 90 min. Absorbance was measured at 765 nm on the TECAN Infinite® 200 PRO microplate reader. The standard curve was performed with gallic acid in a concentration range of 0 to 100 µg/mL (v/v) from a stock solution at 0.2 mg/mL (w/v).

2.8.4. Determination of total flavonoids

The methodology described by Zhishen et al., (1999) was used for the determination of flavonoid content in leaves, with minor adjustments to perform the reaction in microplates. Firstly, 80 µL of extract described before was diluted in 60 µL of Milli-Q water. Then, 8 µL of 0.73 M $NaNO_2$ was added. The reaction time was set at 5 min. After this, 8 µL of 0.75 M $AlCl_3$ was added and incubated for 6 min and subsequently, 60 µL of 1 M NaOH was added. The absorbance was measured at 510 nm. The standard curve was performed using concentrations from 0 to 90 µg/mL (v/v) from a stock of 0.1 mg/mL (w/v) catechin dissolved in methanol.

2.9. Antioxidant enzymatic determinations

Antioxidant enzymes were extracted from 300 mg (FW) of frozen plant material (leaf, root and head), disrupted with pistil and liquid nitrogen, and using 50 mM potassium phosphate (PK) buffer (pH 7.8), containing 0.1 mM Na_2–EDTA and 1 % (w/v) insoluble polyvinylpolypyrrolidone (PVPP). This buffer was used to determine the activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and oxidative damage to lipids (OLD) (Gogorcena et al., 1995). The same medium supplied with 10 mM β-mercaptoethanol was used for glutathione reductase (GR) (Moran et al., 1994), and supplied with 4 mM ascorbic acid was used for ascorbate peroxidase (APX). The supernatants were stored at −20 °C for subsequent enzymatic assays.

The supernatant obtained was divided according to the subsequent analysis to be performed: the samples for the measurement of ascorbate peroxidase (APX) were added 4 mM ascorbic acid and measured according to Nakano and Asada (1981), those destined to GR were added 10 mM β-mercaptoethanol and measured according to Foyer and Halliwell (1976), while no additional reagent was added to the fraction of supernatant used for the measurement of CAT, SOD and protein quantification. All samples were stored at −20 °C until use. SOD was measured according to Beyer and Fridovich (1987); CAT according to Aebi (1984), GR according to Carlberg and Mannervik (1985) and APX with respect to (Amako et al., 1994). Total protein concentration was determined using the Bradford method (Bradford, 1976). The antioxidant enzyme results (APX, GR and CAT) were expressed as µmol enzyme/min mg protein except for SOD, whose results were expressed as Units of SOD/min mg protein.

2.10. Determination of oxidative lipid damage (OLD)

For the OLD determination, the procedure described by Minotti and Aust (1987) was used. For it, 100 µL of the extract was reacted with 1 mL of reagent containing 1.22 M trichloroacetic acid (TCA), 40 mM 2-thiobarbituric acid (TBA) and HCl 0.25 N and 0,45 mM butylated hydroxytoluene (BHT). The samples were incubated at 95 °C for 30 min. After this time, they were cooled in an ice bed and centrifuged at 10,000 g for 10 min. Absorbance was measured at 535 nm on the TECAN Infinite® 200 PRO microplate reader. The oxidative damage results to lipids were expressed in nmol malondialdehyde (MDA)/g FW.

2.11. Determination of hormonal content

Hormone extraction and analysis were essentially conducted as described in Durgbanshi et al., (2005), with few modifications (Chevilly et al., 2021a). This hormone analysis was performed on the three tissues collected. Briefly, for gibberellins (GA), ABA, JA, isoleucine JA conjugate (JA-Ile), indole-3-acetic acid (IAA), phaseic acid (PA), 12-oxo-phytodienoic acid (OPDA), isopentenyladenosine (IPR), SA and glucosinolates (GLU) extraction, starting from approximately 10 mg of lyophilized plant tissue. It was used as the Internal standard solution containing 1 mg/L of [$^2H_2$]-GA1, [$^2H_2$]-GA7, [$^2H_6$]-ABA, DHJA, and [$^{13}C_6$]-SA and 0.1 mg/L of [$^2H_2$]-IAA. After aqueous extraction with a ball mill and centrifugation (10,000 rpm and 4°C for 10 min), the pH of the supernatant was adjusted to 3 with 30 % (v/v) acetic acid and subsequently partitioned against an equal volume of diethyl ether twice. The organic layers for each sample were recovered, combined and dried down under vacuum. The dry residues were resuspended in a methanol: water (10:90) solution that was filtered through 0.2 µm PTFE syringe filters. Extracts were analyzed by reversed phase UPLC (Acquity SDS, Waters Corp., Milford, USA) coupled to a mass spectrometer (TQ-S, Micromass Ltd., UK). Phytohormones were detected according to their specific transitions using a multi-residue mass spectrometric method. All data were acquired and processed using MassLynx v4.1 software. Relative quantification was achieved by comparing the areas of the different samples.

2.12. Glucosinolate determination

Extraction was performed in leaf, root and head according to (Zandalinas et al., 2012) with some modifications. Briefly, 400 µL of 80 % (v/v) methanol supplemented with biochanin A at 1 mg/L (internal standard, IS) was added to 10 mg freeze-dried plant material. After 10 min of sonication, samples were centrifuged at 10,000 g for 10 min at 4°C. Prior to analysis, supernatants were filtered through 0.2 µm PTFE syringe filters (Whatman International Inc., Kent, United Kingdom) and diluted 1:4 with 80 % (v/v) methanol. Analysis of glucosinolates was carried out using a liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC/ESI-QTOF-MS), selecting specific mz values for each of the glucosinolates. Relative quantification was attained by recording peak area for each metabolite and normalizing it to IS peak area, the resulting value was divided into sample weight (in g).

2.13. Ion content determination

Ions were determined as described (Gisbert et al., 2020) . Briefly, samples of leaves and roots of lettuce plants were freeze-dried for two days. DW was determined, and ions were extracted by a 30 min incubation in 1 mL of 0.1 M HNO_3 at room temperature. Then, samples were centrifuged, and the supernatant was diluted with 4 mL of Milli-Q water and filtered (0.22 μm). Sodium, potassium, calcium, and magnesium ions were measured in a plasma emission spectrophotometer (Agilent Technologies 700 series ICP-OES, Santa Clara (Ca), USA). Measurements were normalized to DW. Three biological replicates of each treatment were analyzed.

2.14. Statistical analysis

The statistical analysis was performed using the STATGRAPHICS Centurion software (Statgraphics Technologies, Inc., Virginia, USA). Student's t-test was calculated by comparing the results obtained for each treatment with control conditions.

3. Results

3.1. Agronomic parameters: production and plant size

Few studies have addressed the -mechanism of action of biostimulants on broccoli cultivation under field conditions. In this work, different agronomic, physiological, and biochemical parameters were measured to assess the effect of our biostimulant (BS) (Benito et al., 2022) on broccoli production under control, salt or water stress.

The head harvest was carried out during 3 weeks. At the time of harvest, the weight, diameter and height of the edible part of the harvested plants were measured (Fig. 2 **A-C**). As a result of the biostimulant treatment, we observed a significant increase in head weight in those plants treated with biostimulant under salt stress conditions, while weight was significantly lower in non-stressful and drought conditions in treated plants (Fig. 2 **A**). In these two conditions, head diameter was larger (Fig. 2 **B**). This could be because the heads were less compact (Larger height than the control) in the biostimulant treatment under salt stress (Fig. 2 **C**).

The most dramatic effect was observed in the flowering time (Fig. 2 **D**). Our study did not aim to determine the overall agronomical yield. Instead, we focused on studying the impact of the biostimulant on crop biology and agronomical traits under field conditions. However, we also measured total yield and found that the biostimulant increased production per hectare under both control and stress conditions (**Supplementary Figure 1**). To further verify these observations, it is necessary to conduct additional experimental trials in subsequent years or in different locations.

3.2. Effect of the biostimulant on the photosynthetic parameters

We have discovered that our biostimulant is effective at increasing the head weight and diameter in determinate conditions, and also, in preventing premature flowering. In order to characterize the molecular and physiological basis of these observed phenotypes, we measured photosynthetic parameters, as photosynthesis is directly related to plant growth, biomass production and, in agriculture, crop yield (Calzadilla et al., 2022). The SPAD index is a measure of the relative chlorophyll amount present in the leaf, determined by measuring absorbance at two wavelength ranges, which gives an estimate of the plant's health status. We measured the SPAD index twice, once at 14 days and once at 34 days after the onset of stress. In the first measurement, the BS applied in non-stressed plants generated a small decrease in the SPAD index, while in salinity, the application of the biostimulant increased it. On the other

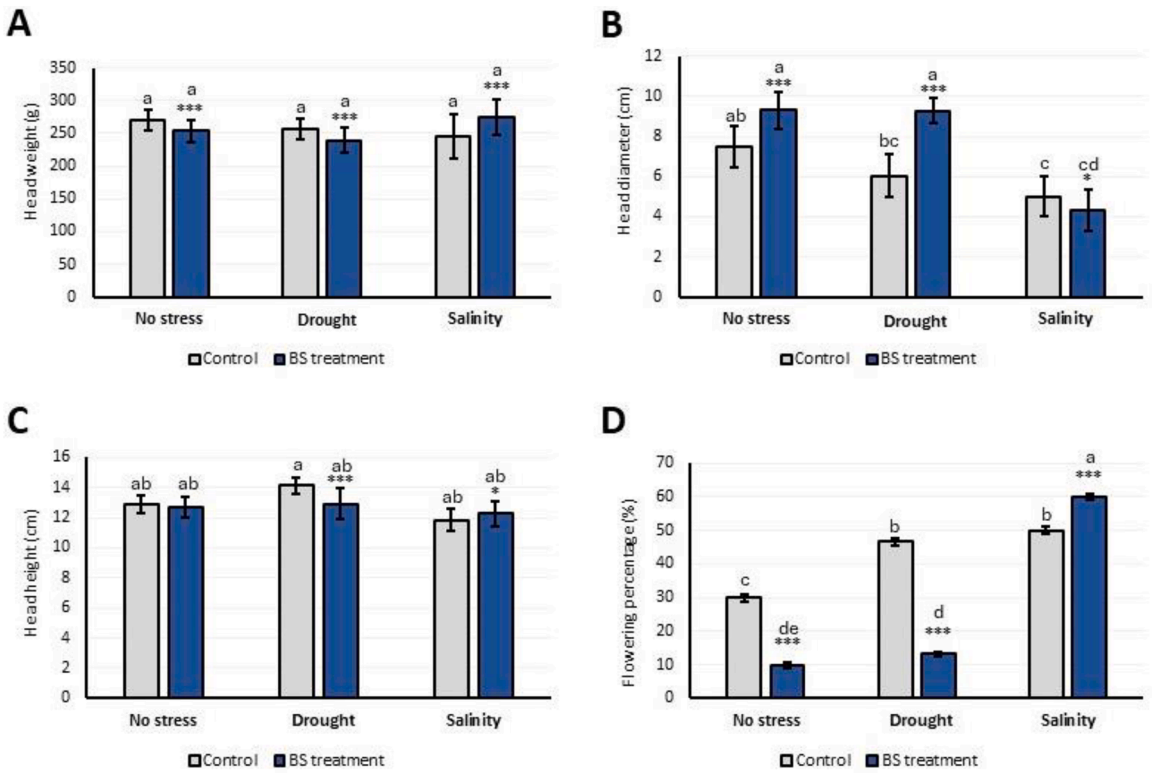

Fig. 2. Agronomical parameters of the broccoli heads. The X-axis shows the different conditions tested, while the Y-axis represents (**A**) the weight (g), (**B**) diameter (cm) and (**C**) height (cm) of heads, and (**D**) percentage of plants with early flowering. Bars represent the standard error ($n = 30$). The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* p-value<0.05; *** p-value<0.001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

hand, 34 days after the onset of salt stress, plants treated with the biostimulant had a significantly higher SPAD index than those not treated (Fig. 3 **A**). We then measured the performance of PSII (Fig. 3 **B**). These measurements indicated that the applied stresses did not decrease the performance of PSII in the control plants under each of the conditions. In the case of plants treated with the biostimulant, a reduction in photosynthetic yield was observed, which was more pronounced in plants stressed with salinity, showing highly significant differences in this case.

Plants grown under stress conditions and treated with BS had showed a modest increase chlorophyll a accumulation, as compared to untreated plants. This increase was observed in all three conditions studied, although it was more elevated when the plants were subjected to salt stress. In the case of chlorophyll b, plants treated with BS under salt stress also presented increased accumulation of this pigment, as compared to untreated plants. This increase was also detected in plants treated with BS under water stress and the same was observed for the total chlorophyll. The carotenoid content increased upon BS treatment, but it was only highly significant under drought and salt stress (Fig. 3 **C**). The concentration of photosynthetic pigments was in accordance with the measured SPAD index (Fig. 3 **A, C**), which could suggest that our biostimulant increased the plant photosynthetic activity under salt stress conditions (Madakadze et al., 1999).

3.3. Relative water content (RWC) and water use efficiency (WUE)

According to the RWC, a measure of the leaf turgor, plants that were grown under normal conditions and treated with biostimulant showed increased water content compared to the control group. The same increase was observed in plants treated with salt, although it was not statistically significant. Under drought conditions, both control and biostimulant-treated plants showed similar results as non-stressed plants (**Supplementary Figure 2 A)**. Another parameter used to evaluate the hydration status of the plant is the Water Use Efficiency (WUE). Our experiment showed that control plants grown under water-stressed conditions had a lower WUE compared to plants grown under non-stressed or salinity conditions (**Supplementary Figure 2 B**). Plants grown under salinity conditions had WUE values similar to non-stressed plants. When we applied the biostimulant (BS) to plants under salt stress, we found that it reduced their WUE compared to control plants.

3.4. Determination of osmolytes

We have observed that the BS treatment ameliorates several standard stress parameters. This improvement could be because the biostimulant is protecting the plants from stress or boosting the plant's stress response. To help overcome the negative effects of abiotic stress, plants can accumulate osmoprotective solutes, such as Pro and TSS. To differentiate between these two hypotheses, we analyzed TSS and Pro. The most interesting results were found in roots. In this tissue, there was a significant decrease in the concentration of TSS and Pro in the biostimulant-treated plants under drought conditions (Fig. 4 **B, E**). The concentration of TSS in the leaf (Fig. 4 **A**) and Pro in the head (Fig. 4 **F**) increased under drought conditions, as compared to the control. In addition, an increase in Pro concentration was observed in all three tissues analyzed under salinity in the presence of BS (Fig. 4 **D-F**).

3.5. Non-enzymatic antioxidants

Under conditions of high salinity and other types of abiotic stresses, plants activate a series of responses, including the activation of enzymes and the synthesis of antioxidant compounds to combat the generated ROS, among which there are phenolic compounds and the subgroup of

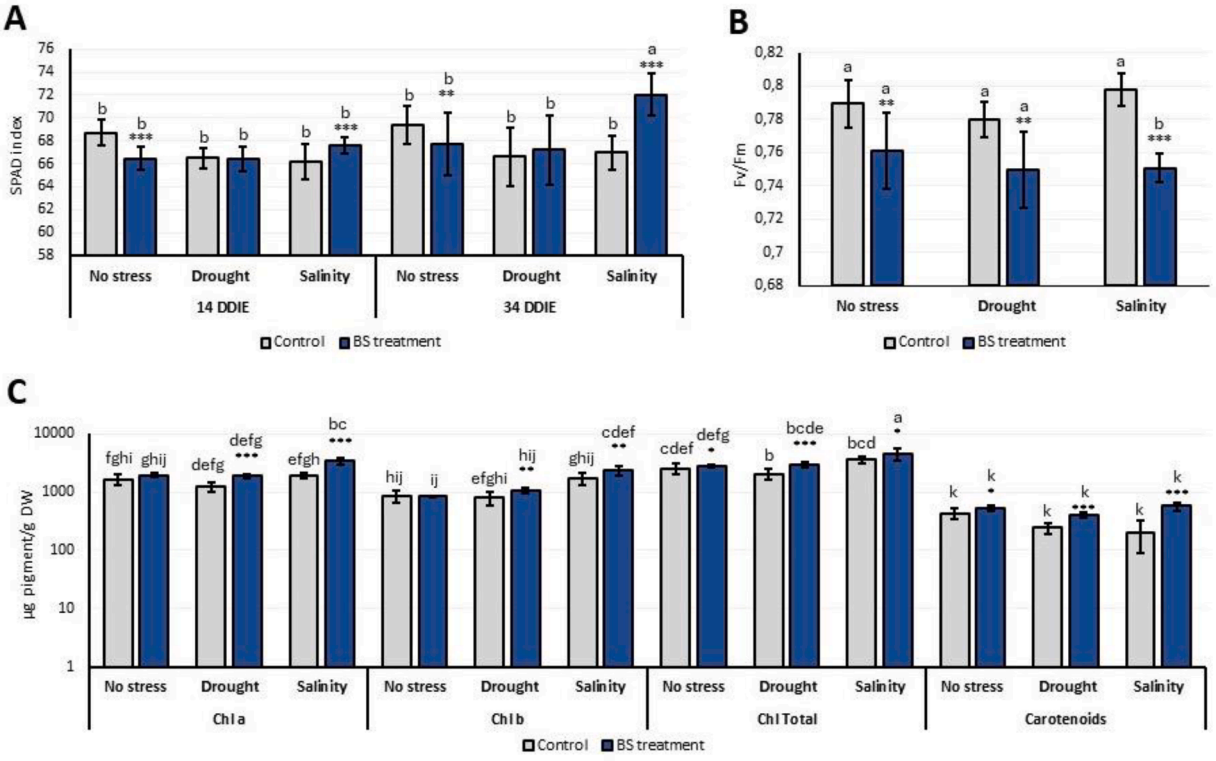

Fig. 3. Effect of the biostimulant on the photosynthesis. The X-axis shows the different conditions tested, while the Y-axis represents (**A**) SPAD index taken at 14 and 34 days after stress (DAS), (**B**) the efficiency of photosystem II (Fv/Fm) presented as a ratio of variable fluorescence (Fv) over maximum fluorescence value (Fm), and (**C**) Concentration of the photosynthetic pigments: chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (total Chl) and carotenoids (Car) represented as µg per gram of dry weight on a base 10 logarithmic scale. The bars represent the standard error with n=9. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* *p*-value<0,05; ** *p*-value<0,01; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

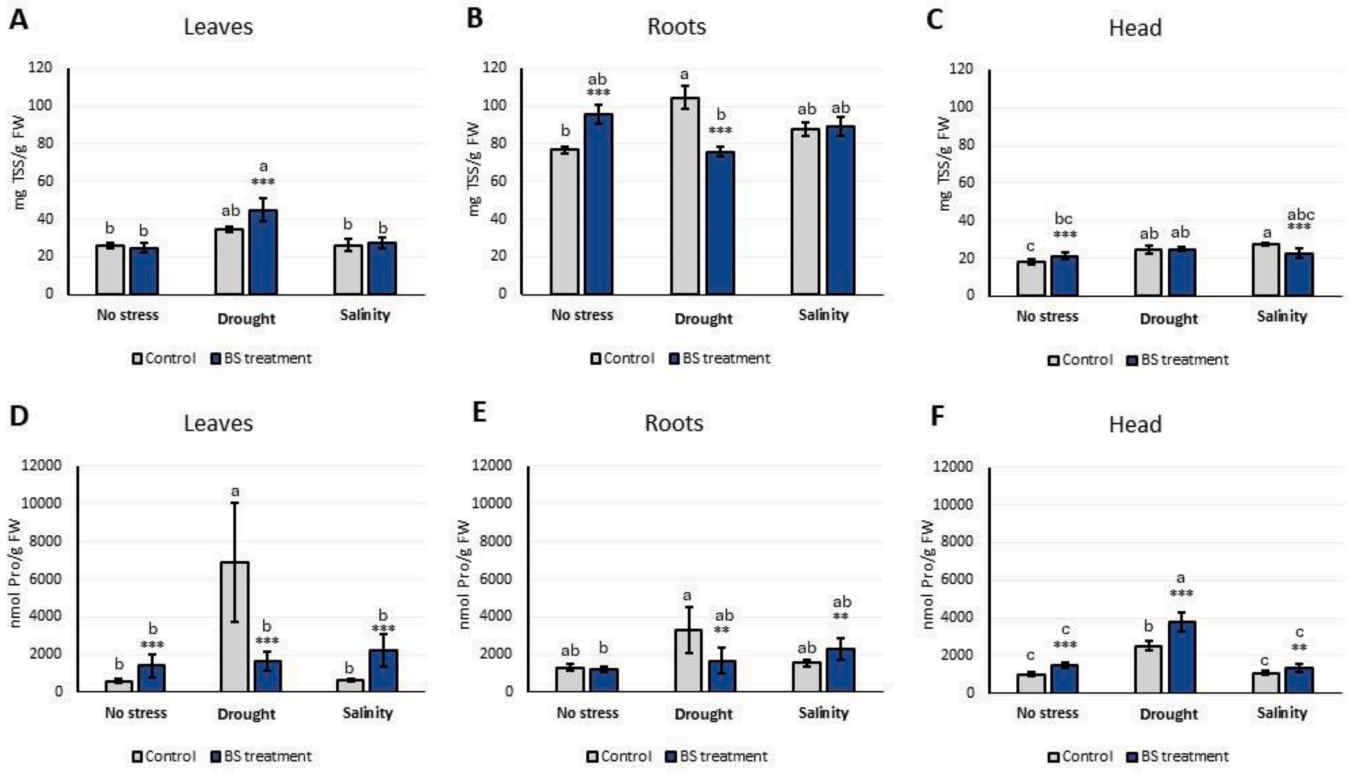

Fig. 4. Accumulation of total sugars (TSS) and proline (Pro). The X-axis shows the different conditions tested, while the Y-axis represents (**A-C**) the mg of TSS per g of fresh weight and (**D-F**) the μmoles of Pro per g of fresh weight. The bars represent the standard error with $n = 9$. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (** p-value<0,01; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

flavonoids (Zuzunaga-Rosas et al., 2022). We further investigated whether the effect of the BS was based on a better protective response against oxidative stress, or in diminishing the stress. The activation of the non-enzymatic antioxidant system was studied by determining representative antioxidant compounds, such as phenols and total flavonoids in broccoli leaf tissues. Regarding the total phenolic compounds (TPC) analysis, a slight increase in phenol concentration was observed in plants treated with BS in non-stressed conditions. However, plants treated with BS under drought showed the greatest increase in phenolic compounds. On the contrary, those plants treated with BS subjected to salt stress showed a reduction in the content of total phenols compared to untreated plants (**Supplementary Figure 3 A**). Concerning the analysis of total flavonoid content (TFC) in leaves, no statistically significant results were obtained (**Supplementary Figure 3 B**).

3.6. Antioxidant enzymes

We also determined the activation of the enzymatic antioxidant system in leaf, root and head. A common pattern was observed for all the enzymes tested in roots under drought. In this case, a highly significant increase in the four antioxidant enzymes (SOD, CAT, ascorbate peroxidase (APX) and GR) was detected in plants treated with BS (Fig. 5 **B, E, H, K**). The same trend was observed in leaves (Fig. 5 **A, G, J**) except for the APX enzyme (Fig. 5 **D**). However, in the broccoli heads, a higher concentration of APX, as compared with control plants, was only observed in the plants treated with BS under drought conditions (Fig. 5 **F**). At the same time, in salinity, decreases in the concentrations of SOD in leaves (Fig. 5 **A**), APX in the head (Fig. 5 **F**) and CAT in the root (Fig. 5 **H**), and an increase in APX in the root (Fig. 5 **E**) was observed.

3.7. Oxidative lipid damage (OLD)

To further characterize the effect of the stress in plants, we determined the level of oxidative lipid damage, which is a standard marker of the cellular damage caused by stress. Under control and salt stress we observed higher MDA level in leaves, but it was the opposite under drought stress (Fig. 6 **A**). It was observed that, in roots, plants grown under drought conditions showed a much higher content of MDA compared to non-stressed plants (Fig. 6 **B**). On the other hand, the application of BS in plants grown under drought conditions produced a decrease in lipid oxidation to levels comparable to those obtained in plants without stress, obtaining highly significant differences. Under salinity and control conditions, a slight increase in MDA concentrations detected in BS treated plants was observed, except in broccoli head, where a significant decrease in MDA was observed in plants treated with BS (Fig. 6 **C**).

3.8. Ion content

Drought or salinity stress alters the ionic content in plant tissues. For this reason, we determined the effect of our biostimulant on the content of Na^+, K^+, Ca^{2+} and Mg^{2+} under standard and stress conditions (drought and salinity). An increase in K^+ was observed in the presence of our biostimulant in roots under drought and salinity conditions. In the case of Na^+, an increase in this ion was observed in leaves under salinity and in roots under drought, but a decrease was observed in heads under saline stress (Fig. 7).

3.9. Content of phytohormones and glucosinolates

The effect of the biostimulant on the hormonal profile of broccoli under drought, salinity and control conditions was also determined. The

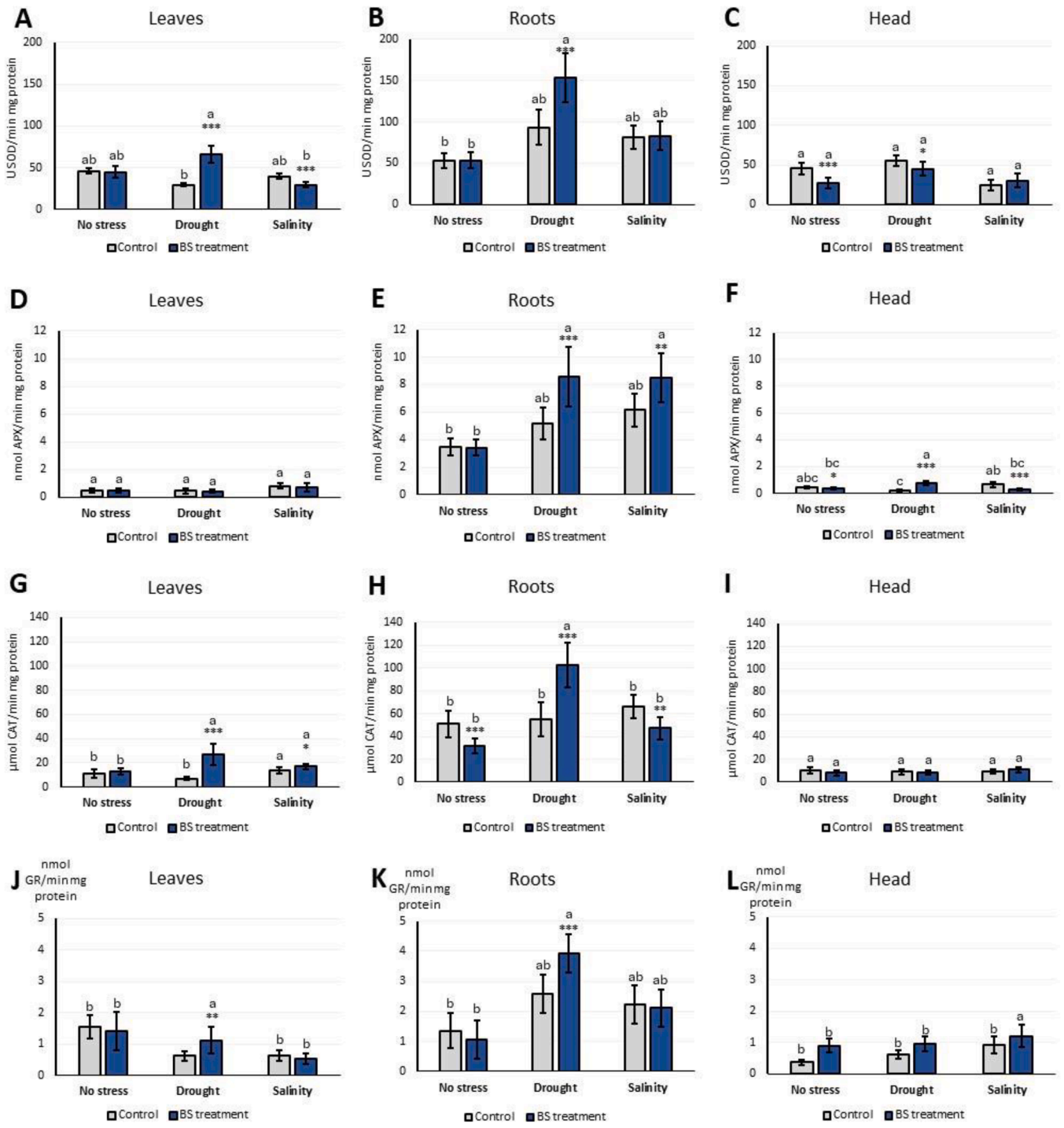

Fig. 5. Activity of enzymatic antioxidants. The X-axis shows the different conditions tested, while the Y-axis represents (**A-C**) the superoxide dismutase activity in units / (min mg protein) (SOD), (**D-F**) ascorbate peroxidase activity in nmol/(min mg protein) (APX), (**G-I**) the catalase activity in µmol/(min mg protein) (CAT) and (**J-L**) the glutathione reductase enzyme in nmol/(min mg protein) (GR). The bars represent the standard error with $n = 9$. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* p-value<0,05; ** p-value<0,01; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

most striking result is the increase in the concentration of the hormones IAA, ABA, JA, IPR, giberellic acids (GA3 or GA4) in the roots of plants treated with BS under salt stress conditions (Fig. 8). These plants showed a greater than 20 % increase in hormonal concentration compared to the untreated control. Furthermore, a significant decrease in IAA, ABA, JA and GA4 was observed in leaves under salt stress (Fig. 8 **A-C, F**).

Glucosinolates have been related to the health promoting properties of broccoli consumption (Olayanju et al., 2024) and the abiotic stress response (Martínez-Ballesta et al., 2013). The analysis of glucosinolates showed a differential presence of aliphatic and indolic glucosinolates in the different plant tissues evaluated. 1-methoxyindole-3-methyl glucosinolate (1-MeO-I3M), 4-methoxyindole-3-yl-methyl glucosinolate (4-MeO-I3M), and indole-3-yl-methyl glucosinolate (I3M) were detected in the three tissues; while 4-hydroxyindol-3-yl-methyl glucosinolate (4-OH-I3M), glucocheirolin and glucoraphanin were detected in the leaf and head. In addition to these glucosinolates, the presence of

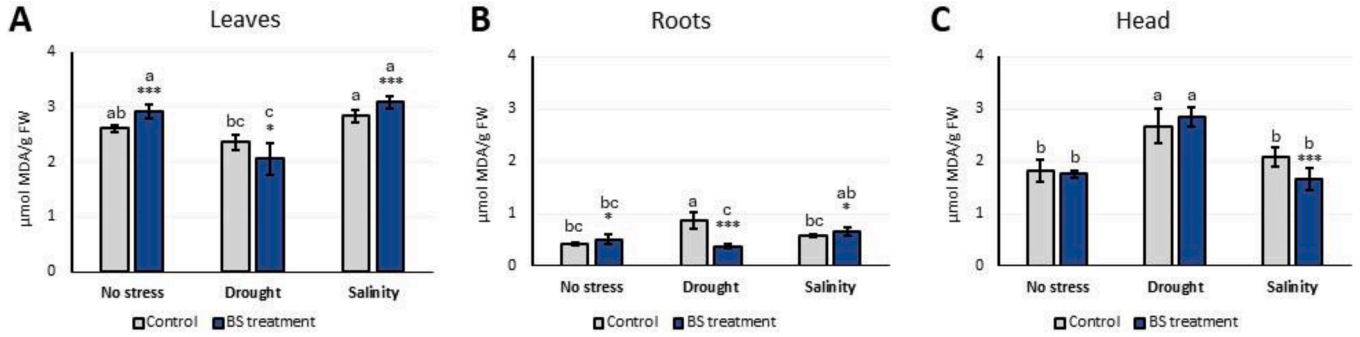

Fig. 6. Lipid oxidative damage. The X-axis shows the different conditions tested, while the Y-axis represents µmol of MDA per mg of fresh weight. The bars represent the standard error with $n = 9$. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* p-value<0,05; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

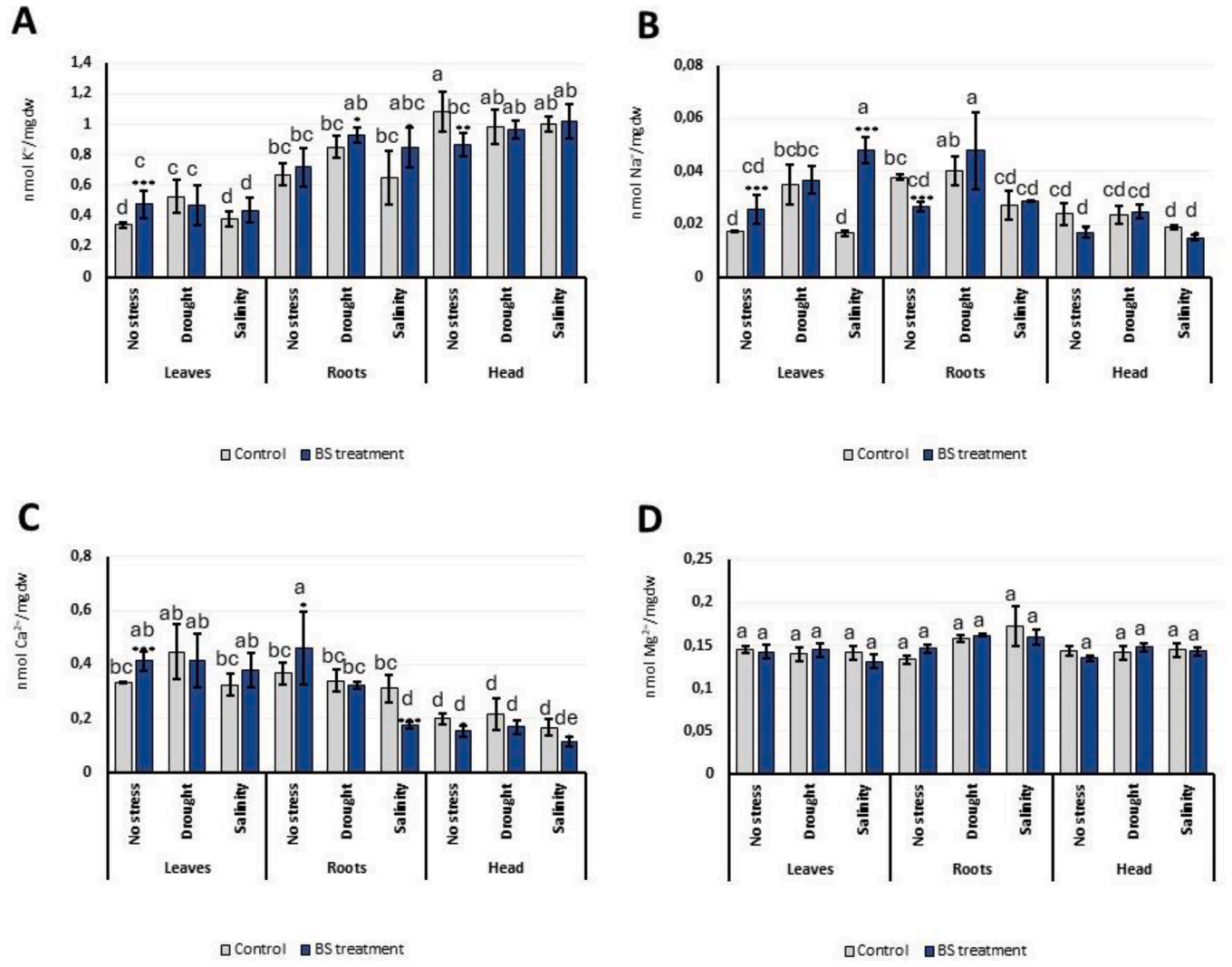

Fig. 7. Determination of (**A**) potassium (**B**) sodium (**C**) calcium and (**D**) magnesium. The X-axis indicates the different concentrations of abiotic stressors. The Y-axis represents the mMol of the indicated ion per milligram of dry weight (mgdw). The bars represent the standard error with n=9. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* p-value<0,05; ** p-value<0,01; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

8-methylsulfinyloctyl glucosinolate (8-MSO) was detected in the leaf, while 3-phenylpropyl glucosinolate (3-PPG) was detected in the head (Fig. 9).

4. Discussion

4.1. Effect of the biostimulant on agronomical traits

Our first objective was to confirm in field the results previously

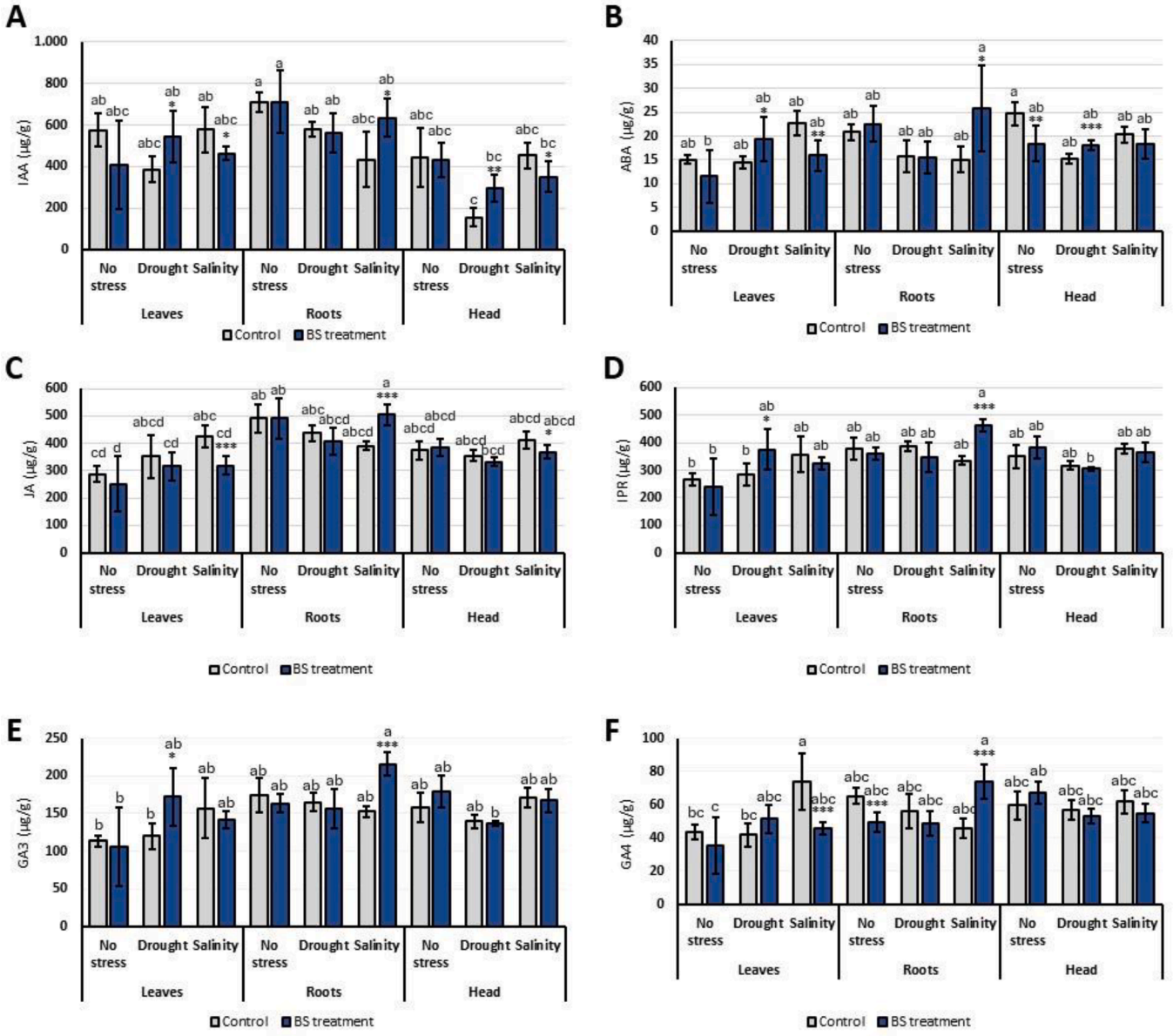

Fig. 8. Concentration of phytohormones in leaf, root and head of broccoli. The X-axis shows the different conditions tested, while the Y-axis represents µg/g of (A) indole acetic acid (IAA), (B) abscisic acid (ABA), (C) jasmonic acid (JA), (D) isopentenyl adenosine (IPR), (E) gibberellic acid 3 (GA3) and (F) gibberellin A4 (GA4). The bars represent the standard error with $n = 9$. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* p-value<0,05; ** p-value<0,01; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

obtained in laboratory (Benito et al., 2022). The assayed biostimulant is able to increase yield in lettuce when applied together with a microbial biostimulant by increasing the CK content (Benito et al., 2024). In the present study we wanted to confirm if the mechanism in a different plant, without the use of microbes, is similar, or if the biostimulant triggers different defense mechanisms in different plants. Broccoli is an ideal system to look for different mechanisms as it does not establish symbiotic associations with the most common symbiotic microorganism, and the edible part is not the leaf, but the inflorescence (head). In addition, in phylogenetic terms, broccoli is closer to the model plant *Arabidopsis thaliana* than lettuce, so the biostimulant is likely to be effective, without any symbiotic associations, as the laboratory tests in Arabidopsis were performed in the absence of microorganisms. We confirmed that our biostimulant was effective at increasing the head weight and the diameter, particularly under salt stress conditions (Fig. 1). Conducting the experiments in field allowed us to monitor parameters which cannot be determined in laboratory or in model organisms. Early flowering is an unwanted trait for farmers as it spoils the head. In addition, early flowering depends on diurnal temperature cycling and is aggravated by drought and salt treatment (Miller, 1988). In control conditions, the percentage of flowering was elevated due to the high temperatures recorded during the last days of the crop cycle in the experimental field (**Supplementary table 1**). This percentage increased in plants grown under stress conditions. However, it was observed that the plants treated with BS presented a lower percentage of plants with flowers, both in control and drought conditions (Fig. 2 **D**), so our biostimulant was also very effective in preventing premature flowering under control and drought conditions.

4.2. *Effect of the biostimulant at the physiological and biochemical level*

Abiotic stress damages the plant at the physiological and molecular levels. Standard symptoms of stress damage are a decrease of the plant's photosynthetic capacity, biomass accumulation, growth rate and,

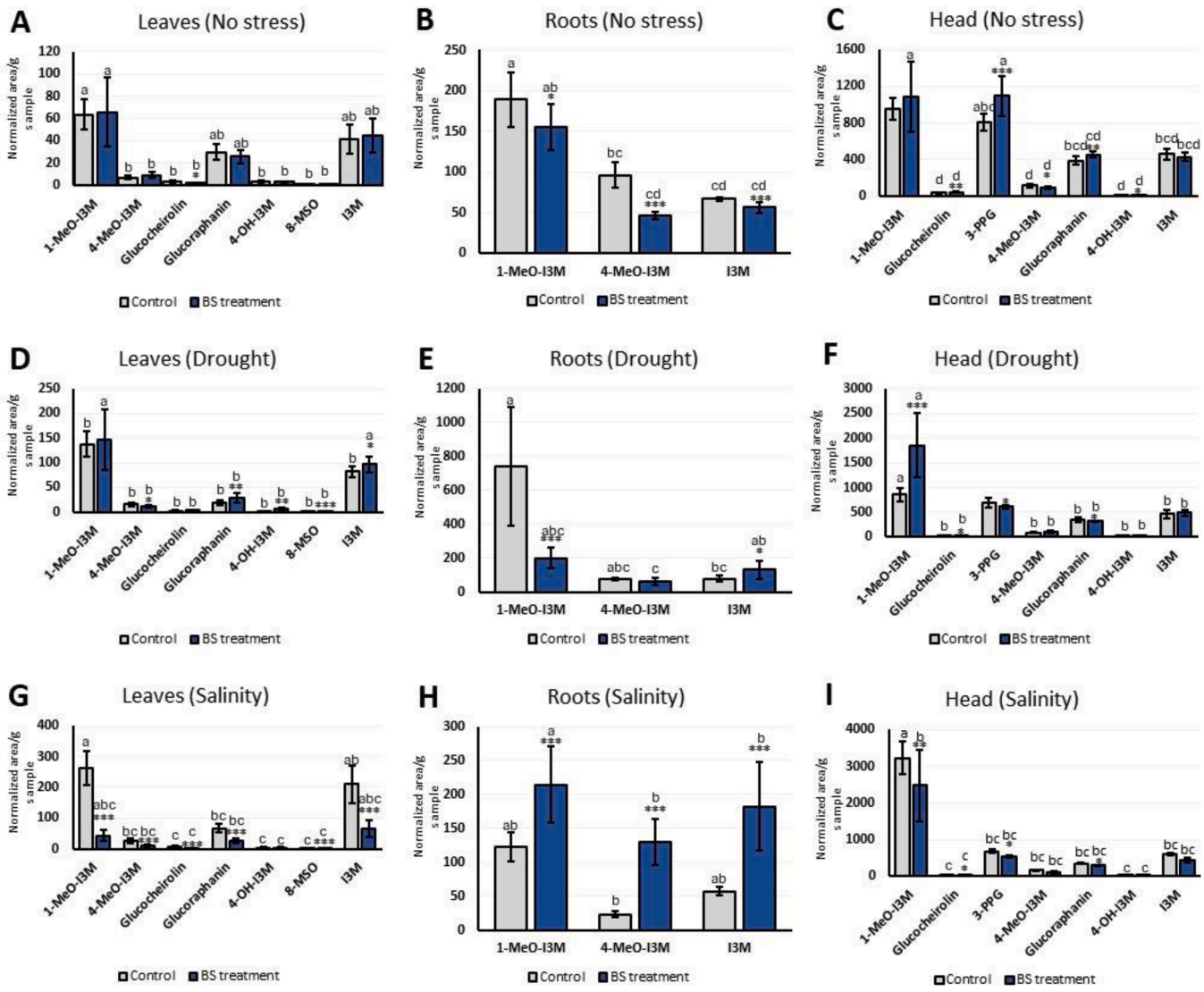

Fig. 9. Concentration of glucosinolates in leaf (A, D, G), root (B, E, H) and head (C, F, I) of broccoli under non-stressful (A-C) and, water deficit (D-F) and salt (G-I) stress conditions. The X-axis shows the different conditions tested, while the Y-axis represents normalized area/g sample of the different glucosinolates. 1-methoxyindole-3-methyl glucosinolate (1-MeO-I3M), 4-methoxyindol-3-yl-methyl glucosinolate (4-MeO-I3M), indole-3-yl-methyl glucosinolate (I3M), 4-hydroxyindol-3-yl-methyl glucosinolate (4-OH-I3M), 8-methylsulfinyloctyl glucosinolate (8-MSO), 3-Phenylpropyl glucosinolate (3-PPG). The bars represent the standard error with $n = 9$. The effect of the biostimulant was statistically tested against the same treatment without biostimulant by a Student's t-test (* p-value<0,05; ** p-value<0,01; *** p-value<0,001). Different lowercase letters indicate significant differences between treatments (Duncan test $p \leq 0.05$).

therefore, agronomic yield. One of the plant's multiple responses to abiotic stress is the reduction of the synthesis of photosynthetic pigments, including chlorophyll a and b, and carotenoids. Our data indicate that, despite the reduction of the content of photosynthetic pigments in control plants grown in drought conditions, none of these pigments were reduced in plants treated with the biostimulant, but they showed higher values than plants grown in the absence of stress (Fig. 3 **C**). In the case of carotenoids, we also observed an increase in the accumulation of these molecules in plants treated with BS under salt stress (Fig. 3 **C**).

One plant's strategy to prevent the loss of water caused by salt or drought stress is the synthesis of osmolites such as TSS (Martínez-Ballesta et al., 2004). In *Arabidopsis thaliana* it has been shown that some biostimulants can mobilize the starch pools an increase the TSS (Benito et al., 2023). We have determined that in roots and heads there are higher levels of TSS in the absence of stress. Under drought levels TSS are higher than the control in leaves and lower in roots. This could mean that the root is having a higher energy demand, and the leaves are mobilizing more starch upon drought (Fig. 4 **A, B and C**). However, our results showed significant differences between the conditions. In leaves, plants treated with the biostimulant (BS) showed an increase in the TSS concentration during drought. This suggests that the biostimulant increased the accumulation of osmoprotectors, enhancing the plant's ability to adapt to stress. However, in the roots, the TSS content of BS-treated plants decreased upon water stress while increased in non-stressed plants. This indicates that the biostimulant may increase the accumulation of osmoregulatory solutes before the onset of stress, specifically in the tissue where the first signaling occurs supporting the hypothesis that activates the stress response.

We further investigated the effect of our biostimulant in osmolyte accumulation. Certain amino acids, like Pro, are also classified as protective osmolytes. Pro accumulation in the cytoplasm decreases the water potential and improves water flow into the cells to maintain the turgor of plant cells (Hasegawa et al., 2000). The analysis conducted in this study showed that plants treated with biostimulant and grown under salinity conditions increased the Pro content in leaf, head, and root tissues. An increase was also detected in plants treated with biostimulant and not stressed in leaf and head tissues, which supports the hypothesis that the biostimulant was activating the stress defense system

and inducing the Pro accumulation, in this case, in the aerial part of the plant. These results are consistent with data obtained in previous research on the relationship between Pro and salt stress (Hare and Cress, 1997; Chevilly. et al., 2021b).

Osmolyte biosynthesis is not the only mechanism induced under abiotic stress. Plants may activate different responses to cope with abiotic stress, including the activation of enzymes and the synthesis of antioxidant compounds to counteract the harmful effects of ROS generated under adverse environmental conditions (Zuzunaga-Rosas et al., 2022). Our data indicate that under all conditions tested, the root generally exhibited higher enzymatic activity than the leaf and head. This suggests that the activity of these enzymes is significantly increased in the root tissue as the first line of defense against free ROS. Interestingly, the application of the biostimulant was found to increase the activity of all enzymes in plants subjected to water deficit. These findings are consistent with previous research that shows the activation of antioxidant enzymes in response to oxidative stress (Espinosa-Diez et al., 2015). The increase in enzyme activity in the root supports the hypothesis that the biostimulant promotes the plant's defense response by boosting the activity of enzymes in the tissue that acts as a stress sensor (Rajput et al., 2021; Zuzunaga-Rosas et al., 2022). These results are consistent with those obtained in the analysis of carotenoids, especially in the determination of TPC under control conditions and drought (**Supplementary Figure 3 A**). This may be because carotenoids, along with phenolic compounds and flavonoids, are considered non-enzymatic antioxidants (Leiva-Ampuero et al., 2020). These secondary metabolites are frequently synthesized in response to environmental changes and accumulate in various plant tissues to act as free radical scavengers, enabling the plant to tolerate different abiotic stress such as salt and water deficit stress (Şirin and Aslım, 2019). This further confirms the model in which our biostimulant promotes the activity of the antioxidant defense system in broccoli plants subjected to abiotic stress conditions, mainly water deficit conditions. To have a general perspective of the joint effect of the biostimulant and the stress on plant physiology, we have performed PCA analysis of all the parameters determined in different organs, with and without biostimulant (Supplementary Figure 4). In leaves, the effect of the biostimulant is mainly quantitative, as the association for the different parameters is similar with or without biostimulant, but the magnitudes are greatly reduced (**Supplementary Figure 4 A, B**). In roots and heads the effect was not only quantitative but qualitative, as the biostimulant induced dramatic changes in the ratio and position of the different groups, thus confirming that the biostimulant was indeed having an effect on plant physiology (**Supplementary Figure 4 C-F**).

4.3. Effect of the biostimulant on the ion content

Increasing K^+ content is a physiological strategy to prevent damage against drought and salt stress, as K^+, besides being an essential mineral nutrient, can act as an osmolyte and keeping a high K^+/Na^+ ratio prevents sodium toxicity (Mulet et al., 2023). Another explanation is the vacuolar sequestration of Na^+. The increase of sodium concentration in leaves under salt stress upon BS treatment was notorious. Given that the BS induces a yield increase under salt stress (**Supplementary Figure 1**), one plausible explanation is that the plant is activating the defense system and increasing Na^+ sequestration in the vacuoles of leaf cells, probably through the NHX system (Jiang et al., 2010) (Fig. 7 **A and B**). The samples under drought stress also present an increase in sodium concentration. This could be due to the salt naturally present in the soil where the field experiment was performed. Under drought stress the plant loses turgor. As mentioned before, potassium is an osmolyte and one strategy to prevent water loss upon drought stress is to increase the potassium uptake. If sodium is present in the soil, the uptake by the plant may be facilitated as sodium can enter through some of the potassium uptake systems. In agreement with this explanation, we observed that the sodium increase is similar to the potassium increase and that the K^+/Na^+ ratio remains unaltered.

4.4. Effect of the biostimulant on the hormone and glucosinolate content

Different phytohormones are known to act as bioactive compounds that govern the stress response in vegetable crops (Altaf et al., 2023). Previously, several authors have described that biostimulants upregulate the production of phytohormones to adapt to abiotic stress in horticultural crops. It has been shown that different seaweed extracts and botanicals possess activities similar to CK and auxins (Stirk et al., 2014), while the application of humic substances stimulates the endogenous production of auxins and GA (Aremu et al., 2015). Furthermore, the application of microbial biostimulants, seaweed extracts and humic substances increaseed auxin, GA and CK content in sorghum plants under salt stress conditions (Desoky et al., 2018). The application of seaweed extracts improved the growth and functioning of *Vitis vinifera* by increasing resistance to water deficit stress (Samuels et al., 2022; Monteiro et al., 2022). Other research indicates that different classes of biostimulants can trigger the reprogramming of hormones and secondary metabolism, as has been shown in pepper (Popko et al., 2018). This information correlates with our data. Our biostimulant mainly promoted the production of IAA, ABA, IPR, JA and GA in roots under salinity conditions and IAA, ABA, IPR and GA3 in leaves under drought conditions. ABA has been related to abiotic stress tolerance as is able to induce the stomata closure to prevent water loss and induce a downstream signalling. In addition, a known target of ABA is the sodium sequestration in the vacuole via the NHX1 sodium exchanger (Yokoi et al., 2002), so the ABA upregulation may explain the observed increased sodium content in BS treated plants under salt stress (Fig. 7 **B**). IPR is a bioactive cytokinin that has also been related to stress response in several crops, and similar results have been obtained for JA (Fahad et al., 2015).

Finally, we determined the concentration of glucosinolates, which are a class of sulfur-rich secondary metabolites typically produced by plants of the *Brassicaceae* family, such as broccoli. These secondary metabolites play an important role in the defense of plants against biotic and abiotic stress, and at the same time, are involved in sensory and nutritional properties (Hanschen and Rohn, 2021). The induction of the accumulation of glucosinolates by using biostimulants can represent an agronomic tool to improve the nutritional quality of crops and their resistance to abiotic stress. Treatments with a seaweed extract have been described to induce a more significant accumulation of indole and aliphatic glucosinolates in broccoli (Hellín et al., 2018), while the application of a microbial biostimulant based on *Trichoderma hamatum* was effective to increase the glucosinolate content in different brassica leafy vegetables (Velasco et al., 2021).

As observed in the phytohormone analysis, in roots under saline conditions, a significant difference was observed between the control and biostimulants-treated sample. Under salt stress BS treatment correlated with higher concentrations of 1-MeO-I3M, 4-MeO-I3M and I3M (Fig. 9 **H**). On the other hand, a significant increase in glucoraphanin, I3M and 4-OH-I3M was observed in leaves of plants treated with BS under drought conditions (Fig. 9 **D**), but the concentration of these glucosinolates was lower than the control in salinity (Fig. 9 **G**). In head, lower concentrations of 3-PPG and glucoraphanin was observed in the presence of BS in both drought and salinity (Fig. 9 **F, I**). This could be a consequence that plants are resisting better to abiotic stress due to the BS treatment, and the concomitant increase in antioxidants and hormones triggering stress response.

To summarize all the results, we have represented the significant p-values of all the parameters evaluated in radial diagrams (**Supplementary Figure 5**).

5. Conclusion

The main objective of the present project was to evaluate the effectiveness of a novel biostimulant (Calbio) in a crop (broccoli) under field

conditions and under two different abiotic stress conditions (drought and salt stress). Our results indicate that the BS activates the plant stress response and helps the plant to overcome the stress effects and maintain yield. Our data shows that both the SPAD index and the concentration of chlorophyll a, b and carotenoids increased in the presence of biostimulant under salinity and drought conditions (Fig. 3) and this treatment also delayed early flowering under drought conditions (Fig. 2), thus confirming the effectivity, especially under stress conditions. Under control conditions, our BS increases yield and affects the levels of osmolytes, increasing TSS in roots and concomitantly increasing the available free sugars (Fig. 6). Under drought conditions, the BS improves the antioxidant response (SOD, APX, CAT, GR and TPC) in leaves and roots. However, under salt stress, BS activates the hormonal and the metabolic response, increasing the glucosinolate content and phytohormone concentrations, including the ones triggering the stress response such as ABA, IPR and JA and the sodium accumulation in leaves. Therefore, here we describe that a novel biostimulantis able to increase the antioxidant and hormonal plant response under abiotic stress conditions in field, making it a valuable and sustainable tool for farmers, in particular for organic farmers. Future investigation will describe in depth the molecular mechanisms underlying the physiological and biochemical changes described in the current report.

Glossary

BBCH scale. Scale used to identify the stages of phenological development of plants (Meier, 2018).

BBCH 41 refers to the beginning of the development of the harvestable vegetative parts of the vegetative propagation organs. Heads begin to form: the two youngest leaves do not unfold.

BBCH 49 refers to a stage when the typical size, form and firmness of heads has been reached.

BBCH 51 refers to a stage when the main inflorescence is visible between uppermost leaves and the branches of inflorescence begin to elongate.

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CRediT authorship contribution statement

Carlos Montesinos: Writing – original draft, Methodology, Investigation, Data curation. **Patricia Benito:** Writing – original draft, Software, Investigation, Data curation, Conceptualization. **Rosa Porcel:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Javier Bellón:** Resources, Project administration, Methodology, Funding acquisition. **Miguel González-Guzmán:** Writing – review & editing, Investigation. **Vicent Arbona:** Writing – review & editing, Investigation. **Lynne Yenush:** Writing – review & editing, Methodology. **José M. Mulet:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors also want to declare that have no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2024.113584.

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