Alleviation of drought stress in soybean by applying of biostimulant based on amino acids and macro- and micronutrients

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Abstract

Drought stress is one of the most predominant environmental factors hindering the soybean productivity. This study investigated the effects of an exogenous application of biostimulants, consisting of nitrogen, phosphorous, iron, copper, boron, manganese, zinc and amino acids, in the physiological, biochemical and productive responses of soybean cultivated under drought stress. Findings showed that applying 0.5 kg ha\(^{-1}\) of the biostimulant improved soybean tolerance to drought. The biostimulant application maintained the leaf photosynthetic rate (\(A\)), stomatal conductance (\(g_s\)), transpiration rate (\(E\)), leaf temperature, water use efficiency (WUE) and carboxylation efficiency (CE), in addition to increasing the SPAD index. Moreover, the biostimulant heightened the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) and maintained the activity of the nitrate reductase enzyme. Regarding osmoprotectant, the biostimulant application enhanced proline accumulation, which could improve the soybean's osmotic adjustment under drought conditions. In addition, foliar application of the biostimulant maintained the biometric and production characteristics, stem diameters, number of branches, number of pods with 1, 2 and 3 grains, and significantly increased leaf area, number of primary stem nodes, dry matter mass in the aerial part and roots, ultimately increasing yield. Based on the aforementioned beneficial properties, the biostimulant based on macro- and micronutrients and amino acids, particularly in the dose 0.5 kg ha\(^{-1}\), has proven to effectively relieve the adverse effects of drought stress in soybean.

1 Introduction

Plants are often submitted to adverse environmental conditions, resulting in stresses that negatively impact their growth, development and/or yield (Van Oosten et al. 2017; Mousavi-Derazmahalleh et al. 2019). Lack of water is the main limiting factor for soybean production worldwide (Anda et al. 2020; Dong et al. 2019). Drought hinders the global production of soybean (\textit{Glycine max} L. (Merr.)), which provides for 71% and 29% of the world's protein and oil consumption, respectively (Wijewardana et al. 2019a).

The effects of water deficit on soybean germination (Kosturkova et al. 2014), physiological processes (Farooq et al. 2009), seed development and quality (Bellaloui et al. 2012; Wijewardana et al. 2019b) and yield (Wijewardana et al. 2018; Wijewardana et al. 2019c) have been reported in the literature. However, to meet the growing demand for food, it is necessary to increase soybean yield, even in environments with low water availability (Rosa et al., 2021).

In the photosynthetic process, the lack of water leads to deleterious effects on important enzymes, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase, pyruvate phosphate dikinase, NADP-malate dehydrogenase and NADP-malic enzyme (Farooq et al. 2009a; Taiz and Zeiger 2017; Shukla et al. 2018). This is due to imbalances of molecules or ions in cells, particularly in oxygen reactive oxygen species (ROS) (Schmidt et
al. 2018), since these molecules are highly unstable and have a high reaction capacity, mainly damaging lipids, proteins, nucleic acids, and affecting the cell physiology (Podgórska et al. 2017).

Under drought stress conditions, there is an increase in ROS levels in the apoplast, due to the activity of the NADPH oxidase enzymes in the respiratory burst of plants (Podgórska et al. 2017). For such, plants have a machinery of antioxidant molecules and enzymes that helps to mitigate the deleterious effects, particularly the oxidative stress (Das and Roychoudhury 2014). Among these, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) stand out, which act in the maintenance of homeostasis redox (Skukla et al. 2018).

An alternative to improve crop yield under water deficiency conditions is the application of biostimulants that act to protect plants, minimizing the adverse effects caused by environmental stresses (Calvo et al. 2014). Biostimulants are classified as products containing active ingredients capable of directly or indirectly enhancing plant development (Maćik et al. 2020), consisting of macro and micronutrients, as well as phytohormones and other beneficial substances for plant metabolism (Kocira et al. 2018). Their primary characteristic is the supplementation of nutrients and activation of physiological functions throughout the plant development process, and may be applied via soil, irrigation systems or foliar spraying (Mariani et al., 2017; Wozniak et al. 2020).

Biostimulants are innovative agricultural techniques to protect plants (Povero et al. 2016). The use of biostimulants in agriculture has been gradually increasing, showing positive effects under stress conditions (Calvo et al. 2014; Fleming et al. 2019). These products have been used in soybean crops to enhance plant response to stress and increase yield by preserving metabolism, nutrient and water absorption, as well as the activation of antioxidant activity mechanisms.

It is important to understand how biostimulants affect plants under water stress in order to understand the specific mechanisms of action. Therefore, the hypothesis of this study is that the use of biostimulants mitigates the effect of water deficit through the maintenance of plant metabolism, which is mainly reflected in productivity. To answer the question, biometric, physiological, biochemical and productive parameters were analyzed to investigate the capacity of a biostimulant based on essential nutrients and amino acids to mitigate the effects of water stress in soybean.

2 Materials And Methods

2.1 Experimental location and setup, and treatments

The experiment was conducted in greenhouse of the Department of Crop Production of the School of Agricultural Sciences of the São Paulo State University (UNESP), in Botucatu, São Paulo, Brazil, during the crop year of 2019/20. The location's geographical coordinates are 22°50'31"S, 48°25'29"W at an altitude of 795 m. The experiment used seeds of the soybean cultivar 95R95-IPRO, 2018/19 season crop, sown in 24 pots on December 7, 2018, obtaining five plants per 14 L pot. The soil used was classified as Red-
Yellow Latosol (RYL), consisting of 61% clay, 18% silt, and 21% sand, and its nutritional characteristics were corrected and the physicochemical characteristics are shown in Table 1.

| pH | O.M. | P<sub>resin</sub> | K | Ca | Mg | H + Al | SB | CEC | V | Clay | Silt | Sand |
|----|------|------------------|---|----|----|--------|----|-----|---|------|------|------|
| 5.4 | 24 | 15 | 6.7 | 36 | 14 | 32 | 57 | 89 | 64 | 614 | 196 | 190 |

OM: Organic matter, SB: sum of bases, CEC: cation exchange capacity, V: base saturation.

Fertilization occurred according to the chemical analysis for fertility purposes (Table 1) and recommendation for the cultivation of soybean (Raij et al. 1996). All tested treatments received a standard seed treatment with the recommended dose of *Bradyrhizobium*-based inoculant. In the sowing, 50 kg ha<sup>-1</sup> of simple super phosphate and 20 kg ha<sup>-1</sup> of potassium chloride were applied.

Greenhouse climate conditions were logged throughout the experiment using Datalogger (Instrutherm, HT-500, São Paulo, Brazil) (Fig. 1). Photosynthetically active radiation (PAR) within the greenhouse was monitored by a quantometer (QMSS-E Quantum Apogee PAR Meter, Logan, UT, USA), with an average daily reading of 833.5 µmol m<sup>-2</sup> s<sup>-1</sup>.

The adopted experimental design was casualized blocks, with six treatments and four repetitions. During the V4 growth stage of soybean cultivation, all treatments – except T1 – were submitted to a continuous water deficit of 50% of field capacity until the moment of analysis.

The maintenance of the water requirements of the treatments was performed daily by the method of the soil water retention curve and weighing of the pots. Thus, water deficit was imposed by weighing the pots, saturating the sampling of pots with water, draining for 12 hours to reach the field capacity (FC) and weighing again to determine the mass of water in this situation. From then on, and with the aid of a table of maximum soil retention capacity and of the equation:

\[ W = W_{fc} - W_{d} \]

Where:

- \( W \) = water to be added to the pot (mL);
- \( W_{fc} \) = initial pot weight with soil moisture at field capacity or 50% (g);
- \( W_{d} \) = daily pot weight (g).

The pots were watered according to the treatment, that is, 100% of the FC for treatments without water deficiency and 50% of the FC with water deficiency. As a result, daily weighing and rehydration of the pots...
were carried out so that they reached the desired levels again.

Treatments consisted of five biostimulant doses. Foliar applications occurred during the R1 growth stage. Applications were carried out using a high-pressure backpack sprayer (CO$_2$) equipped with a spraying boom with two nozzles 0.5 m apart, with a spray volume of 200 L ha$^{-1}$, constant pressure of 1.5 bar.

Treatments consisted of T1- dose 0.0 kg ha$^{-1}$ (without water deficit stress); T2 - dose 0.0 kg ha$^{-1}$ (with water deficit stress); T3 - dose 0.25 kg ha$^{-1}$; T4 - dose 0.5 kg ha$^{-1}$; T5 - dose 0.75 kg ha$^{-1}$; T6 - dose 1.0 kg ha$^{-1}$.

The biostimulant is formulated with mineral components and organic like macronutrients and micronutrients chelated with EDTA, and the amino acid glycine betaine according shown in the Table 2.

| Composition                  | (w/w) |
|------------------------------|-------|
| Nitrogen (N)                 | 4.0%  |
| Phosphorous (P$\text{\textsubscript{2}}$O$\text{\textsubscript{5}}$) | 21.0% |
| Iron (Fe EDTA)               | 0.5%  |
| Copper (Cu EDTA)             | 0.3%  |
| Boron (B)                    | 0.5%  |
| Manganese (Mn EDTA)          | 3.0%  |
| Zinc (Zn EDTA)               | 3.0%  |
| Glycine betaine              | 12.0% |
| pH (1%)                      | 3.6%  |

*Biostimulant in solid formulation; no granulometric specification

2.2 Determination of physiological variables

Physiological evaluations were carried out during the R6 phenological growth stage (plant stress peak) and consisted of the following variables: leaf gas exchanges, based on the net CO$_2$ assimilation rate ($A$), stomatal conductance ($g_s$), transpiration rate ($E$), leaf temperature (Tl) and intercellular CO$_2$ concentration ($C_i$), using an Infrared Gas Analyzer (IRGA) (LI-COR Biosciences Inc., Li-6400xt, Lincoln, Nebraska, USA), with measurements taken between 9:00 a.m. and 11:30 a.m., using the atmospheric CO$_2$ concentration, with room temperature and humidity, and constant photosynthetically active radiation (PAR) (1500 µmol photons m$^{-2}$ s$^{-1}$). Water-use efficiency (WUE) was calculated based on the $A/E$ ratio, and carboxylation
efficiency (CE) was calculated based on the $A/C$ ratio. The SPAD index was measured through a portable chlorophyll meter (SPAD-502®, Minolta, Konica Minolta Sensing, Inc., Osaka, Japan).

### 2.3 Determination of antioxidant compound and enzymes

To analyze the antioxidant enzymes SOD, APX, CAT, POX, reductase nitrate (RN), and the non-enzymatic compound proline (Prol), samples were collected during the R6 phenological growth stage (plant stress peak).

For the activity of enzymes SOD, CAT, POX and APX, 300-mg samples of expanded leaves were milled in liquid nitrogen and added to a homogenization medium. The medium consists of a potassium phosphate buffer 0.1 M, pH 6.8, ethylenediaminetetraacetic acid (EDTA) 0.1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, polyvinylpyrrolidone (PVPP) 1% (p/v). Next, homogenized samples were centrifuged in a refrigerated centrifuge (Hettich, Universal 320R, Tuttlingen, Germany) at 12,000 g at 4°C for 15 minutes and the supernatant was used as crude enzyme extract.

For SOD activity, an aliquot of 50 µL of crude extract was added to 2,950 µL of reaction medium, consisting of sodium phosphate buffer 50 mM (pH 7.8) containing methionine 13 mM, p-nitroblue tetrazolium (NBT) 75 µM, EDTA 0.1 mM and riboflavin 2µM. The reaction was performed in a chamber with fluorescent light of 15 W at 25 ºC for 10 minutes (Del Longo et al. 1993). Subsequently, lighting was interrupted and the absorbance of blue formazan resulting from NBT photoreduction was determined in a spectrophotometer at 560 nm (Shimadzu, UV-2700, Kyoto, Japan). The reaction blank consisted of a mixture between the plant sample and the reaction medium kept in the dark, under the same temperature and time conditions. A SOD unit was defined as the quantity of enzyme required to inhibit NBT photoreduction by 50%. Results were expressed in U min$^{-1}$ mg$^{-1}$ protein.

For CAT activity, in turn, an aliquot of 50 µL of crude extract was added to 950 µL of reaction medium, consisting of sodium phosphate buffer 50 mM (pH 7.0) and H$_2$O$_2$ 12.5 mM (Havir and Mchale 1987). Absorbance was obtained in a spectrophotometer (Shimadzu, UV-2700, Kyoto, Japan) at the wavelength of 240 nm after 1 minute. Enzymatic activity was determined by using the absorbance and absorption coefficient of 36 M$^{-1}$ cm$^{-1}$ and results were expressed in µmol of H$_2$O$_2$ min$^{-1}$ mg$^{-1}$ protein.

For POD activity, an aliquot of 100 µL of crude extract was added to 4900 µL of reaction medium, consisting of sodium phosphate buffer 25 mM (pH 6.8), pyrogallol 20 mM and H$_2$O$_2$ 20 mM (Kar and Mishra 1976). The production of purpurogallin was determined by the measure of spectrophotometer absorbance (Shimadzu, UV-2700, Kyoto, Japan) at the wavelength of 420 nm, at 25°C. Enzymatic activity was calculated using absorbance and the molar extinction coefficient of 2.47 mM$^{-1}$ cm$^{-1}$ (Chance and Maehley 1955) and expressed in µmol of purpurogallin min$^{-1}$ mg$^{-1}$ protein.

For APX activity, an aliquot of 100 µL of crude extract was added to 900 µL of reaction medium, consisting of sodium phosphate buffer 0.05 M (pH 7.0), ascorbic acid 0.8 Mm and H$_2$O$_2$ 1.0 Mm (Nakano and Asada 1981). Enzymatic activity was determined by the measure of spectrophotometer absorbance
(Shimadzu, UV-2700, Kyoto, Japan) at the wavelength of 290 nm, at 25°C, considering the molar extinction coefficient of \(2.8 \text{ Mm}^{-1} \text{ cm}^{-1}\). Results were expressed in µmol of ascorbic acid min\(^{-1}\) mg\(^{-1}\) protein.

For Prol determination, 100 mg of leaf tissue were homogenized in 2 mL of sulfosalicylic acid 3% (p/v) and placed in the refrigerated centrifuge (Hettich, Universal 320R, Tuttlingen, Germany) at 6300 \(g\) for 10 min. Samples of 100 µL of the extract were added to 200 µL of acid ninhydrin solution (1.25 g ninhydrin, 30 mL glacial acetic acid, and 20 mL of phosphoric acid 6M) and the mixture was incubated at 100 ºC for 1 hour. The reaction was paralyzed in ice bath and supernatant absorbance was measured in a spectrophotometer (Shimadzu, UV-2700, Kyoto, Japan) at the wavelength of 520 nm. Absorbance results were compared to the standard curve of proline (0 to 100 µg mL\(^{-1}\)) (Bates 1973) and results were expressed in µmol proline g\(^{-1}\) fresh matter (FM)\(^{-1}\).

To determine RN activity, 200 mg of leaf sample was placed in a tube with penicillin and added 10 mL of the extraction solution; subsequently, plants were vacuum-incubated for 3 cycles of 2 minutes each. After incubation, samples were placed in water bath for another 30°C for 1 hour. Next, 1 mL of the extracted solution was collected and transferred to tubes, where 1 ml of the sulfanilamide solution and 1 mL of the N-Naphthyl solution were added; readings were made through spectrometry at 540 nm, in accordance with the methodology proposed by Jaworski (1971).

2.4 Determination of biometric parameters of plants

Biometric evaluations were collected during R6 phenological growth stage (plant stress peak) and consisted of variables of leaf area (LA) (cm² plant\(^{-1}\)), number of branches (NB); shoot dry matter mass (SDM) (g plant\(^{-1}\)); root dry matter mass (RDM) (g plant\(^{-1}\)); height of plants (PH) (cm); diameter of plant stem (SD) (cm) and number of primary stem nodes (NN).

LA was quantified using a meter (Li-COR Biosciences Inc., Li-3100C, Lincoln, NE, USA). The height of plants was calculated using measuring tape from the base to the apex of plants. NB by direct count. The diameter of the base stem was obtained with a digital caliper (MeterMall, 150 mm and reading 0.1 mm, Marysville, OH, USA).

SDM and RDM was obtained by collecting a plant sample and inserting it in a forced air circulation drying incubator (Fanem, 330/5, São Paulo-SP, Brazil) at 65 ºC until reaching constant mass, and each sample was later weighed separately in a precision analytical scale (Shimadzu, BL-3200H, Kyoto, Japan).

NN was counted after washing the roots with water.

2.5 Determination of production components

The evaluations of production components were collected during harvesting stage, when grains had a humidity of approximately 13% and consisted in counting the average number of pods per plant (NPP), average number of pods with 1 grain (NP1), average number of pods with 2 grains (NP2), and average
number of pods with 3 grains (NP3), and productivity (P). P (g plant⁻¹) was obtained through the mass of grains measured in a precision analytical scale (Shimadzu, BL-3200H, Kyoto, Japan), adjusting humidity to 13%.

2.6 Statistical analysis

Results were submitted to variance analysis, polynomial reduction to assess product doses under water deficit and mean test to compare doses with the control without water deficit and without biostimulant application, at a level of 0.05 of probability. The non-significance of the regression deviation and/or higher value of the determination coefficient (R²) express the significance of parameters of the statistical model, using the statistics software SISVAR® (Ferreira 2014). Pearson's correlation analysis was performed with normalized data from the treatments adopted to verify the relationship among analyzed variables. Pearson's correlation heatmap was generated with software RStudio® (R Software (R Development Core Team)).

3 Results

3.1 Physiologicfal variables

Highest A highest was observed without water deficit. However, under water deficit conditions, the dose of 0.5 kg ha⁻¹ of the biostimulant reached similar A in compared to 0, 0.75 and 1.00 kg ha⁻¹ doses. Under water deficit conditions and biostimulant application conditions, A results were adjusted to the quadratic model and increased by 34.75% up to a dose of 0.5 kg ha⁻¹ compared to a dose of 0 kg ha⁻¹, with subsequent reduction in larger doses (Fig. 2a).

There was no significant difference in gs between the different amounts of biostimulant tested, except between the dose of 0.25 kg ha⁻¹ and 0 kg ha⁻¹ without water deficit, with a reduction of 266.38% in gs (Fig. 2b).

The use of biostimulant under water deficit conditions increased Ci by 197.96% with the dose of 0.5 kg ha⁻¹ compared to the dose 0 kg ha⁻¹ (Fig. 2c). However, there was no significant effect of biostimulant application on E (Fig. 2d), Tl (Fig. 2e) and WUE (Fig. 2f).

Higher CE was observed under the application of 0 kg ha⁻¹ of biostimulant under water deficit conditions, but without significant difference from the control and at doses 0.25 kg ha⁻¹ and 0.75 kg ha⁻¹. Lower CE was observed under the effect of the 0.5 kg ha⁻¹ and 1.0 kg ha⁻¹ doses, which were similar to the 0.25 kg ha⁻¹ and 0.75 kg ha⁻¹ doses (Fig. 2g).

The relative chlorophyll content increased by 24% under the 0.5 kg ha⁻¹ dose compared to the 0 kg ha⁻¹ dose of the biostimulant in plants subjected to water deficit conditions (Fig. 2f).

3.2 Antioxidant compound and enzymes
SOD activity was higher under application of 0.5 kg ha\(^{-1}\) under water deficit conditions, with an increase of 420% compared to plants that did not receive biostimulant and 86.57% compared to the control (Fig. 3a).

CAT activity increased 167.24% under application of 0.5 kg ha\(^{-1}\) of biostimulant and water deficit compared to 0 kg ha\(^{-1}\), but it did not differ statistically from the control and doses 0.25, 0.75 and 1.00 kg ha\(^{-1}\) (Fig. 3b).

Higher APX activity was observed under the application of 0.75 kg ha\(^{-1}\) of biostimulant, with an increase of 695.04% in relation to the 0 kg ha\(^{-1}\) dose, but with no significant difference with the 0.5 kg ha\(^{-1}\) dose (Fig. 3c). While the POX activity was not influenced by the evaluated treatments (Fig. 3d).

Under water deficit, RN activity increased by 134.15% with application of 0.5 kg ha\(^{-1}\) compared to the dose of 0 kg ha\(^{-1}\), however, it did not differ from control and dose 0.25 kg ha\(^{-1}\) (Fig. 3e).

Higher accumulation of Prol was observed at the dose of 0.5 kg ha\(^{-1}\), with an increase of 105.79% in relation to the dose of 0 kg ha\(^{-1}\), but it was similar to the dose of 0.75 kg ha\(^{-1}\) (Fig. 3f).

### 3.3 Biometric components

The highest PH was seen in the control without water deficiency. Under water deficit conditions, the highest plant height was observed in the biostimulant dose of 0.5 kg ha\(^{-1}\), which did not differ from the doses of 0 kg ha\(^{-1}\) and 0.75 kg ha\(^{-1}\) (Fig. 4a).

SD was not affected by treatments (Fig. 4b). However, NB reduced as the biostimulant dose increased. Highest NB was observed in the sample with no water deficit, which did not differ from samples under water deficit and biostimulant application from dose 0 to dose 0.75 kg ha\(^{-1}\). Smallest NB was observed in the dose of 1.0 kg ha\(^{-1}\) (Fig. 4c).

The largest LA was observed under dose of 0.5 kg ha\(^{-1}\), with increase of 278.75% compared to the dose of 0 kg ha\(^{-1}\) (Fig. 4d). While NN was highest under application of 0.5 kg ha\(^{-1}\), with an increase of 90% compared to dose 0 kg ha\(^{-1}\) and 73% compared to the control (Fig. 4e).

SDM under dose of 0.5 kg ha\(^{-1}\) under water deficit conditions increase of 66.35% compared to plants that did not receive biostimulant, with subsequent reduction at higher doses, and an increase of 44.74% compared to the control (Fig. 4f). This performance was also observed in RDM, however the increase in this case was 26.33% in the dose of 0.5 kg ha\(^{-1}\) compared to plants that did not receive biostimulant, and of 12.00% compared to the control (Fig. 4g).

### 3.4 Production components

NPP was negatively impacted by water deficit conditions even with the application of the biostimulants, thus the control treatment presented higher NPP (Fig. 5a). The NP1 was not affected by treatments,
resulting in an average of 2.11 pods plant\(^{-1}\) (Fig. 5b).

NP2 increased 105.18% with the dose of 0.5 kg ha\(^{-1}\) compared to the dose of 0 kg ha\(^{-1}\), however, it did not differ of the control and dose 0.25 (Fig. 5c). This tendency was also observed in NP3, with increase of 65.09% compared to the dose of 0 kg ha\(^{-1}\), but without significant difference in relation to control, doses 0.25 and 0.75 kg ha\(^{-1}\) (Fig. 5d).

P increased 22.15% under 0.5 kg ha\(^{-1}\) of biostimulant, compared to dose 0 kg ha\(^{-1}\), in addition to 19.55% compared to the control (Fig. 5e). There was a greater correlation among productivity and LA, NN, SOD, SDM, RDM, SPAD index, \(C_i\) and CAT (Fig. 6).

4 Discussion

Photosynthesis is one of the processes most impacted by drought stress (Pinheiro and Chaves 2011). The ability of plants to adapt to stress conditions is an important factor to mitigate the effects of drought stress (Basu et al. 2016). Such adaptation can influence the maintenance or increase in productivity and biological processes, such as photosynthesis and, as a result, the growth and development of plants. In this study, under water deficit conditions, the use of biostimulant in the dose of 0.5 kg ha\(^{-1}\) increased the assimilation of CO\(_2\) compared to the sample not treated with biostimulant. In fact, the correct biostimulant dosage directly contributes to enhancing the efficiency of the physiological process (Fleming et al. 2019).

The relief of water deficiency effects in soybean plants promoted by the biostimulant was also seen in stomatal conductance. The increase of stomatal conductance based on biostimulant dose resulted in keeping stomata open, which enabled the continuous addition of CO\(_2\) in the Calvin cycle, contributing to a higher flow of Ca\(^{2+}\) and K\(^+\) at stomatal level, essential ions to protect ionic and osmotic stress, in addition to the improvement of the water rate and photosynthesis through photosynthetic efficiency (Van Oosten et al. 2017). On the other hand, under conditions of scarce water supply, abscisic acid quickly accumulates and closes the stomata, reducing mesophyll conductance, CO\(_2\) assimilation and the electron transport rate (Siddique et al. 2016; Taiz and Zeiger 2017).

Despite the maintenance of \(A\) and \(g_s\) under water deficit conditions and with biostimulant application at the dose of 0.5 kg ha\(^{-1}\), there was an increase in \(C_i\) and reduction of CE. Lower CE values associated to higher \(C_i\) reflect biochemical alterations in the photosynthesis machinery of soybean, and lower activity of the RUBISCO enzyme (Zhang et al. 2016). Therefore, despite the potential damage to the photosystem, the biostimulant used in this study helped to maintain the stomatal control of plants and gas exchange, mainly under the effect of the 0.5 kg ha\(^{-1}\) dose. The action of biostimulants varies according to the species, growth stage and doses (Du Jardin 2015).
The similar effect on plant transpiration in the different treatments may be related to the maintenance of photosynthetic efficiency and, consequently, to the production of sugars that play an important role in the osmotic balance in conditions of water deficit. In fact, under water deficit conditions, plants promote changes in the accumulation of soluble sugars, in order to maintain the hydric potential, mitigate oxidative damage and other functions to help maintain plasma membrane stability during extended stress periods (Arabzadeh 2012).

However, the application of biostimulant had a beneficial effect on plants under water deficit stress, which showed WUE values similar the control sample without stress (Santaniello et al. 2017). This effect could be associated to the maintenance of $g_s$, without prejudice to $E$, by maintaining the osmotic potential due to the biostimulant's application. Prado et al. (2013) demonstrated that the leaf hydric conductance is regulated by the osmotic permeability coefficient of cell membranes. In addition, applying the biostimulant could reduce the concentration of abscisic acid (ABA) and help prevent ABA production from resulting in stoma closure, under stress conditions, ultimately reducing $g_s$, directly influencing water use efficiency (Cohen et al. 2011).

The reduction of chlorophyll content in plants under water deficit conditions is broadly known (Farooq et al. 2009b; Silva et al. 2014). The biostimulant's mitigating effect in the dose of 0.5 kg ha$^{-1}$ in plants submitted to water deficit stress can be seen by the increase in the SPAD index. This increase may be related to the composition of the biostimulant, which consists of N, Mn, Fe, Zn, Cu and Glycine betaine, which provide substrates for metabolic syntheses, such as chlorophyll molecules, which may be related to the increase of activity of antioxidant complex enzymes (Van Oosten et al. 2017).

According to Oliver et al. (2020), plants use different mechanisms simultaneously, such as antioxidant enzymes and protein, to keep the photosynthetic machinery active even during drought stress conditions. In this study, the biostimulant induced an increase in antioxidant enzymes SOD, CAT and APX in soy plants submitted to water deficit stress. SOD acts in the dismutation of $O_2^•−$ into $O_2$ and $H_2O_2$, as one of the first enzymes in ROS elimination in plants under stress conditions (Das and Roychoudhury 2014). This increase is possibly related to a higher availability of ions in the biostimulant's composition, since SODs are categorized from a connection with ions in different cell compartments.

The use of biostimulant improved the activity of antioxidant enzymes, especially when using 0.5 kg ha$^{-1}$. SOD is the first line of defense against accumulation of ROS resulting from stresses, acts in the dismutation of the superoxide anion ($O_2^−$) to form $H_2O_2$ in order to reduce the level of ROS generated by oxidative stress (Caverzan et al., 2016). While CAT is responsible for removing $H_2O_2$, reducing it to two molecules of $H_2O$ and its activity is associated with increased levels of photorespiration (Caverzan et al. 2016; Rivas et al. 2017). APX breaks down this ROS using ascorbic acid + $H_2O$ as a reducing agent and is part of the glutathione cycle pathway which, together with NAPDH, forms redox pairs that are essential for maintaining homeostasis and combating oxidative damage (Das and Roychoudhury 2014; Noctor et al. 2016).
Our results demonstrate that the use of biostimulant similarly influenced the activity of POX in all tested treatments, suggesting a greater effect of the product on the other enzymes of the antioxidant complex mentioned above. In fact, POX was more correlated to Prol (Fig. 6).

Biostimulants activate mechanisms plants, increasing the activity of antioxidant compounds and enzymes. Therefore, plants show greater tolerance to stress throughout their life cycle, as well as improved growth (Calvo et al. 2014). Kocira (2019) reported a considerable increase in soy antioxidant activity and productivity following the foliar application of biostimulant based on amino acids and micronutrients.

The application of biostimulant at the dose of 0.5 kg ha\(^{-1}\) promoted a nitrogen use under water deficit conditions similar to the samples with no drought stress conditions, due to the maintenance of the nitrate reductase enzyme's activity. Prolonged water deficit periods can be harmful to plants, since they reduce the absorption and transport of water and nutrients, altering the concentration of several metabolic processes, such as the production of amino acids and carbohydrates (Dat et al. 1998). The maintenance of \(A\) under water deficit conditions promoted by the dose of 0.5 kg ha\(^{-1}\) of the biostimulant may be related to the exogenous application of nutrients and amino acids (Teixeira et al. 2019). Therefore, plants showed a reduction in the stress effects and, as a result, improved growth conditions.

During a drought, the accumulation of osmoprotective molecules helps maintain cell osmotic balance and reduces the plant hydric potential, maintaining soil water absorption and therefore assuring the continuity of metabolic and growth processes (Sandres and Arndt 2016). The increased proline concentration in soy plants that received the biostimulant dose of 0.5 kg ha\(^{-1}\) indicates an increased tolerance to drought and may be associated to the number of compounds of the proline biosynthesis metabolic pathway, based on the use of the biostimulant. Proline biosynthesis generally occurs based on the phosphorylation of glutamate, which is dependent upon nitrogen (Meena et al. 2019), a nutrient present in the biostimulant composition. Proline is an excellent cleaner of OH\(^{-}\) and \(^{1}\)O\(_2\) and can efficiently mitigate the damages caused by lipid oxidation (Das and Roychoudhury 2014; Meena et al. 2019).

Water deficit conditions change the physiological and biochemical processes of plants, reduces plant growth and, as a result, the productive capacity of crops. Stem diameter did not show any significant difference compared to the control sample, corroborating the preventive effect of the biostimulant under drought conditions. On the other hand, the lower plant height resulted in a greater leaf area under drought conditions and with biostimulant application (i.e. there was a biomass partition with investment in photosynthesizing tissues, evidenced by the greater leaf area). In general, plants presented a reduction of leaf area under drought conditions. However, the use of biostimulant at the dose of 0.5 kg ha\(^{-1}\) induced the increase of leaf area, potentially assisting in the stress adaptation process, reducing energy demand, despite the increase in leaf area.

The number of pods per plant was significantly affected without the application of biostimulant, presenting values below the control condition. The mitigating effect of the biostimulant resulted in a
production increase under drought stress conditions, related to the increase of tolerance mechanisms due to the modulation of ROS concentration and regulation of the concentration of phytohormones and lipids (Rouphael and Colla 2020).

The use of biostimulant at the dose of 0.5 kg ha\(^{-1}\) contributed to an increase in productivity, also exceeding the conditions with no drought stress, as it provided an increase in leaf area to increase the surface for absorbing sunlight and transforming it into energy chemical, SPAD index, number of nodules for better use in N assimilation, increased activity of antioxidant enzymes and dry matter mass.

The effect on production components was most expressive in the number of pods with two and three grains, indicating that the product maintained the pod formation period similar to that of the control sample with no drought stress, contributing to plant productivity. Final plant productivity depends on the remobilization of photosynthetic products to form pods and fill grains (Wijewardana et al. 2018), evidenced by the increased antioxidant activity and accumulation of the metabolic osmoprotective agent proline (Nishikawa et al. 2005). Therefore, the use of biostimulant helps increase drought tolerance in a more sustainable and economically feasible manner compared to genetic enhancement (Fleming et al. 2019).

Our results are in agreement with Rosa et al. (2021) who observed induction of soybean plants to recovery after water deficit under the action of a biostimulant based on *Ascophyllum nodosum* (L.) seaweed extract and fulvic acids, through improvements in the physiological and biochemical aspects of the plants, which was reflected in income gains.

Soybean plants can enhance the efficiency of physiological, biochemical and yield characteristics when treated with biostimulant based on macro- and micronutrients and amino acids under drought conditions. Our findings demonstrate that the use of biostimulant can mitigate the effects caused by water deficit, by increasing leaf area, plant dry matter, in addition to maintaining the assimilation of carbon and stomatal conductance, reducing leaf temperature, and effectively producing antioxidant compounds, reflecting in the accumulation of biomass and production of grains in soybean crop. This positive effect was observed in plants treated with the dose of 0.5 kg ha\(^{-1}\) of biostimulant, which improved soy plants’ process of adaptation to drought stress. It is important to note that our findings shed new light onto the processing of biostimulants based on macro and micronutrients and amino acids in soybean crop, which is extremely important for sustainable use in agriculture considering the current climate change scenario.

Declarations

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Authors Contribution RAR, DMRS and JCCS performed the experiments, collected and analyzed data and wrote the original draft; RAR and MAS contributed to the conceptualization of research goals,
experimental design, acquisition of funding, project administration, and manuscript review.

**Data availability** Contact corresponding author.

**Conflict of interest** The authors declare that they have no conflict of interest.

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Figures

**Figure 1**

Minimum (Minimum T), maximum (Maximum T) and average (Average T) temperature and minimum (Minimum H), maximum (Maximum H) and average (Average T) air relative humidity in the greenhouse during the experiment.

**Figure 2**

Physiological characteristics of soybean plants subjected to the application of different doses of biostimulant under water deficit. CO₂ assimilation - \( A(A) \), stomatal conductance - \( g_s(B) \), internal CO₂ concentration - \( C_i(C) \), transpiration - \( E(D) \), leaf temperature (E), water use efficiency - \( WUE(F) \), carboxylation efficiency - \( CE(G) \) and SPAD index (H). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. NS = Not significant.
Figure 3

Activity of antioxidant enzymes and compound in soybean plants subjected to the application of different doses of biostimulant under water deficit. Superoxide dismutase - SOD (A); catalase - CAT (B); ascorbate peroxidase - APX (C); peroxidase - POX (D); reductase nitrate (E) and proline (F). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. NS = Not significant.

Figure 4

Biometric characteristics of soybean plants subjected to the application of different doses of biostimulant under water deficit. Plant height (A), stem diameter (B), number of lateral branches (C), leaf area (D), number of nodules in the main root (E), shoot dry matter weight (F) and root dry matter weight (G). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. NS = Not significant.
Figure 5

Components of production of soybean plants subjected to the application of different doses of biostimulant under water deficit. Number of pods per plant (A), number of pods with 1 grain (B), number of pods with 2 grains (C), number of pods with 3 grains (D) and productivity (E). Means followed by the same letter do not differ from each other by the Tukey test at 5% probability. NS = Not significant.
Figure 6

Pearson’s correlation between CO$_2$ assimilation rate ($A$), stomatal conductance ($g_s$), transpiration rate ($E$), leaf temperature ($T_l$), intercellular CO$_2$ concentration ($C_i$), Water-use efficiency (WUE), carboxylation efficiency (CE), SPAD index (SPAD), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidases (POX), nitrate reductase (RN), proline (Prol), leaf area (LA), number of branches (NB), shoot dry matter mass (SDM), root dry matter mass (RDM), height of plants (PH), diameter of plant stem (SD), number of primary stem nodes (NN), number of pods per plant (NPP), average number of pods with 1 grain (NP1), average number of pods with 2 grains (NP2), and average number of pods with 3 grains (NP3), and productivity (P). **Significant at 5% *Significant at 1%