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Biostimulant potential in mitigation of damage from herbicides applied in post-emergency in soybean crops

Potencial de bioestimulante na mitigação de danos de herbicidas aplicados em pós-emergência na cultura da soja

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Highlights

Herbicides increased the photosynthetic rate of soybean plants.
ALS and PROTOX inhibitor herbicides do not inhibit gas exchange in soybeans.
Ascophyllum nodosum reduces damage caused by Cloransulam and Lactofen herbicide.
Biostimulants have the potential to mitigate the effects of herbicide application.
Cloransulam and lactofen altered chlorophyll a fluorescence parameters.

Abstract

An alternative to minimize the effects of herbicide resistance induction is the rotation of active ingredients. Among the widely used herbicides, inhibitors of acetolactate synthase (ALS) and protoporphyrinogen oxidase (PROTOX) enzymes stand out. However, the use of these herbicides can cause a series

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of damages to the photosynthetic metabolism of soybeans and compromise crop development. A potential alternative to mitigate these effects is the application of a biostimulant derived from the algae Ascophyllum nodosum. Therefore, the objective of this study was to assess the potential of a biostimulant derived from A. nodosum in mitigating damage to the photosynthetic process of soybean plants treated with herbicides in a greenhouse and post-emergence. To achieve this, an experiment was set up in a greenhouse with nine treatments arranged in a factorial design with 3 herbicide applications (no herbicide, Cloransulam, and Lactofen) × 3 biostimulant applications (no application, 3, and 6 days after herbicide application (DAA)). Herbicide applications were carried out 25 days after sowing, at the vegetative stage 3 (V3). Gas exchange evaluations, chlorophyll indices, and chlorophyll a fluorescence were measured at 2 and 10 DAA of the herbicides. Based on the results obtained, it was possible to conclude that 2 days after herbicide application, the herbicides did not cause inhibition of gas exchange in soybean plants. Although there was no isolated effect of the biostimulant on fluorescence parameters, its application at 3 DAA of the herbicides led to increases in photosynthetic rate, stomatal conductance, and carboxylation efficiency of the soybean plants. On the other hand, at 10 DAA of the herbicides, it was found that Cloransulam and Lactofen altered all evaluated chlorophyll a fluorescence parameters, but no protective effect of the biostimulant was observed. It can be concluded that the biostimulant has potential for use in soybean crops to mitigate the effects of selective herbicide application.
Key words: Ascophyllum nodsosum. Glycine max. Cloransulam. Extrato de algas. Fotossíntese. Lactofen.

Resumo

Uma alternativa para minimizar os efeitos da indução de resistência aos herbicidas é a rotação de ingredientes ativos. Entre os herbicidas amplamente utilizados, destacam-se os inibidores das enzimas acetolactato sintase (ALS) e da protoporfirinogênio oxidase (PROTOX). No entanto, a utilização destes pode causar uma série de danos ao metabolismo fotossintético da soja e comprometer o desenvolvimento da cultura, tendo como alternativa para mitigar os efeitos a aplicação de bioestimulante derivado da alga Ascophyllum nodsosum. Portanto, objetivou-se com o presente estudo verificar o potencial de um bioestimulante derivado de A. nodosum em mitigar danos ao processo fotossintético de plantas de soja tratadas com herbicidas em casa de vegetação e pós-emergência. Para isto, foi instalado um experimento em casa de vegetação com nove tratamentos, em arranjo fatorial com 3 aplicações de herbicidas (sem herbicida; Cloransulan; Lactofen) X 3 aplicações de biostimulante (sem aplicação; 3 dias após a aplicação dos herbicidas (DAA); 6 DAA). Foram realizadas avaliações de trocas gasosas, índices de clorofilas e fluorescência da clorofila a. Por meio dos resultados obtidos foi possível concluir que aos 2 dias após a aplicação dos bioestimulantes, os herbicidas não proporcionaram inibição das trocas gasosas das plantas de soja. Por outro lado, aos 10 DAA dos herbicidas foi possível constatar que o cloransulam e lactofen alterou os parâmetros da fluorescência da clorofila a, porém não foi constatado efeito protetor do bioestimulante. Embora não tenha efeito isolado do bioestimulante nos parâmetros da fluorescência, sua aplicação aos 3 DAA dos herbicidas proporcionou incrementos na taxa fotossintética, condutância estomática e na eficiência da carboxilação das plantas de soja. É possível concluir que o bioestimulante tem potencial de uso na cultura da soja com o propósito de mitigar os efeitos da aplicação de herbicidas seletivos.
Palavras-chave: *Ascophyllum nodsosum. Glycine max.* Cloransulam. Extrato de algas. Fotossíntese. Lactofen.

Introduction

Soybeans are recognized as an economically important crop worldwide and are a predominant source of high-quality plant protein. However, soybean yield losses due to weeds exceed 50% (Datta et al., 2017). The interference caused by weeds can compromise physiological performance and, consequently, soybean grain productivity (Lamego et al., 2013). For this reason, the Roundup Ready RR® technology has been widely accepted, as it allows the use of glyphosate herbicide in post-emergence, given that the molecule provides broad-spectrum control and high efficacy against weeds (Garcia et al., 2024; Merotto et al., 2015; Bontempo et al., 2016).

As a side effect, the successive use of the same herbicide without adopting integrated weed management favors the emergence of herbicide-resistant biotypes (Beckie, 2011). In this context, an alternative to minimize the effects of resistance induction is the use of selective herbicides (R. S. de Oliveira et al., 2011), such as protoporphyrinogen oxidase (PROTOX) and acetolactate synthase (ALS) enzyme inhibitors, which are also registered for soybean cultivation. However, the use of these herbicides in certain applications can cause a series of damages to the soybean metabolism and compromise the crop's development and productivity (Karpstein & Waureck, 2020).

Lactofen acts by inhibiting the PROTOX enzyme, which is essential for the biosynthesis of chlorophylls and cytochromes (R. S. de Oliveira et al., 2011). Even though soybeans are considered a tolerant plant (Zanatta et al., 2020), they may experience oxidative stress when exposed to this herbicide. Oxidative damage affects the structure and functionality of the photosynthetic machinery by reducing chlorophyll levels and inactivating the reaction centers of photosystems. Oxidative damage in plants, including soybeans, occurs mainly due to the excessive accumulation of reactive oxygen species (ROS), which are normal byproducts of cellular metabolism, especially during the photosynthetic process. ROS include various types of reactive molecules such as superoxide and hydroxyl radicals, hydrogen peroxide, and singlet oxygen. In soybean plants, the excessive accumulation of these ROS can lead to a series of cellular damages such as lipid peroxidation, protein damage, and DNA mutation (Mansoor et al., 2022). Consequently, they reduce photosynthesis and plant productivity (Tripathy et al., 2007). On the other hand, the herbicide Cloransulam inhibits the activity of the acetolactate synthase (ALS) enzyme, which is essential for the biosynthesis of branched-chain aliphatic amino acids, leucine, isoleucine, and valine (Christoffoleti & Nicolai, 2016). This leads to a reduction in protein synthesis, interfering with cellular enzymatic reactions and the structural composition of the plants (Christoffoleti & Nicolai, 2016).

An alternative to mitigate the deleterious effects of these herbicides on the photosynthetic apparatus of soybeans is the use of biostimulants derived from the marine algae *Ascophyllum nodosum* (Du Jardin, 2015). Derivatives of *Ascophyllum nodosum* are attributed with promoting morphophysiological changes in the development of soybean plants (V. M. dos Santos et al., 2017; Silva et al., 2017; Mącik et al., 2020) due to their composition, which includes plant hormones, amino acids, vitamins, mineral nutrients, phenols, and carbohydrates (Cavalcante et al., 2020). Thus, the composition of the algae extract gives the biostimulant the ability to alter hormonal regulation, cell division, as well as chlorophyll content and nutrient partitioning in plants (Mahmoud et al., 2019; El-Samad et al., 2019). Several authors have reported the positive effect of biostimulants on physiological parameters of soybean plants (Kulkarni et al., 2019; Joshi-Paneri et al., 2020; Anli et al., 2020). This occurs because the presence of hormones and carbohydrates allows the biostimulant to act as an osmoregulator and modulator of stomatal opening, as well as influence protein and amino acid production (Yakhin et al., 2017; El-Katony et al., 2020; Chittora et al., 2020). Therefore, biostimulants are natural compounds or products that, when applied to plants, promote growth, enhance nutrient absorption, and improve tolerance to biotic (such as pests and diseases) and abiotic stresses (such as drought, salinity, heat, cold, and excessive radiation). In soybean plants, the use of biostimulants can positively impact metabolism, providing greater resilience and performance under adverse conditions. The main effects of biostimulants are improvements in nutrient absorption and transport, stimulation of plant hormone production, increased antioxidant activity, stimulation of carbon and nitrogen metabolism, osmotic regulation, protection against water stress, and increased resistance to pathogens and pests (Coelho et al., 2019; Mącik et al., 2020; Mrid et al., 2021), potentially minimizing the adverse effects of selective herbicides on soybean crops.

Based on the information presented, it is known that herbicides can negatively influence the physiological performance of soybean plants. However, it is believed that biostimulants derived from *A. nodosum* have the potential to mitigate the deleterious effects of these compounds (Guan et al., 2020; M. C. Oliveira et al., 2020). At the same time, the response of soybeans treated with biostimulants may vary according to the crop's developmental stage (Dourado et al., 2014). Therefore, the present study aimed to assess the potential of a biostimulant derived from *A. nodosum* in mitigating damage to the photosynthetic process of soybean plants treated with herbicides, as well as to determine the crop developmental stage most responsive to the biostimulant.

Materials and Methods

Experimental area and climatic characteristics of the region

The experiment was conducted in a greenhouse at the Instituto Federal Goiano – Campus Rio Verde (17°48'08" south latitude and 50°54'20" west longitude, at an altitude of 780 m) in the municipality of Rio Verde, Goiás, from November 2018 to January 2019. During the experiment, the climatic conditions were partially controlled to maintain air temperature at a maximum of 30°C and relative humidity at 70%, using the greenhouse's climate control system.

Experimental design and soil characteristics of the cultivation area

The experimental units consisted of polyethylene pots with a capacity of 12 dm^3, filled with soil collected from a depth of 0-0.20 m in an uncultivated Cerrado area, classified as dystroferric Red Latosol (H. G. Santos et al., 2018). The soil was broken up, sieved through a 2 mm mesh, and prepared using the TFSA (air-dried fine soil) method.

A sample of the collected material was analyzed and revealed the following chemical and physical characteristics: pH in $CaCl_2$: 5.95; Ca: 5.24 cmolc dm^{-3}; Mg: 2.28 cmolc dm^{-3}; Al: 0.05 cmolc dm^{-3}; H + Al: 2.97 cmolc dm^{-3}; K: 0.24 cmolc dm^{-3}; P: 71.56 mg dm^{-3}; CEC: 10.84 cmolc dm^{-3}; base saturation (V): 72.59%; aluminum saturation (m): 0.63%; organic matter (OM): 63.38%; clay: 265 g kg^{-1}, silt: 62 g kg^{-1}, and sand: 673 g kg^{-1}. The micronutrient analysis revealed Fe, Mn, Cu, and Zn levels of 131.0, 80.4, 10.1, and 20.4 mg dm^{-3}, respectively.

Based on the results of the soil chemical analysis, interpretation, and recommendations for the crop, liming was carried out to correct soil acidity. The planting fertilization was performed with the equivalent of 60 kg ha^{-1} of K_2O and 80 kg ha^{-1} of P_2O_5, according to Sousa and Lobato (2004). The soil foundation fertilization was carried out, as recommended by Novais et al. (1991), through dilution, using MAP and KCl in the amounts of 0.86 and 0.58 g kg^{-1} of soil, respectively, with urea applied in two doses, 20 and 40 days after emergence, with each application consisting of 0.9 g per pot in the experiment.

Water replenishment was performed daily in a localized manner, maintaining soil moisture at levels equivalent to 60% of the total pore volume. The water retention capacity of the substrate (field capacity) was determined by the gravimetric method, weighing all pots with dry soil and after saturation and subsequent drainage. The experiment was set up in a randomized block design with four replications, with treatments arranged in a factorial design (3 × 3), totaling 9 treatments. The first factor corresponded to the application of the herbicides lactofen (180 g a.i. ha^{-1}) and cloransulam (39.48 g a.i. ha^{-1}) in post-emergence, along with a treatment without herbicides; while the second factor consisted of two application times of the biostimulant, in addition to a treatment without biostimulant application.

Treatment application

The biostimulant used was Megafol® (Organic Carbon: 109.8 g L^{-1}; N: 36.6 g L^{-1}; K_2O: 97.6 g L^{-1}; Valagro®), which was applied at a dose of 1.0 L c.p. ha^{-1}. The commercial products of the herbicides were Pacto® (cloransulam, concentration 840 g kg^{-1}, WG, Corteva AgroSciences®) and Cobra® (lactofen, concentration 240 g L^{-1}, EC, Bayer®).

The soybean cultivar used in the study was Brasmax Power® (73710 IRF IPRO), characterized by indeterminate growth habit and early cycle, with a maturity group of 7.3 for the experiment's microregion. Initially, five soybean seeds were sown per experimental unit (pot), and subsequently, thinning was performed, leaving only two seedlings.

The herbicide application was performed when the soybean plants had three fully expanded trifoliate leaves (V3), twenty-five days after sowing. The biostimulant applications occurred when the soybean plants had four and five fully expanded trifoliate leaves (V4/V5), respectively at 3 and 6 days after herbicide application.

The treatments were applied using a CO_2-pressurized backpack sprayer, equipped with a six-nozzle boom using TT 110-02 double flat fan nozzles. The equipment was positioned 0.50 m above the soybean plants, set to 300 kPa, providing a spray volume equivalent to 200 L ha^{-1}. The applications were performed with the soil moist, and after the operation, the pots were returned to the greenhouse to ensure absorption of the applied products. The dates and climatic conditions at the respective treatment application times are described in Table 1.

Table 1
Climatic conditions recorded at the time of application of the different treatments. Rio Verde - GO

	Herbicides	Biostimulant	
Stage	V3	V4	V5
Wind speed (km h^{-1})	1,0	1,8	0,9
Temperature (oC)	26	28	27
Relative humidity (%)	65	60	55

[1] Neutral detergent fiber (NDFap) corrected for ash and protein; [2] Neutral detergent fiber free of nitrogen; [3] Nitrogen insoluble in neutral detergent (NIDN); [4] Nitrogen insoluble in acid detergent (NIAD).

The plants were evaluated for gas exchange, chlorophyll a fluorescence, and chlorophyll a, b, and total indices. The evaluations were carried out at 2 and 10 days after herbicide application (DAA) in soybean plants, when the plants had three and six fully expanded trifoliate leaves, respectively, between 8:00 a.m. and 12:00 p.m., always on the central leaflet of the youngest fully expanded trifoliate. The methodologies for each evaluated characteristic are described below.

Gas exchange, chlorophyll a fluorescence, and chlorophyll content

Gas exchange, chlorophyll a fluorescence, and chlorophyll content were evaluated at 2 and 10 days after herbicide application (DAA). Gas exchange was assessed using a portable infrared gas analyzer (IRGA), model 6800 (Li-Cor, Inc., Lincoln, Nebraska, USA) to obtain the photosynthetic rate (A) (μmol CO_2 m^{-2} s^{-1}), transpiration rate (E) (mmol H_2O m^{-2} s^{-1}), stomatal conductance (g^- in mol H_2O m^{-2} s^{-1}), and internal CO_2 concentration (Ci) (μmol CO_2 mol^{-1} air). From these data, the ratio between the internal (Ci) and external (Ca) CO_2 concentration (Ci/Ca) and the instantaneous carboxylation efficiency (EiC) (A/Ci) [(CO_2 m^{-2} s^{-1} kPa^{-1}) (μmol mol^{-1})$^{-1}$] were estimated.

Values for chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Chl T) were obtained using a portable chlorophyll meter (Brand: Falker, Model: ClorofiLog

CFL1030) (Porto Alegre, Rio Grande do Sul), and the values were expressed as the Falker Chlorophyll Index (FCI).

Chlorophyll a transient fluorescence was determined using a portable fluorometer (FluorPen FP100, Photon Systems Instruments) (Průmyslová, Drásov, Czech Republic) on the same leaf used for gas exchange evaluation, which was previously dark-adapted for 30 minutes to ensure complete oxidation of the photosynthetic electron transport system. Subsequently, the leaf was exposed to a pulse of 3000 μmol m^{-2} s^{-1} of blue light, measuring the minimal fluorescence (F_0) at 50 μs when all PSII (photosystem II) reaction centers are open, known as the O-step, followed by the J-step (at 2 ms), the I-step (at 30 ms), and the maximum fluorescence (F_v) when all PSII reaction centers are closed, known as the P-step. These values were used to estimate various bioenergetic indices of PSII, as described by Strasser et al. (2000). The F_v/F_0 ratio was calculated according to the formula $F_v/F_0 = (F_v\text{-}F_0)/F_0$. Specific energy fluxes were obtained for absorbed light per reaction center (ABS/RC); energy trapped per reaction center (TRo/RC); electron transport flux per reaction center (ETo/RC); energy dissipation per reaction center (DIo/RC); and the photosynthetic performance index based on absorption (PI).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the F-test ($p<0.05$) to determine significance between the evaluated factors. When a significant effect was detected, the SNK test ($p<0.05$) was applied to compare the means between the levels of each factor, using the SISVAR statistical software (Ferreira, 2011).

Results and Discussion

To support the understanding of the effects of abiotic stress on the photosynthetic apparatus, the evaluation of chlorophyll a fluorescence is a widely used method in physiological investigations of plants (Eullaffroy et al., 2009), including soybeans. In this study, among the chlorophyll a fluorescence parameters analyzed, only the energy capture flux per reaction center (TRo/RC) was significantly altered by herbicide application (Table 2). In this case, it was observed that the plants treated with cloransulam showed higher TRo/RC compared to the others, indicating that they did not experience photoinhibitory damage in PSII. Cloransulam, by inhibiting the ALS enzyme and blocking the synthesis of essential amino acids, may lead to an increase in TRo/RC due to an adaptive plant response. This adjustment may redistribute resources to optimize the photosynthetic apparatus, allowing for more efficient light capture and increased electron transport, especially when the herbicide does not directly damage the photosystems. In the case of lactofen, which generates reactive oxygen species by inhibiting PROTOX, the treated plants are able to activate antioxidant mechanisms that prevent damage to PSII. This defense process allows PSII to remain intact and functional, avoiding photoinhibition and preserving the efficiency of TRo/RC, even under oxidative stress caused by the herbicide (Traxler et al., 2023).

Table 2

Significance and mean values for chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl T), absorbed energy flux per active reaction center (ABS/RC), captured energy flux per reaction center (TRo/RC), electron transport flux per reaction center (ETo/RC), dissipated energy flux per active reaction center (DIo/RC), and performance index based on absorption (PIABS) at 2 DAA of herbicides, according to the treatments. Rio Verde - GO, 2018/2019 season

FV	Chl *a*	Chl *b*	Chl T
Treatments	3,305^{ns}	0,128^{ns}	4,591^{ns}
No Herbicide	25,17	5,77	30,95
Cloransulan	24,90	5,61	30,51
Lactofen	23,95	5,52	29,47
CV (%)	9,80	17,10	11,09

FV	ABS/RC	TRo/RC	ETo/RC	DIo/RC	PIABS
Treatments	0,050^{ns}	0,035*	0,015^{ns}	0,005^{ns}	0,144^{ns}
No Herbicide	2,38	1,89 b	0,95	0,50	1,75
Cloransulam	2,54	2,00 a	1,03	0,51	1,76
Lactofen	2,45	1,88 b	1,02	0,56	1,98
CV (%)	7,42	4,86	6,60	20,61	20,37

FV - source of variation; CV - coefficient of variation. **; * Significant at 1% and 5% probability, respectively, by F-test. ^{ns} not significant. Means followed by the same letters do not differ from each other by the SNK test at 5% probability.

When examining the chlorophyll a, chlorophyll b, and total chlorophyll indices, no significant differences were observed at 2 days after herbicide application (DAA) (Table 2). For herbicides that do not directly inhibit photosynthesis, their damage may not be evident immediately after plant exposure (Merotto et al., 2015), which likely occurred in this study. At 2 DAA, the maintenance of TRo/RC suggests that, despite lactofen being a PROTOX inhibitor, there was no significant increase in reactive oxygen species (ROS) to the point of causing damage to PSII, considering that the TRo/RC of plants treated with lactofen was similar to the control. Additionally, this similarity indicates that lactofen did not significantly induce ROS production, or that the plant activated antioxidant mechanisms to prevent photoinhibitory damage (Choudhury et al., 2017).

In parallel with the evaluations of chlorophyll indices and chlorophyll a fluorescence, gas exchange measurements were conducted to assess the potential of PROTOX and ALS inhibitors to cause damage to the photosynthetic capacity of soybean plants. In this case, it was observed that the treatments only did not alter the carboxylation efficiency (EiC) and the photosynthetic rate (A) of the soybean plants (Table 3).

Plants treated with lactofen and cloransulam showed a reduction in stomatal conductance (g_0), and lactofen also reduced the transpiration rate (E), though without significantly affecting the photosynthetic rate (A) (Table 3). This stomatal closure can be considered a negative effect, as it reduces the plant's efficiency in regulating CO_2 uptake. However, despite the reduction in g_0 and E, photosynthesis and pigment concentration were not altered, nor were the fluorescence parameters (Carretero, 2008). This behavior indicates that at the developmental stage in which the herbicides were applied and evaluated, there was no significant negative impact on gas exchange (Vargas et al., 2014). It is important to note that, although lactofen may induce deleterious effects, as previously mentioned, it did not directly harm the photosynthetic apparatus and allowed the continuation of photosynthetic metabolism, even with reduced g_0 and E. Therefore, the herbicides induced a physiological response involving stomatal closure without compromising A, but this response could limit water-use efficiency and affect growth under prolonged conditions. Consequently, there may have been a decrease in the transpiration rate and CO_2 concentration in the stomatal chamber, which reduced the Ci/Ca ratio.

Table 3

Mean values for transpiration rate (E), photosynthetic rate (A), Ci/Ca ratio (Ci/Ca), stomatal conductance (g_0), and instantaneous carboxylation efficiency (A/Ci) at 2 DAA of herbicides, according to the treatments. Rio Verde - GO, 2018/2019 season

FV	*E*	*A*	*Ci/Ca*	g_0	*A*/Ci
Treatments	21,55*	9,29^{ns}	0,01*	0,30**	0,000037^{ns}
No Herbicide Herbicida	9,18 a	17,19	0,87 a	0,81 a	0,051
Cloransulam	7,14 ab	15,23	0,83 ab	0,58 b	0,047
Lactofen	5,93 b	15,44	0,79 b	0,42 b	0,050
Means	7,42	15,95	0,83	0,60	0,049
CV (%)	31,45	31,78	5,75	34,79	31,61

FV - source of variation; CV - coefficient of variation; **; * Significant at 1% and 5% probability, respectively, by F-test. ^{ns} not significant. Means followed by the same letters do not differ from each other by the SNK test at 5% probability.

At 10 DAA of the herbicides, after the biostimulant applications, the chlorophyll indices, chlorophyll a fluorescence variables, and gas exchange were again evaluated. The results showed that the biostimulant alone did not influence chlorophyll a, b, total chlorophyll indices, or the maximum quantum yield of PSII (F_v/F_0) (Table 4). On the other hand, the analysis of variance showed a significant result for the interaction between biostimulant and herbicides, indicating that these factors are dependent, as the variables F_v/F_0, ABS/RC, TRo/RC, ETo/RC, DIo/RC, and PIABS were significant.

Table 4
Summary of the analysis of variance for the variables chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl T), maximum quantum yield of PSII (F_v/F_0), absorbed energy flux per active reaction center (ABS/RC), captured energy flux per reaction center (TRo/RC), electron transport flux from QA to QB per active reaction center (ETo/RC), dissipated energy flux per active reaction center (DIo/RC), and performance index based on absorption (PI_{ABS}) at 10 DAA in post-emergence of herbicides in soybean plants. Rio Verde - GO, 2018/2019 season

FV	GL	Chl *a*	Chl *b*	Chl T
Biostimulant (B)	2	2,65^{ns}	0,54^{ns}	4,95^{ns}
Herbicide (H)	2	6,96^{ns}	6,06^{ns}	29,16^{ns}
B x H	4	3,68^{ns}	1,35^{ns}	7,19^{ns}
Block	3	3,01^{ns}	0,76^{ns}	5,24^{ns}
CV (%)	-	5,56	14,99	7,49

FV	GL	F_v/F_0	ABS/RC	TRo/RC	ETo/RC	DIo/RC	PI_{ABS}
Bioestimulante (B)	2	0,002^{ns}	0,083^{ns}	0,002^{ns}	0,044^{ns}	0,060^{ns}	0,218^{ns}
Herbicide (H)	2	0,017**	0,557**	0,011^{ns}	0,716**	0,408**	3,161**
B x H	4	0,014**	0,734**	0,065*	0,165**	0,383**	1,398**
Block	3	0,00^{ns}	0,283^{ns}	0,060^{ns}	0,013^{ns}	0,084^{ns}	0,048^{ns}
CV (%)	-	4,60	10,02	6,90	19,01	21,31	47,10

FV - source of variation; CV - coefficient of variation; **; * Significant at 1% and 5% probability, respectively, by F-test. ^{ns} not significant. Means followed by the same letters do not differ from each other by the SNK test at 5% probability.

Although it was expected that the application of herbicides, particularly lactofen, in combination with the biostimulant could influence chlorophyll levels, this did not occur significantly for any of the treatments (Table 4). These results are consistent with those from the first evaluation (2 DAA of herbicides) and also align with observations by Carretero (2008). This author states that soybeans have considerable tolerance to PROTOX-inhibiting herbicides, indicating that the effects caused by their application are more related to the generation of reactive oxygen species than to direct effects caused by reduced chlorophyll synthesis (Moles et al., 2016; Dong et al., 2019). Furthermore, it was found that the ALS-inhibiting herbicide did not result in decreases in chlorophyll levels. Possibly, the reduction in protein synthesis due to the restriction in the production of the amino acids leucine, valine, and isoleucine was not intense enough to reduce ALA (5-aminolevulinic acid) levels and, consequently, chlorophylls (Fraga et al., 2019).

With the application of herbicides, there was a decrease in F_v/F_0 compared to untreated plants (Table 5). This variable represents the fraction of energy that is effectively used in the synthesis of ATP and

NADPH₂ during photosynthesis, which can indicate the integrity of PSII (Tripathy et al., 2007). Thus, it can be stated that PROTOX and ALS inhibitors caused a reduction in energy transfer to the photochemical stages of photosynthesis. In this case, the reduction in the F_v/F_0 ratio indicates an increase in energy dissipation in the form of fluorescence and heat, or an interruption in the electron flow to QA (Yusuf et al., 2010).

In the absence of the biostimulant, low F_v/F_0 values were observed when lactofen was applied (0.68) (Table 5). As mentioned earlier, this may be related to the oxidative stress caused by the herbicide, due to the accumulation of protoporphyrinogen (R. S. de Oliveira et al., 2011). Although it has been suggested that herbicides, especially ALS inhibitors like cloransulam, may cause effects quickly after application, the reduction in F_v/F_0 was not significant at 2 DAA, as shown in Table 2. However, the combination of cloransulam and the biostimulant did not appear to provide benefits to PSII performance, as F_v/F_0 in the presence of the biostimulant did not differ significantly. This behavior suggests that the deleterious effect of the cloransulam-bioestimulant combination, mentioned earlier, may have impacted the F_v/F_0 parameter, but not significantly in the early phase (2 DAA), which is confirmed in Table 5, where the effect is observed in later stages. This indicates that the application of the biostimulant, performed at 3 and 6 DAA, may not be effective when combined with a fast-acting herbicide, as reinforced by R. S. de Oliveira et al. (2011), who notes the sensitivity of plants to ALS inhibitors. Additionally, Craigie (2011) highlights that the response of plants treated with biostimulants is highly dependent on environmental conditions, which may explain the observed differences.

Table 5

Breakdown of the interaction between Herbicides × Biostimulants with mean values of maximum quantum yield of PSII (F_v/F_0), absorbed energy flux per active reaction center (ABS/RC), captured energy flux per reaction center (TRo/RC), electron transport flux from QA to QB per active reaction center (ETo/RC), dissipated energy flux per active reaction center (DIo/RC), and performance index based on absorption (PIABS) at 10 DAA in post-emergence of herbicides in soybean plants. Rio Verde - GO, 2018/2019 season

Biostimulant	Herbicides			
	Absence	Cloransulam	Lactofen	Means
		F_v/F_0		
Absence	0,75 aA	0,76 aA	0,68 aB	0,73
3 DAA	0,74 aA	0,69 bA	0,73 aA	0,72
6 DAA	0,78 aA	0,63 cC	0,68 aB	0,70
Means	0,76	0,69	0,70	0,72
CV (%)		5,71		

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		ABS/RC		
Absence	2,71 aAB	2,52 cB	3,04 aA	2,74
3 DAA	2,62 aA	2,95 bA	2,77 aA	2,78
6 DAA	2,48 aB	3,37 aA	2,76 aB	2,87
Means	2,60	2,95	2,86	2,80
CV (%)			11,89	
		TRo/RC		
Absence	2,03 aA	1,89 bA	2,09 aA	2,00
3 DAA	1,95 aA	2,04 abA	2,03 aA	2,00
6 DAA	1,96 aB	2,20 aA	1,92 aB	2,03
Means	1,98	2,04	2,01	2,01
CV (%)			6,90	
		ETo/RC		
Absence	0,98 aA	0,94 aA	0,49 aB	0,80
3 DAA	0,93 aA	0,57 bB	0,64 aB	0,71
6 DAA	1,07 aA	0,43 bB	0,54 aB	0,68
Means	0,99	0,65	0,55	0,73
CV (%)			21,68	
		DIo/RC		
Absence	0,67 aB	0,59 cB	0,97 aA	0,74
3 DAA	0,67 aA	0,94 bA	0,74 aA	0,78
6 DAA	0,52 aC	1,26 aA	0,83 aB	0,86
Means	0,62	0,92	0,85	0,79
CV (%)			27,31	
		PIABS		
Absence	1,07 bA	1,44 aA	0,23 aB	0,92
3 DAA	1,17 bA	0,54 bB	0,46 aB	0,72
6 DAA	1,82 aA	0,15 bB	0,34 aB	0,77
Means	1,35	0,71	0,35	0,81
CV (%)			47,01	

CV - coefficient of variation; Means followed by the same uppercase letters in the row and lowercase letters in the columns do not differ from each other by the SNK test at 5% probability.

The absorption flux per reaction center (ABS/RC) increased with the application of herbicides (Table 5). As the plant is subjected to stress conditions that affect its photochemical machinery, there is a tendency for ABS/RC values to increase. This is justified because the number of active reaction centers decreases, concentrating absorption on those that are still available. Additionally, in the absence of the biostimulant, a higher ABS/RC was observed in plants exposed to lactofen. When the biostimulant was applied at 6 DAA, the effect was reversed, with the highest ABS/RC value found in the presence of cloransulam, indicating that this herbicide reduced the number of active reaction centers.

In the absence of herbicide and with the application of the biostimulant at 6 DAA, there was a lower absorption flux, indicating the presence of a greater number of active reaction centers, as well as greater integrity of the reaction centers.

Regarding ABS/RC, in the presence of cloransulam, there was a reduction in the number of active reaction centers and, consequently, an increase in this variable with the application of the biostimulant at 6 and 3 DAA. It has been reported that cloransulam application reduced the electron transport rate by 5 to 8% in common beans (Krenchinski et al., 2019). Additionally, it is noted that among all treatments, the absence of herbicides combined with the application of the biostimulant at 6 DAA resulted in the lowest averages for this characteristic (2.48), indicating the biostimulant's potential to protect the reaction centers. For the energy capture flux in the reaction center (TRo/RC), there was no isolated effect of the herbicides and the biostimulant; however, a combined effect between the herbicides and the biostimulant was observed (Table 5). Contrary to what occurred for the F_v/F_0 ratio in the presence of the biostimulant at 6 DAA, the interaction with cloransulam resulted in higher TRo/RC (2.20). The application of cloransulam may have contributed to the reduction in the number of active reaction centers, inducing an increase in capture flux, as another effect of the herbicide is the depletion of pathway intermediates due to the interruption in the transport and use of photosynthesis products (Zhou et al., 2019). This effect was not observed for lactofen.

Considering photosynthetic efficiency and the functioning of the Calvin cycle, one of the most important stages within the overall process is the electron transport flux from QA to QB (ETo/RC). Plants that did not receive herbicide applications showed the highest electron transport rate (Table 5), indicating greater PSII integrity, fewer oxidative damages, and less energy dissipation in the form of heat (Vanlerberghe, 2013). In the absence of the biostimulant and at 3 and 6 DAA, the highest values were observed in the absence of herbicides. Additionally, increases in ETo/RC were observed with the presence of the biostimulant, though without significant differences.

The biostimulant was not able to reverse the injury caused by cloransulam application. In general, reductions in ETo/RC values can occur when plants are subjected to adverse climatic conditions or some type of abiotic stress (Perboni et al., 2015). Similarly, the electron flux in photosystem II generally represents the first manifestation of stress in the plant's leaf (Maxwell & Johnson, 2000). This reinforces that herbicide application is detrimental to the photochemical efficiency of soybean plants, even with the application of a biostimulant.

When the photochemical utilization of light energy is reduced, for example, during stress situations, other forms of energy dissipation tend to increase, such as fluorescence and heat (Stirbet & Govindjee, 2011; Chen et al., 2016; Zhou et al., 2019). In this regard, there was an increase in energy dissipation flux per RC (DIo/RC) in the presence of both herbicides (Table 5). Similar to what was observed for ABS/RC, in the absence of the biostimulant and in the presence of lactofen, plants exhibited higher DIo/RC, followed by the absence of herbicides and the presence of cloransulam, respectively. The same trend was observed with the biostimulant application at 6 DAA, where higher values were recorded in the presence of cloransulam, followed by lactofen and the absence of herbicides, respectively. Simultaneously, in the presence of cloransulam, a beneficial effect of the biostimulant application at 3 and 6 DAA was noted, with a significant increase in energy dissipation capacity. It is known that these indices reflect the disorganization of the plant chloroplasts, which showed lower performance index (PIABS), as the higher absorption flux (ABS/RC), capture (TRo/RC), and electron transport (DIo/RC) did not result in increased photochemical efficiency but rather in energy loss in the form of fluorescence, indicating poor utilization of the electrons generated in the water-splitting process at the start of the electron transport chain (Buchanan et al., 2015). In this study, the absence of herbicides provided higher PIABS values (1.82), while lactofen application (0.34) resulted in the greatest decreases at 6 DAA (Table 5). This result is consistent with what was observed for F_v/F_0 and also corroborates the findings for ABS/RC, as both are directly related, and as electron transport increases, F_v/F_0 improves (Zobiole et al., 2011). This result indicates the herbicides' ability to reduce the photochemical performance of the plants, regardless of their mechanisms of action (R. S. de Oliveira et al., 2011).

In the absence of the biostimulant, lactofen resulted in the lowest PIABS value (0.23), while at 3 DAA of the herbicides, both reduced this variable. However, when the biostimulant was applied at 6 DAA, a significant reduction in PIABS was observed in association with lactofen (0.34) and cloransulam (0.15). When evaluating the absence of herbicides, the biostimulant showed the ability to increase PIABS when applied at 6 DAA. On the other hand, in plants that received cloransulam, the opposite effect was observed.

Chlorophyll a fluorescence parameters have been directly related to CO_2 assimilation and, consequently, to the plant's photosynthetic efficiency. Thus, in parallel with the evaluation of fluorescence parameters, gas exchange measurements were conducted to verify the effect of the biostimulant on the photosynthetic apparatus of soybean plants.

Based on the data obtained, it was found that the biostimulant had no effect on stomatal conductance (g_0). However, it was observed that the herbicides had an effect on all evaluated characteristics. On the other hand, no significant differences were observed for the combined action of the biostimulant and herbicide, except for g_0 in soybean plants (Table 6).

Table 6
Summary of the analysis of variance for the variables transpiration (E), photosynthetic rate (A), stomatal conductance (g_0), Ci/Ca ratio (Ci/Ca), and instantaneous carboxylation efficiency (A/Ci) at 10 DAA in post-emergence of herbicides in soybean plants. Rio Verde, 2018/2019 season

FV	GL	*E*	*A*	*Ci/Ca*	g_0	*A/Ci*
Biostimulant (B)	2	2,63 *	19,228*	0,0061**	0,037ns	0,00043**
Herbicide (H)	2	11,01**	23,287*	0,0098**	0,252**	0,00040**
B x H	4	2,31**	17,741*	0,0030**	$0,025^{ns}$	0,00030**
Block (B)	3	$0,77^{ns}$	$1,062^{ns}$	$0,0002^{ns}$	$0,020^{ns}$	$0,00002^{ns}$
CV (%)	-	9,18	8,62	2,97	15,63	10,19

FV - source of variation; CV - coefficient of variation; **; * Significant at 1% and 5% probability, respectively, by F-test. ^{ns} not significant. Means followed by the same letters do not differ from each other by the SNK test at 5% probability.

The second evaluation of g_0 presented contrasting results compared to the first. Soybean plants at 3 DAA of lactofen showed higher g_0 than the other treatments (Table 7). This result suggests that shortly after herbicide application, the oxidative stress triggered also promoted an increase in the synthesis of antioxidant enzymes and stress-responsive gene regulators, optimizing the plant's metabolism (Calvo et al., 2014; Du Jardin, 2015). This allowed the plants to reach their maximum potential for gas exchange at 10 DAA of the herbicide, increasing g_0 values (Hasanuzzaman et al., 2021).

Similar to g_0, the treatments that received the biostimulant application at 3 DAA in the absence of herbicide showed a lower Ci/Ca ratio (Table 7). On the other hand, when analyzing the herbicide levels within each biostimulant level, it was noted that for the cloransulam and lactofen applications, there were no differences that could be attributed to the treatments. However, in the absence of herbicides, a higher Ci/Ca ratio was observed with the biostimulant application at 6 DAA, followed by the absence of biostimulant and the applications at 3 DAA (Table 7).

Table 7
Mean values for transpiration rate (E), photosynthetic rate (A), Ci/Ca ratio (Ci/Ca), stomatal conductance (g_0), and carboxylation efficiency (A/Ci) at 10 DAA in post-emergence of herbicides in soybean plants. Rio Verde - GO, 2018/2019 season

Biostimulant	Herbicides			
	Absence	Cloransulam	Lactofen	Means
		E		
Absence	9,24 aB	10,32 aAB	10,79 aA	10,12
3 DAA	7,48 bC	9,62 aB	10,94 aA	9,36
6 DAA	10,01 aA	9,80 aA	10,78 aA	10,16
Means	8,91	9,91	10,84	9,89
CV (%)		9,18		
		A		
Absence	19,68 aA	19,87 aA	17,43 abA	18,99
3 DAA	22,38 aA	19,78 aA	19,9 aA	20,67
6 DAA	17,92 bB	20,96 aA	15,60 bB	18,16
Means	19,97	20,20	17,64	19,27
CV (%)		11,32		
		Ci/Ca		
Absence	0,84 bA	0,87 aA	0,88 aA	0,86
3 DAA	0,78 cB	0,87 aA	0,88 aA	0,85
6 DAA	0,89 aA	0,87 aA	0,91 aA	0,89
Means	0,84	0,87	0,89	0,87
CV (%)		3,51		
		g_0		
Absence	0,92 aA	1,00 aA	1,14 aA	1,02
3 DAA	0,78 aB	0,79 aB	1,16 aA	0,91
6 DAA	0,98 aA	0,90 aA	1,11 aA	0,99
Means	0,89	0,89	1,14	0,97
CV (%)		16,24		
		A/Ci		
Absence	0,060 bA	0,059 aA	0,051 aB	0,053
3 DAA	0,077 aA	0,059 aB	0,058 aB	0,064
6 DAA	0,055 bB	0,062 aA	0,044 bB	0,053
Means	0,063	0,060	0,051	0,06
CV (%)		11,95		

CV - coefficient of variation; * Significant at 1% and 5% probability, respectively, by F-test. ns not significant. Means followed by the same uppercase letters in the row and lowercase letters in the columns do not differ from each other by the SNK test at 5% probability.

Unlike the first evaluation, the presence of lactofen resulted in plants with a higher transpiration rate, which can be attributed to the time that elapsed between the application and the second evaluation. When analyzing the biostimulant breakdown within each herbicide level, a higher transpiration rate was observed in the absence of herbicides and with the application at 3 DAA. On the other hand, in the absence of herbicides, an increase in the transpiration rate was observed with the biostimulant application at 6 DAA. Therefore, this result indicates the potential of the biostimulant to act as an osmoregulator, signaling molecule, and stomatal opening modulator, triggering positive responses in the plants' photosynthetic rate (Yakhin et al., 2017). Plants treated with lactofen, despite having higher g_0, a higher Ci/Ca ratio, and higher E, showed lower A values (Table 7). Additionally, photosynthesis depends on the plant's ability to move electrons to the final acceptors in PSII; in this case, plants with inhibited PROTOX enzyme showed the lowest PIABS values.

The results showed that in the breakdown of the biostimulant within each herbicide level, a significant interaction was observed only with the applications at 6 DAA in the absence of herbicides. In this case, the highest photosynthetic rate occurred in the presence of cloransulam (Table 7). Regarding the breakdown of herbicides within each biostimulant level, it was noted that in the absence of herbicides, the highest photosynthetic rate occurred with biostimulant applications at 3 DAA. Similarly, in the presence of lactofen, the highest photosynthetic rate also occurred at 3 DAA (Table 7).

Regarding the instantaneous carboxylation efficiency (A/Ci), it was noted that the application of the biostimulant provided increases in the values of this characteristic (Table 7), with particular emphasis on the application at 3 DAA. Furthermore, in the absence of the biostimulant, lower A/Ci was observed in plants treated with lactofen, while at 3 DAA, this trend was noted in the treatments with lactofen and cloransulam, respectively. However, when the biostimulant was applied at 6 DAA, the highest A/Ci ratio was observed in combination with cloransulam. It is known that the instantaneous carboxylation efficiency (EiC) is closely related to the intracellular CO_2 concentration and the rate of carbon dioxide assimilation (Machado et al., 2005).

Conclusions

The herbicides lactofen and cloransulam negatively affected the photosynthetic system, particularly the quantum yield of PSII and photochemical efficiency. Despite this, the biostimulant derived from *Ascophyllum nodosum* partially mitigated the damage caused by the herbicides, protecting the reaction centers and improving electron transport, especially when applied 6 days after the herbicide.

Despite changes in stomatal conductance and transpiration rate, photosynthesis was not significantly affected in the short term.

The most responsive phase to the biostimulant was 6 days after herbicide application, showing greater efficacy at that time.

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