

Article

Impact of Nodulation Efficiency and Concentrations of Soluble Sugars and Ureides on Soybean Water Deficit During Vegetative Growth

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Abstract: Drought is the primary limiting factor affecting soybean productivity, and is exacerbated by climate change. In legumes like soybeans, biological nitrogen fixation (BNF) is the main form of nitrogen acquisition, with nitrogen being converted into ureides. A greenhouse experiment was conducted using the soybean cultivar BMX Zeus IPRO, with two water treatments applied during the vegetative phase: control (C) and water deficit (D). The relative water content and number of nodules were reduced in the D plants. Ureide concentrations (allantoin and allantoic acid) were higher in nodules under D conditions. However, no differences were observed in allantoin, total ureide, and soluble sugar concentrations in leaves. Our results suggest that reducing the number of nodules may be a key strategy for maintaining BNF under drought conditions and that ureide accumulation could be the primary metabolic response in this soybean cultivar. These findings indicate that the effects of water restriction on BNF are likely associated with local metabolic responses rather than a systemic ureide feedback mechanism inhibiting BNF.

Keywords: biological nitrogen fixation; climatic changes; *Glycine max*; nitrogen metabolism; nitrogen support



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1. Introduction

Soybean is regarded as the primary source of plant-based protein for human and animal consumption, playing a crucial role in food production and food security [1]. Water stress, whether isolated or in combination with other biotic and abiotic factors, is the main limiting factor affecting soybean productivity [2,3]. Crop losses due to water deficiency have profound economic impacts, which are increasingly exacerbated by climate change. In the last two growing seasons (2021/2022, 2022/2023), soybean production in the state of Rio Grande do Sul has been severely affected by the La Niña climatic phenomenon [4].

Nitrogen absorption in legumes, such as soybeans, can occur through the symbiosis between nitrogen-fixing bacteria and plant roots via the process of biological nitrogen fixation (BNF) [5,6]. BNF accounts for approximately 65% of annual nitrogen input [7], and

the higher efficiency of BNF contributes to reducing nitrogen losses and greenhouse gas emissions from agriculture [8]. Additionally, BNF provides ecosystem services with high economic value, contributing to more sustainable agriculture [9].

In legumes, nitrogen fixed by BNF is transported via the xylem to the shoot primarily as ureides (allantoin and allantoic acid) [10–12]. In the leaves, ureides are converted into amino acids and proteins and are used for plant growth [13]. BNF in soybean is particularly susceptible to water stress, and the key processes limiting nodule function and inhibiting BNF are associated with carbohydrate availability, decreased sucrose synthase activity [14,15], reduced nodule nitrogenase activity [16], the accumulation of nitrogenous compounds (ureides) in the nodules and/or shoot [16,17], decreased oxygen permeability, and the accumulation of reactive oxygen species (ROS).

The reduction in N₂ fixation, along with the increase in ureide and free amino acid concentrations in plant tissues, is associated with a decrease in photoassimilate partitioning [16,18,19], causing an energy limitation in the nodule. The accumulation of ureides in shoots and nodules is considered to be the primary factor associated with the inhibition of BNF under drought conditions [20]. The differential accumulation of ureides in soybean cultivars is considered to be an indicator of drought susceptibility [19].

Given the scenario of climate change, with increasingly frequent and prolonged drought periods in southern Brazil, studying the physiological behavior of soybean plants during water deficit periods is crucial for adopting sustainable agricultural management practices. In this study, we evaluated the responses of the BMX Zeus IPRO cultivar, which is widely grown in the state of Rio Grande do Sul and has a high productivity potential. Specifically, a reduction in productivity was observed in this state for two consecutive years (2021/2022 and 2022/2023) due to the La Niña climate phenomenon, with the most severe drought periods occurring during the vegetative development stage of the crop. We explored the effects of soil water restriction during the vegetative phase on biochemical traits associated with the biological nitrogen fixation (BNF) process. We tested the hypothesis that a lower availability of soluble carbohydrates due to water limitation would lead to a reduction in energy input, thereby decreasing BNF efficiency, as assessed by the number and mass of nodules, which would negatively impact the concentration of ureides in both the nodules and shoots.

2. Materials and Methods

2.1. Experimental Conditions

The experiment was conducted in a greenhouse from November 2022 to January 2023, using soybean plants (*Glycine max* (L.) Merr.) of the cultivar BMX Zeus IPRO. During the experimental period, the average temperature and relative humidity were 24 °C and 72.2%, respectively. Five inoculated seeds were sown in each 8 L pot (2 mL/kg of seeds) with the inoculant Rizoliq® (Rizobacter, Londrina, Brazil), containing a minimum bacterial concentration of 5×10^9 CFU/mL, with the strains *Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA 5080. At the time of sowing, the seeds were also treated with a commercial seed treatment containing chelated micronutrients (Co and Ni), non-chelated micronutrients (Mo), and algae extract, following the manufacturer's technical recommendation. The pots were filled with 6.5 kg of soil and commercial substrate in a 2:1 ratio. The chemical analysis of the soil revealed the following characteristics: P = 4.9 mg/dm³; organic matter = 6.5%; pH H₂O = 5.7; K = 480.0 mg/dm³; Ca²⁺ = 12.2 mmolc/dm³; Mg = 2.3 mmolc/dm³; H + Al = 5.8 mmolc/dm³; Al³⁺ = 0.0 mmolc/dm³; CEC = 15.7 mmolc/dm³.

After germination and cotyledon leaf emergence, thinning was carried out to maintain two plants per pot. At the V4 development stage, the plants received foliar treatment with macronutrients (P, Mg, S), chelated micronutrients (Co, Ni, and Zn), and non-chelated micronutrients (Mo), according to the manufacturer's technical recommendations. The plants were irrigated daily using an automatic irrigation system and were kept well-watered, at 89% of pot capacity, until the imposition of water treatments.

2.2. Experimental Design and Imposition of Treatments

The water treatments were applied when the plants reached the V6 vegetative stage, with the plants being divided into two groups, a control group (C) and a water deficit treatment group (D), in which irrigation was suspended for a period of eight days, according to the methodology proposed by Schneider et al. [21]. The control plants continued to receive daily irrigation with sufficient water to maintain soil water content at approximately 89% of field capacity. Water stress was imposed by suspending irrigation. To monitor the reduction in soil water content, the pots were weighed daily during the eight-day irrigation suspension period to track the gravimetric water content, which resulted in a 76% reduction by the end of the period. Additionally, tensiometers were installed at a depth of 0.15 m in three pots for each water treatment, and the replenishment of evapotranspired water was based on tensiometric readings. The suspension of irrigation led to soil matric potential values of -40 kPa, at which point the assessments were conducted.

The experimental setup was a randomized completely design with two treatments (C and D). The treatments consisted of 10 replicates (pots); with two plants per pot, totaling 20 pots and 40 plants. Nodulation efficiency (nodule number and mass) was evaluated in each pot, with a total of 10 pots per water treatment. To evaluate the morphological and biochemical traits, one plant from each pot was used, totaling 10 plants for the assessment of morphological traits and 6–10 plants for the biochemical traits (soluble sugars and ureides, respectively) for each water treatment.

2.3. Water Status, Specific Leaf Area and Chlorophyll Index

To determine the plant water status, leaves were collected and immediately transferred to the laboratory in thermal boxes to prevent excessive water loss. Initially, the leaves were weighed on a precision balance to determine the fresh weight (FW). The fresh leaves were then immersed in distilled water in Petri dishes, kept refrigerated, and protected from light. After 24 h, the plant material was weighed again to determine the turgid weight (TM) and subsequently dried in an oven at 60°C for one week to determine the dry weight (DW). The relative water content (RWC) was calculated using Equation (1):

$$\text{RWC (\%)} = ((\text{TM} - \text{DW}) / (\text{FW} - \text{DW})) \times 100 \quad (1)$$

Leaf area (cm^2) was determined using scanned images analyzed with the ImageJ software (<https://imagej.net/ij/>). The specific leaf area (SLA, $\text{cm}^2 \text{g}^{-1}$) was estimated using the ratio between the leaf area (LA, cm^2) and the dry weight of the leaves. The chlorophyll index (CI) was estimated using a portable chl meter, model ClorofiLog (Falker, Porto Alegre, Brazil), at the central leaflet.

2.4. Nodulation

To evaluate the nodulation attributes, the plants were removed from the pots, and the roots were washed. The number of nodules per plant was manually counted for each pot. The nodules were then weighed to determine the fresh weight and subsequently dried in an oven at 60°C until a constant weight was reached, allowing for the determination of the dry weight.

2.5. Concentration of Total Soluble Sugars in Leaves and Roots

The extraction and quantification of soluble sugars were performed following the method of Dubois et al. [22], with minor modifications. Samples of approximately 10 mg of dry weight, obtained after homogenization in liquid nitrogen and drying in an oven at 40°C , were extracted with 1 mL of 80% (v/v) ethanol and incubated in a water bath at 75°C for 15 min. The extracts were centrifuged at 12,000 rpm for 15 min, and the supernatant was collected. The sediment was re-extracted with 500 μL of 80% (v/v) ethanol and combined with the supernatant from the first extraction.

For quantification, 250 μL of the samples was mixed with 250 μL of 80% (*v/v*) ethanol, 2.5 mL of concentrated sulfuric acid, and 0.5 mL of 5% (*v/v*) phenol. After mixing, the solutions were allowed to stand at room temperature for 20 min. The absorbance at 490 nm was measured using a spectrophotometer. A standard curve was established using D-glucose.

2.6. Concentration of Ureides in Leaves and Roots (Nodules)

For the extraction and determination of ureides (allantoin and allantoic acid), 1 g of fresh tissue was added to 10 mL of MCW solution (60% *v/v* methanol, 25% *v/v* chloroform, and 15% *v/v* H_2O). After 48 h of extraction in the refrigerator, 4 mL of the supernatant was collected, and 1 mL of chloroform and 1.5 mL of deionized water were added. The mixture was then transferred to another tube. Following phase separation, the ureides were determined according to Vogels and Van der Drift [23]. Ureide concentrations ($\mu\text{mol g}^{-1}$ FW) were calculated based on a standard allantoin curve.

2.7. Data Analysis

The variation in nodulation efficiency and biochemical parameters (concentration of sugars and ureides in the shoots and nodules) between the water treatments was assessed using the *t*-test. All analyses were performed using R software version 4.2.0 [24], with a significance level set at $p < 0.05$.

3. Results

The suspension of irrigation resulted in a 44% reduction in the relative leaf water content (RWC) in D plants (40.97 ± 4.43) compared to the C plants (67.07 ± 1.26) (Table 1). In WD plants, a 30% increase in specific leaf area (SLA) and a 17% increase in the chlorophyll index (CI) were observed compared to the C group (Table 1).

Table 1. Relative leaf water content (RWC), specific leaf area (SLA), and (CI) for soybean plants (*Glycine max* (L.) Merr.) measured using Cultivar BMX Zeus IPRO at V6 vegetative stage after eight days of the imposition of control (C) and water deficit (D) treatments. Values represent means $\pm$ standard error ($n = 10$).

Parameter	C	D	<i>p</i>
RWC	67.07 ± 1.26	40.97 ± 4.43	<0.0001 ***
SLA	43.79 ± 0.8	57.32 ± 1.74	<0.0001 ***
CI	33.17 ± 0.49	38.89 ± 1.19	0.0001 ***

Asterisk (***) indicates a significant difference between treatments by the *t*-test ($p \leq 0.001$).

The WD plants showed an approximately 35% reduction in the number and a 55% reduction in the fresh weight of nodules (Figure 1) compared to the control plants. However, no differences were observed in the dry weights of the nodules.

The concentration of total soluble sugars in both leaves and roots did not differ between the C and D treatments (Table 2). For ureide concentrations, a significant increase was observed in allantoin acid in both leaves and nodules, and allantoin and total ureides increased in the nodules in D plants (Figure 2). The concentration of both allantoin and total ureides in leaves did not differ between the water treatments.

Table 2. Concentration of total soluble sugars in leaves (TSSL) and total soluble sugars in roots (TSSR) in soybean plants (*Glycine max* (L.) Merr.) measured using a Cultivar BMX Zeus IPRO at V6 vegetative stage after eight days of the imposition of control (C) and water deficit (D) treatments. Values represent means $\pm$ standard error ($n = 6$).

Parameter	C	D	<i>p</i>
TSSL	8.55 ± 0.31	9.57 ± 0.89	0.58
TSSR	5.27 ± 1.3	5.4 ± 0.53	0.96

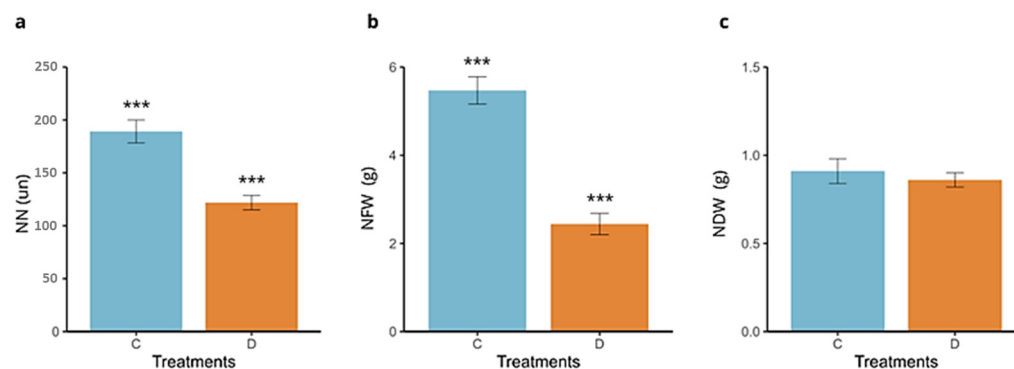


Figure 1. Number of nodules (NN) (a), fresh weight of nodules (NFW) (b), and dry weight of nodules (NDW) (c) in soybean plants (*Glycine max* (L.) Merr.) measured using a Cultivar BMX Zeus IPRO at V6 vegetative stage, after eight days the imposition of control (C) and water deficit (D) treatments. Bars represent means $\pm$ standard error ($n = 10$). Asterisk (***) indicates a significant difference between treatments by the t -test ($p \leq 0.001$).

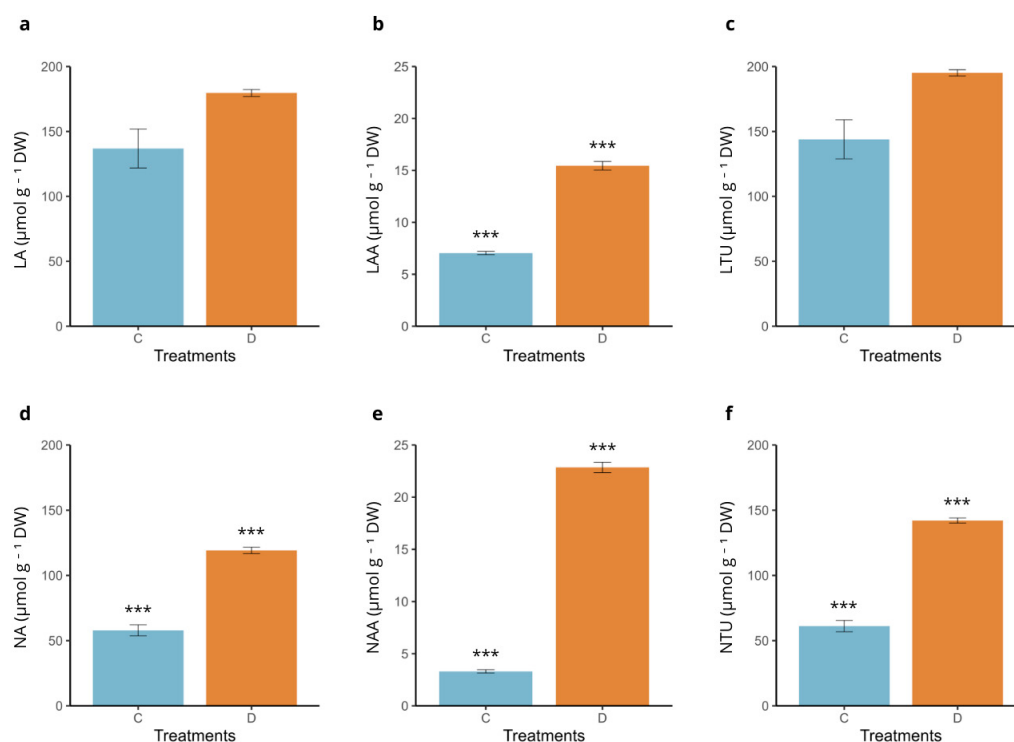


Figure 2. Concentration of allantoin in leaves (LA) (a), allantoic acid in leaves (LAA) (b), total ureides in leaves (LTU) (c), and concentration of allantoin in nodules (NA) (d), allantoic acid in nodules (NAA) (e), and total ureides in nodules (NTU) (f) in soybean plants (*Glycine max* (L.) Merr.) measured using a Cultivar BMX Zeus IPRO after eight days of imposition of control (C) and water deficit (D) treatments. Bars represent means $\pm$ standard error ($n = 10$). Asterisk (***) indicates a significant difference between treatments according to the t -test ($p \leq 0.001$).

4. Discussion

In this study, we investigated the effects of soil water restriction during the vegetative phase of soybean growth on biochemical traits related to the biological nitrogen fixation (BNF) process. In southern Brazil, the La Niña climate phenomenon occurred more intensely during the soybean vegetative development stage in two consecutive years (2021/2022 and 2022/2023). It is important to note that these water deficit situations are becoming increasingly common in soybean production areas across Brazil. Therefore, we

focused on the biochemical responses associated with BNF efficiency, specifically analyzing sugar and ureide concentrations under water restriction.

After eight days of irrigation suspension, we observed a 44% reduction in the relative leaf water content (RWC), accompanied by a significant increase in the concentration of ureides (allantoin and allantoic acid) in nodules. The reduction in/inhibition of biological nitrogen fixation (BNF) under water deficit conditions is associated with a negative feedback mechanism due to the accumulation of ureides [19,25–28]. Ureides, which are products of BNF in soybeans, have a low solubility [13,29], and an inverse relationship is observed between soil water availability and the transport of ureides to the shoot [19,30,31]. Some studies have linked cultivar sensitivity to water deficit with ureide accumulation in nodules when assessing the effects of water restriction across different cultivars. The results observed in this study may suggest a degree of sensitivity of the BMX Zeus IPRO cultivar under more severe restriction conditions, especially during the vegetative phase.

The accumulation of ureides observed in the nodules may also be associated with various biochemical processes affected by reduced soil water availability and xylem water flux, such as decreased nitrogenase activity due to increased resistance to oxygen diffusion and a decline in sucrose synthase activity [32]. However, BNF under drought conditions is regulated at a local level and does not involve a systemic response [14,33]. A systemic response might involve reduced energy availability due to a lower translocation of photoassimilates to the nodules. Our results showed that the concentration of total soluble sugars in leaves and nodules did not differ between treatments. Although estimates suggest that 12–15% of the photoassimilates produced by plants are allocated to maintain nodular activity [34], we believe that BNF was not limited by energy restriction and that a smaller nodule size was likely essential to sustain BNF during periods of water scarcity.

Thus, the reduced number of nodules, but without a difference in nodule dry weight in water-deficient plants, is consistent with the idea that there was still adequate capacity to maintain BNF [35]. The reduced number of nodules appears to be a key strategy, as it requires fewer carbohydrates to maintain the energy of the bacteroids and for the conversion of N_2 into nitrogenous molecules (ureides). It is important to note that despite the higher concentration of ureides in nodules, the transport of ureides to leaves does not seem to be significantly affected, as we did not observe differences in allantoin and total ureides in the leaves. However, the concentration of allantoic acid showed a significant increase in both leaves and nodules of water-deficient plants. These differences in ureide concentrations (allantoin and allantoic acid) may be due to the rapid turnover rate of allantoin to allantoate [36].

In addition to adjustments to the number and dry weight of nodules, we also observed that the chlorophyll index was higher in water-deficient plants. The increased chlorophyll concentration may be an important indicator of the maintenance of photosynthetic capacity during periods of water restriction [37]. We also observed an increase in specific leaf area (SLA) in plants under water deficit. SLA is a trait associated with photosynthetic capacity. Thus, the data on these foliar traits (RWC, chlorophyll index, and SLA) suggest that water-deficient plants adjusted their growth to maintain physiological activity in response to water restriction.

Maintaining biological nitrogen fixation (BNF) under drought conditions is crucial for helping plants cope with water stress. Our results suggest that after eight days of irrigation suspension, ureide accumulation may be the primary metabolic response in the evaluated cultivar. Although the transport of nitrogenous compounds is affected to a lesser extent, with minimal effects on nitrogen metabolism in leaves (ureide concentration and chlorophyll), our findings indicate that the effects of water restriction on BNF appear to be linked with local metabolic responses rather than a systemic response associated with ureide feedback inhibiting BNF.

The study of the impacts of water deficit on soybean cultivars is particularly important given the climate scenario in southern Brazil. Research that evaluates the physiological responses of cultivars widely grown in this region is essential for developing management

practices for crops facing predicted extreme weather events. Specifically, the state of Rio Grande do Sul stands out in this climate context due to the frequent and severe occurrence of extreme events, including both floods and droughts, the latter often linked to the La Niña phenomenon during the soybean growing season.

The findings of this study provide evidence that, under extreme climatic conditions (prolonged droughts combined with other stress factors) and field conditions, negative effects on biological nitrogen fixation (BNF) can occur on a larger scale, with a significant reduction in both the number and mass of nodules, limiting the supply of ureides to the shoots. Understanding the dynamics of carbohydrate and nitrogen availability for soybean plants, as demonstrated in this study, significantly contributes to the potential for developing plant management strategies. These strategies may include stimulating existing nodulation with nutrients such as cobalt, molybdenum, and nickel [38]. Additionally, in situations where nodule mortality occurs, inoculation reinforcement via aerial spraying near rainfall events may be beneficial [39].

Studies on carbon and nitrogen metabolism in nodulated soybean plants are essential for a comprehensive understanding of the effects of water deficit periods. Additionally, it is crucial to determine whether the reduction in BNF efficiency observed during the vegetative phase affects the reproductive phase and overall productivity. Such research forms a solid foundation for developing agricultural management strategies to address the increasingly frequent challenges posed by extreme climate conditions.

5. Conclusions

Water restriction during the vegetative phase of the BMX Zeus IPRO cultivar, which is widely grown in the state of Rio Grande do Sul, resulted in a reduction in the number of nodules but did not affect nodule dry mass, indicating the maintenance of the biological nitrogen fixation (BNF) process. Moreover, the ability to adjust photosynthetic metabolism, as evidenced by leaf traits such as specific leaf area (SLA), chlorophyll index (CI), and relative water content (RWC), along with the absence of differences in soluble sugar concentrations, seems essential for the energetic maintenance of the nodules. Additionally, this study highlights that the accumulation of ureides in the nodules was the primary metabolic response of the cultivar under water deficit conditions.

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Data Availability Statement: The authors declare that data supporting the findings of this study are available within the article.

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