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Chapter 10

Role of Boron Nutrient in Nodules Growth and Nitrogen Fixation in Soybean Genotypes Under Water Stress Conditions

Nacer Bellaloui, Alemu Mengistu,
My Abdelmajid Kassem, Craig A. Abel and
L.H.S. Zobiole

Additional information is available at the end of the chapter

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1. Introduction

Boron is an essential nutrient for plant growth, development, and seed quality [1-4]. Previous research indicated the involvement of B in cell wall structure [5,6]; cell membrane integrity [2, 7]; sugar metabolism [2], especially sugar alcohols [3,8]; nitrogen assimilation and fixation [9, 10]; nodules [11,12], nodullin protein (ENOD2) and malfunction of oxygen diffusion barrier [13]; phenolic metabolism [2,14,15]; ion uptake [2,16]; and plasma membrane-bount H+ ATPase [7,17,18].

Boron is required for nodules growth and nitrogen fixation [9,11-13], and boron deficiency can occur under certain environmental stress factors even when boron level in soil is adequate [19], leading to yield loss. Among the environmental stress factors that can lead to boron deficiency in plants is drought or water stress. Drought is a major environmental stress factor limiting crop yields worldwide [20], and maintaining boron levels within plants under drought conditions is critical. Boron has low mobility in the phloem [21], although boron mobility in the phloem depends on plant species [3, 22]. Under water stress conditions, plant increases abscisic acid (ABA) production [23], possibly affecting photosynthetic rate in drought-stressed plants, leading to stomata closure and transpiration rate reduction. Under these conditions boron uptake and translocation, and boron movement from leaves to seed is reduced, decreasing seed boron concentration [10]. Although soybean nodule growth and symbiotic N_2 fixation are sensitive to drought [20,24,25], we hypothesized that drought can result in boron deficiency within the plant, impacting nodule growth, N_2 fixation, and CO_2 accumulation. The

objective of this research was to investigate the effects of foliar boron on nodules growth and nitrogen fixation rates in several soybean genotypes under water stress. To avoid the confounding effects of multi-environmental factors in the field on boron application effects, the experiment was conducted under greenhouse conditions. In addition to the current research findings, the present chapter will also highlight previous and current major research findings in boron nutrition and the role of B in nodule growth and symbiotic nitrogen fixation in soybean.

2. Materials and methods

A repeated greenhouse experiment was conducted. Cultivars of maturity group (MG) III Pella, Williams 82, Hutcheson, and Forest, were used. Seeds of soybean cultivar were germinated in flat trays in vermiculite, and then uniform size seedlings at V1 stage were transplanted into 9.45 L size pots. Soil in pots was a Dundee silt loam (fine-silty, mixed, active, thermic Typic Endoqualfs) with pH 6.3, 1.1% organic matter, a cation exchange capacity of 15 cmol/kg, and soil textural fractions of 26% sand, 56% silt, and 18% clay, average B concentration was 0.72 mg kg^{-1}. The soil contained an abundant native population of *B. japonicum*. Water stress was introduced as reported by Bellaloui [20,26]. Briefly, soil in pots were weighed and then saturated with deionized water and left to drain and weighed again to obtain the water field capacity using soil water sensors inserted in pots and measured by Soil Moisture Meter (WaterMark Company, Inc., Wisconsin, USA). Plants were divided into well watered (soil water potential between –15 to –20 kPa) (this was considered field capacity for the control plants), moderate water stress (soil water potential between -90 and -100 kPa), and severely water stressed (soil water potential between –150 to –200 kPa). Boron was foliar-applied as boric acid at a rate of 1.1 kg ha^{-1} once at flowering stage (R1-R2) and once at seed-fill stage (R5-R6). Combined treatments were well watered plants with no B (W-B), well watered plants with B (W+B); water stressed plants with no B (WS-B); water stressed plants with B (WS+B); severely water stressed plants with no B (SWS-B); severely water stressed plants with B (SWS+B). Samples were taken five days after the second B application for nitrate reductase assay to measure the rate nitrate reductase activity (NRA), nitrogenase, and leaf B. Mature seed were weighed at R8 (harvest maturity stage). Plants were considered fully matured when they reached R8 according to [27]. Greenhouse conditions were about 34°C ± 9°C during the day and about 28°C ± 8°C at night with a photosynthetic photon flux density (PPFD) of about 800 - 2300 μmol m^{-2} s^{-1}, as measured by Quantum Meter (Spectrum Technology, Inc., Illinois, USA). The big range of light intensity reflects a bright, sunny, or cloudy day. The source of lighting was a mixture of natural light, bulb light (60 W), cool white (250 W). To avoid differences in the day-length between the two experiments, the two experiments were conducted simultaneously at the same time and during the normal growing season (from April to September) for the Early Soybean Production System in the midsouth USA, and this is to be consistent with the normal photoperiod for soybean growth [10].

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2.1. Nitrate reductase assay

The rate of nitrate reductase activity (NRA) was determined according to [28, 29]. Briefly, NRA was measured in the fully expanded leaves and nodules. Nodules were gently and carefully separated from roots and placed in NRA assay buffer solution. A fresh leaf sample of about 0.3 g was placed in 10 mL of potassium phosphate buffer at a concentration of 100 mM, pH 7.5, containing 1% (v/v) 1-propanol, in the flask. The incubation solution was vacuum-filtered for 1 min, and then flashed with nitrogen gas for 30 s, and then incubated at 30°C. A samples of 0.5 mL was taken at regular intervals (0, 60, 120, 180, and 300 min) for nitrite measurement. Samples were extracted with 5 mL of deionized water and reacted with 1.0 mL of 1% (w/v) sulfanilamide in 10% v/v HCl and 1.0 mL of *N*-naphthyl-(1)-ethylenediamine dihydrochloride (0.1%). Nitrite concentration in samples was measured by reading the absorbance at 540 nm after 30 minutes using a Beckman Coulter DU 800 spectro- photometer (Fullerton, CA). A standard curve was produced using KNO_2 as a source of NO_2 in the tested samples according to (Bellaloui et al., 2006). To measure the enzyme activity where there was no limiting concentration of NO_3 in the incubation culture solution (potential nitrate reductae activity, PNRA), exogenous NO_3 was added at a concentration of 10 mM as KNO_3.

2.2. Acetylene reduction assay

Destructive method for acetylene reduction assay was used. Three plants from each replicate were harvested five days after the second B application (at seed-fill stage). Nitrogenase activity was assayed using the acetylene reduction assay as described elsewhere [29-31]. Roots with nodules intact were excised and incubated in 60 mL plastic syringes. Roots from each replicate and from each treatment were placed in the syringes in the Mason jars and sealed. A 10% volume of air was then removed and replaced with an equal volume of acetylene. After 1 h of incubation at room temperature, duplicate 1.0 mL gas samples were removed and analyzed by gas chromatography for ethylene formation and carbon dioxide evolution. The gas chromatography (Agilent HP6960, Agilent Technologies, Wilmington, DE) was equipped with manual injector, injector loop, and sample splitter. A flame ionization detector (FID) and a thermal conductivity detector (TCD) were used. Using the sample loop and splitter, 0.25 mL of gas was directed into a 30 m length × 0.53 mm i.d. alumina megabore column (115-3532) connected to the FID, and 0.25 mL of sample was injected into a HP-PLOT D column (30 m length × 0.53 mm i.d. megabore with 40 μm film; 1905D-Q04) connected to the TCD using helium as a carrier gas. Chromatographs were integrated using Chem Station software. Standard curves for ethylene and carbon dioxide were updated and produced for each day. Samples having <9% acetylene were not used in the analysis.. Nodules were carefully removed and counted, and then oven-dried at 60°C for 4 - 5 days.

2.3. Boron determination

The concentration of total B was measured in the fully expanded leaves after the second foliar B application, and in seeds at harvest maturity stage. Boron concentration was determined according to Azomethine—H method [19,32-34]. Briefly, 1 g of dry sample was placed in a porcelain crucible for ashing at 500°C for 8 hr. Samples then were extracted with 20 mL of 2

M HCl at 90°C for 10 min and azomethine-H solution containing 0.45% before the analysis (John et al., 1975) with a buffer solution contained 25% ammonium acetate, 1.5% EDTA, and 12.5% acetic acid. The concentration of B in the samples were determined spectrophotometrically by using color development after 45 minutes. Samples were read at 420 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, California). Boron analysis in soil was conducted using Inductively Coupled Plasma spectrometry (ICP) using Thermo Elemental, Thermo Jarrell-Ash model 61E ICP, USA [10].

2.4. Analysis of $\delta^{15}N$ ($^{15}N/^{14}N$ ratio) and $\delta^{13}C$ ($^{13}C/^{12}C$ ratio) using natural abundance

Natural abundance of $\delta\ ^{15}N$ and ^{13}C isotopes was determined using about 0.9 mg of ground seeds. Isotopic analysis was conducted using a Thermo FinniGlyn Delta Plus Advantage Mass Spectrometer with a FinniGlyn ConFlo III, and Isomass Elemetal Analyzer (Bremen, Germany) according to [26, 35,36]. Isodat software version 2.38 was used to obtain Delta values [26]. The elemental combustion system was Costech ECS 4010 with an autosampler (Bremen, Germany).

2.5. Determination of seed sucrose

Seed sucrose concentration was measured in mature seeds. Sucrose concentration was measured according to [37,38] using an AD 7200 diode array feed analyzer (Perten, Springfield, IL). Briefly, about 25 g of seed were ground using a Laboratory Mill 3600 (Perten, Springfield, IL). Initial calibration equations were developed by the Department of Agronomy and Plant Genetics, University of Minnesota St Paul, MN using Thermo Galactic Grams PLS IQ software, developed by Perten company (Perten, Springfield, IL). Analyses of sugars were performed based on a seed dry matter basis [26, 37, 39].

2.6. Seed glucose determination

Glucose concentration in mature seeds was measured enzymatically using Glucose (HK) Assay Kit from Sigma, USA, Product Code GAHK-20, 2012) [40]. In this reaction, glucose is phosphorylated by adenosine triphosphate (ATP) in a reaction catalyzed by hexokinase. Glucose-6-phosphate (G6P) produced is then oxidized to form 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH, and the increase in absorbance at 340 nm is directly proportional to glucose concentration in the sample. Mature seed samples were ground using a Laboratory Mill 3600 (Perten, Springfield, IL). A dry, ground sample of 0.1 mg was extracted with deionized water. The extraction procedure of glucose from seeds was conducted according to [26], and as instructed by Glucose (HK) Assay Kit from Sigma. The concentration of glucose was measured spectrophotometrically by reading the samples at 340nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The concentration of glucose was expressed as mg g dwt^{-1}.

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2.7. Seed fructose determination

Fructose concentration is mature seeds was determined enzymatically according to Fructose Assay Kit from Sigma, USA, Product Code FA-20, 2012 [41]. In this reaction, fructose is phosphorylated by ATP in a reaction catalyzed by hexokinase, and the produced fructose 6-phosphate is then converted to G6P by phosphoglucose isomerase (PGI). The oxidation of G6P to 6-phosphogluconate takes place in the presence of NAD in the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). An equimolar amount of NAD is reduced to NADH, and the consequent increase in absorbance at 340 nm is directly proportional to fructose concentration in a sample. A sample of 0.1 mg was extracted according to Fructose Assay Kit from Sigma, and is detailed in Bellaloui et al (2013) as instructed by Fructose Assay Kit from Sigma. The concentration of fructose in samples were measured spectrophotometrically by reading the samples at absorbance of 340nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The concentration of fructose was expressed as mg g dwt^{-1}.

2.8. Experimental design and statistical analysis

Treatments were arranged in a split plot design with irrigation as a main block and B treatment as sub-plot. Four replicates were used, and each replicate consisted of a pot containing three plants. Proc Mixed was used for data analysis of variance in SAS [42]. Means were separated by Fisher's least significant difference test at the 5% level of probability using Proc GLM analysis in SAS [42]. Since there were no interactions between the two experiments, the data were pooled and combined.

3. Results and discussion

Analysis of variance showed that boron application (T) and irrigation (IR) were significant for grain weight, nodule mass, nitrogen fixation (NF), nitrate reductase activity (NRA), N, B, and natural abundance of $\delta^{15}N$ and $\delta^{13}C$ (Table 1), and sugars (Table 2). Cultivar (CV) was significant for some parameters and not significant for others, indicating differences in cultivar responses to the B application and water stress. Both B application and IR significantly interacted (T×IR and E×T×IR interactions) for these parameters, indicating that B effects on these parameters depended on IR (watered or water stressed conditions). There were no significant interactions between Experiment (E) and T or IR, indicating that the effect of B application or IR had similar effect in each experiment (Table 1). Therefore, the data were pooled and combined [26].

3.1. Effect of B and water stress on grain weight, nodule mass and nodule number

Foliar B application to watered plants (W+B) resulted in significant increase ($P \leq 0.05$) in grain weight, nodule mass, and nodule number (Table 3). For example, in Pella cultivar the increase of grain weight, nodule mass, and nodule number was 18.7%, 38.5%, and 33.3 %, respectively. These parameters were different between cultivars and each cultivar responded differently to

foliar B (Table 3). Previous research indicated that B plays a major role for plant growth and development [1, 2] and crop quality [4,19]. It was shown that foliar B improved seed set, seed yield, and seed quality of alfalfa [19] and sugar beet [4], and altered seed composition in soybean [10]. Previous research showed that B is an essential nutrient for the development of nitrogen-fixing root nodules in pea (*Pisum sativum*) [9]. A lower level of infection of the host plants with *Rhizobium* was noticed in plants grown in B-deficient medium compared to plants supplied with adequate B [9]. It was shown that that little or no ability to fix N_2 under B-deficient plants [11]. Recently, it was found that FB increased nodule weight under irrigated greenhouse conditions [43]. The current results showed that, even though B concentration in leaves was above the critical level (20 mg B kg^{-1}, critical level of B in leaves for normal plant growth) [44], FB resulted in a positive effect on seed and nodule weights, agreeing with previous research of those of [7, 43, 45]. Our results showed that foliar B increased grain weight, nodule mass and number due to the stimulatory effects of B on growth and development [9,11] and nodule improvement.

3.2. Effect of B and water stress on nitrogen fixation and nitrogen assimilation

Foliar application of B resulted in higher rates of nitrogen fixation (increase of nitrogenase), root respiration, and nitrogen assimilation (increase of nitrate reductase activity, NRA), (Table 4). Foliar B application to moderately water stressed plants (Table 4) increased grain weight, nodule number and mass, nitrogen fixation and assimilation. However, foliar B application to severely water stress plants did not result in an increase in these parameters because of the damaging effects of water stress to nitrogen metabolism enzymes, especially nitorgenase and nitrate reductase (Table 5). Researchers reported that B is an essential micronutrient for the development of nitrogen-fixing root nodules [11], and plants grown in B-deficient medium showed lower infection of the host plants with Rhizobium compared to plants supplied with adequate B [11]. It was reported that nodules showed little or no ability to fix N^2 in B-deficient plants, leading to N deficiency and necrosis of nodulated pea plants [9]. This indicated that nitrogen fixation in soybean was sensitive to B deficiency [9,12], and B deficiency can result in the reduction in early nodullin protein (ENOD2) in nodule parenchyma cells and malfunction of oxygen diffusion barrier [13]. It was hypothesized that B protects nitrogenase against oxygen damage by influencing membrane integrity and function [13] and may interact with membrane glycoproteins and glycolipids to maintain the proper conformation in nitrogen-fixing cells [5]. Although B has been shown to be essential for nodule growth and development, there is no convincing evidence that there is a direct effect of B on nitrogen metabolism [2,13,46,]. Nitrate assimilation, reflected by the key enzyme in nitrogen assimilation, was higher in W+B plants, indicating that B enhanced nitrate assimilation. The stimulatory effects of nitrogen assimilation by B was also reported by others (Bellaloui et al., 2011 AJPS. This is because nitrogen metabolism in legumes is both a result of both symbiotic N_2 fixation and mineral N assimilation processes. During this process, atmospheric N_2 is fixed by the enzyme nitrogenase in the bacteroids of nodules [47], and nitrate reduction (assimilation) is catalyzed by the enzyme nitrate reductase (NR). Both NR and nitrogenase enzymes coexist in nodules competing for reductant [48]. It appears that B may stimulate de novo synthesis and making nitrate (enzyme substrate) available for the enzyme nitrate reductase. Adding 0.5 mM B to the buffer solution

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increased NRA by 30% in WS+B compared with WS-B, and adding 10 mM NO_3 to the buffer solution increased NRA by 55% and 40% in leaves and nodules, respectively (data not shown). In SWS plants, adding B or NO_3 did not enhance NRA (data not shown). This indicated that both B and NO_3 stimulated NR enzyme somehow, maybe by facilitating nitrate availability in the cytoplasm to NR for reduction or enhancing nitrate translocation from the vacuoles to the cytoplasm, leading to higher NRA activity. This hypothesis was supported by the effect of B ion uptake [2,16] and the direct or indirect effects of B on the plasma membrane bound H+ ATPase (plasmalemma H+-ATPase activity) [7,17], cell wall structure and membrane integrity [2,7]. Our results are supported by [2] in that B may have an indirect influence on nitrate uptake and assimilation, and enhance NRA by inducing nitrate availability and increasing protein de novo synthesis as a result of nitrate absorption [49]. This observation is supported by [13] who found that adequate level of B increased NRA and decreased nitrate in xylem sap compared to deficiency level. The relationship between nitrogen fixation and nitrogen assimilation and how this relationship is influenced by foliar B and its impact on seed protein and oil and sugars is still not well established.

Source of variability	Seed weight	Nodule mass	Nodule number	ARA (NF)	Leaf NRA	Nodule NRA	B in leaves	N in leaves	$\delta^{15}N$	$\delta^{13}C$
Experiment (E)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Treatment (T)	**	**	***	**	***	**	***	*	*	**
Water stress (WS)	***	**	***	***	***	*	***	*	*	**
Cultivar (CV)	*	*	*	NS	NS	NS	NS	NS	NS	NS
E×T	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
E×WS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
E×CV	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
T×WS	**	*	*	**	*	*	**	*	*	*
T×CV	*	*	*	*	*	*	*	**	*	*
CV×WS	**	**	**	*	**	*	*	*	*	*
E×T×WS×CV	**	**	*	**	**	**	*	*	*	**

* Significance at $P \leq 0.05$; ** Significance at $P \leq 0.01$; *** Significance at $P \leq 0.001$.

Table 1. Analysis of variance of the effects of foliar boron on seed weight (100 seed weight, g), nodule mass (mg $plant^{-1}$), nodule number $plant^{-1}$, [nitrogen fixation (acetylene reduction assay (ARA), μmol of C_2H_4 $plant^{-1}$ h^{-1})], leaf nitrate reductase activity (NRA, µmol NO_2 $g^{-1}h^{-1}$), and nodule NRA (µmol NO_2 $g^{-1}h^{-1}$), boron (B, mg kg^{-1})) and nitrogen (N, %) in leaves and seeds, and in $\delta^{15}N$ and in $\delta^{13}C$ isotope values in seeds in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under well watered and water stressed conditions (WS) with and without foliar boron (B) treatments (T) under greenhouse conditions [a].

Source of variability	Glucose	Fructose	Sucrose	Raffinose	Stachyose
Experiment (E)	NS	NS	NS	NS	NS
Treatment (T)	**	*	*	*	***
Irrigation (IR)	**	*	*	**	***
Cultivar (CV)	*	*	*	NS	NS
E×T	NS	NS	NS	NS	NS
E×IR	NS	NS	NS	NS	NS
E×CV	NS	NS	NS	NS	NS
T×IR	*	*	*	**	*
T×CV	**	*	*	*	*
CV×IR	*	**	*	*	*
E×T×IR×CV	*	*	*	**	*

* Significance at $P \leq 0.05$; ** Significance at $P \leq 0.01$; *** Significance at $P \leq 0.001$.

Table 2. Analysis of variance of the effects of foliar boron on sugars (mg g^{-1} dwt) in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under well watered and water stressed conditions (IR) with and without foliar boron (B) treatments (T) under greenhouse conditions [a].

Watered soybean								
Variety	**Boron**	**Grain weight (100 seed weight, g)**	**Nodule mass (mg dwt $plant^{-1)}$**	**Nodule number $plant^{-1}$**	**ARA (NF) (μmol of C_2H_4 $plant^{-1}$ h^{-1})**	**Root respiration (mmol of CO_2 evolved/g of root/h)**	**Leaf NRA (µmol NO_2 g^{-1} h^{-1})**	**Nodule NRA (µmol NO_2 $g^{-1}h^{-1}$)**
Pella	W-B	16 a	65 a	33 a	11.6 a	7.5 a	5.6 ab	4.7 ab
W 82		15 a	68 a	28 b	10.5 a	7.8 a	4.9 b	5.2 a
Hutcheson		14 b	61 b	25 b	10.1 a	6.8 b	5.7 ab	3.8 b
Forrest		14 b	64 a	17 c	11.4 a	6.4 b	6.3 a	4.2 ab
Pella		19 a	90 a	44 a	15.4 a	9.7 b	7.5 ab	6.4 a
W 82	W+B	18 a	86 ab	38 abc	14.7 ab	10.5 ab	6.8 b	7.8 a
Hutcheson		16 b	84 b	36 c	13.2 b	10.6 ab	7.4 ab	5.2 b
Forrest		17 b	86 ab	38 abc	13.6 b	11.5 a	8.3 a	5.8 b

[a] Soybean plants were grown at field capacity at -15 to -20 kPa [10]. Soybeans were grown under greenhouse conditions similar to those in Bellaloui et al. (2011). Values within columns and within each B treatment sharing a letter are not significantly different (P>0.05) using Fishers' test. W 82=Williams 82.

Table 3. Effect of foliar boron on soybean seed weight (100 seed weight, g), nodule mass (mg $plant^{-1}$), nodule number $plant^{-1}$, ARA (μmol of C_2H_4 $plant^{-1}$ h^{-1}), root respiration (mmol of CO_2 evolved/g of root/h), leaf NRA (µmol NO_2 $g^{-1}h^{-1}$), and nodule NRA (µmol NO_2 $g^{-1}h^{-1}$) in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under well watered conditions without boron (W-B) and with boron (W+B) under greenhouse conditions [a].

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Watered stressed soybean								
Variety	**Boron**	**Grain weight (100 seed weight, g)**	**Nodule mass (mg dwt plant$^{-1)}$**	**Nodule number plant^{-1}**	**ARA (NF)**	**Root respiration**	**Leaf NRA (µmol NO_2 $g^{-1}h^{-1}$)**	**Nodule NRA (µmol NO_2 $g^{-1}h^{-1}$)**
Pella	WS-B	9.4 a	45.3 b	22.4 a	7.6 a	4.3 b	3.6 a	2.4 b
W 82		10.3 a	51.6 a	24.1 a	8.7 a	4.9 b	2.7 b	3.6 a
Hutcheson		9.7 a	42.7 b	21.5 a	6.5 b	5.4 b	3.3 a	2.7 b
Forrest		10.1 a	46.3 b	14.5 b	5.7 c	6.3 a	4.7 a	2.5 b
Pella		11.1 a	56.7 b	28.6 a	10.5 a	5.4 c	5.4 b	4.7 ab
W 82	WS+B	12.5 a	66.4 a	31.4 a	9.7 a	7.6 b	7.5 a	5.3 a
Hutcheson		12.6 a	57.3 b	30.7 a	10.4a	8.7 a	5.8 b	4.7 ab
Forrest		11.5 a	63.2 a	19.6 b	7.4 b	9.8 a	6.3 a	3.9 b

[a] Soybean plants were grown under water stress (WS) (-90 to -100 kPa soil water potential). Soybeans were grown under greenhouse conditions similar to those previously reported [10]. Values within columns and within each B treatment sharing a letter are not significantly different (P>0.05) using Fishers' test. W 82=Williams 82.

Table 4. Effect of foliar boron on soybean seed weight (g), nodule mass (mg plant^{-1}), nodule number plant^{-1}, ARA ((μmol of C_2H_4 plant^{-1} h^{-1}), root respiration (mmol of CO_2 evolved/g of root/h), leaf NRA (µmol NO_2 $g^{-1}h^{-1}$), and nodule NRA (µmol NO_2 $g^{-1}h^{-1}$) in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under water stress conditions without boron (WS-B) and with boron (WS+B) under greenhouse conditions [a].

Severe watered stressed soybean								
Variety	**Boron**	**Grain weight (100 seed weight, g)**	**Nodule mass (mg dwt plant$^{-1)}$**	**Nodule number plant^{-1}**	**ARA (NF)**	**Root respiration**	**Leaf NRA (µmol NO_2 $g^{-1}h^{-1}$)**	**Nodule NRA (µmol NO_2 $g^{-1}h^{-1}$)**
Pella	SWS-B	4.4 a	21.5 b	20.7 a	4.6 a	2.1 c	2.1 a	0.7 a
W 82		5.5 a	28.5 a	21.6 a	5.3 a	3.3 b	1.5 b	0.9 a
Hutcheson		5.3 a	27.5 a	19.6 a	4.7 a	3.6 b	2.4 a	0.8 a
Forrest		4.7 b	22.6 b	21.5 a	3.2 b	4.7 a	1.7 b	0.8 a
Pella		4.3 c	25.4 a	22.6 b	5.1 a	2.6 b	2.4 a	0.8 a
W 82	SWS+B	6.0 a	30.6 a	26.5 a	4.8 a	2.4 b	0.9 b	0.6 a
Hutcheson		5.5 b	26.5 a	29.7 a	3.4 b	3.8 a	2.1 a	0.8 a
Forrest		4.1 c	21.6 b	19.6 b	3.2 b	4.9 a	1.2 b	0.7 a

[a] Soybean plants were grown under severe water stress (soil water potential between –150 to –200 kPa). Soybeans were grown under greenhouse conditions similar to those previously reported [10]. Values within columns and within each B treatment sharing a letter are not significantly different (P>0.05) using Fishers' test. W 82=Williams 82.

Table 5. Effect of foliar boron on soybean seed weight (g), nodule mass (mg plant^{-1}), nodule number plant^{-1}, nitrogen fixation (ARA, μmol of C_2H_4 plant^{-1} h^{-1}), root respiration (mmol of CO_2 evolved/g of root/h), leaf NRA (µmol NO_2 $g^{-1}h^{-1}$), and nodule NRA (µmol NO_2 $g^{-1}h^{-1}$) in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under severe water stress conditions without boron (SWS-B) and with boron (SWS+B) under greenhouse conditions [a].

Foliar boron application increased B in leaves and seed in watered plants (Figure 1). No significant B concentration differences were observed between leaves and seeds B in each watered treatment in each cultivar, indicating that B movement from leaves to seeds was not limited. In severely water-stressed plants, application of foliar B did not significantly increase B in leaves, and B movement from leaves to seed was limited, indicated by the large accumulation of B in leaves and small accumulation of B in seeds. Similar trend of N in leaves and seed was noticed (Figure 2), indicating a close relationship between B and N.

3.3. Effects of B and water stress on seed sugars

Since B plays an important role in carbohydrate mobility and since carbohydrates are a source of reducing power in nitrogen assimilation, sugar profiling was also investigated. Our research demonstrated that foliar B application resulted in higher sucrose, glucose, and fructose under irrigated conditions, but under severe water stress these mono and disaccharides sugars decreased, but stachyose and raffinose increased (Table 6,7,8).

This indicated that there was a redistribution of sugars under severe water stress, and this shift may provide plants with an adaptive mechanism to tolerate the stress.

Foliar boron resulted in higher seed sucrose, glucose, and fructose concentrations in W+B plants, indicating B involvement in sugar metabolism and synthesis. The involvement of B in sugar synthesis and distribution is not understood, but B involvement in sugar movement and metabolism was previously reported [2,3,50]. The decrease of sucrose, glucose, and fructose in SWS-B and SWS+B plants compared with W+B and W-B indicated that B enhanced accumulation of sucrose, glucose, and fructose concentrations may be due to B role in sugar movement within the plants, and that severe water stress limited sugars movement due reduction of B uptake and stomatal conductance. The increase of seed stachyose concentration in seeds of SWS-B and SWS+B plants indicated that severe water stress affects the distribution of sugar fractions, in our case the increase of stachyose and raffinose and decrease of sucrose, glucose, and fructose concentrations. The increase of stachyose under water stress may indicate the role of stachyose in plant tolerance to biotic and abiotic stress [14,15]. It was also reported that raffinose and galactinol levels may play an important role in plant tolerance to biotic and abiotic stress [14,15], and the accumulation of galactinol and raffinose may protect the plant from drought [51], and the activity of sucrose synthase, the main enzyme involved in sucrose hydrolysis in nodules, was significantly inhibited under drought conditions [52,53]. The biological functions of raffinose and stachyose are not clear [54], but previous research reported that oligosaccharides (sucrose, raffinose, and stachyose) are related to seed quality [55] and the acquisition of desiccation tolerance during seed development and maturation.

Soybean seed sugars are important to soybean seed industry because they determine the quality of seeds beside protein and oil. This is because soybean seed with high raffinose and stachyose concentrations are undesirable and have negative effects on the nutritive value of soymeal and seed consumed by human. Stachyose and raffinose are indigestible by humans and animals, especially monogastric animal such as chicken and pigs, causing flatulence or diarrhea [56]. On the other hand, low raffinose and stachyose levels in soybean seed are desirable [57]. High level of seed sucrose, glucose, and fructose are desirable because it

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improves taste and flavor of tofu, soymilk, and natto [58]. Currently, soybean cultivars with improved sugar profiles have been released to the market [58], and breeding for desirable sugars in soybean or agricultural practices to improve seed sugars are needed.

		Watered soybean				
Variety	**Boron**	**Glucose (mg g^{-1})**	**Fructose (mg g^{-1})**	**Sucrose (mg g^{-1})**	**Raffinose (mg g^{-1})**	**Stachyose (mg g^{-1})**
Pella	W-B	1.5 b	0.73 a	35.4 b	5.4 a	32.6 b
W 82		2.1 a	0.65 b	42.4 a	4.8 b	43.6 a
Hutcheson		1.5 b	0.64 b	32.7 b	5.3 ab	42.8 a
Forrest		2.2 a	0.61 b	30.8 b	5.7 a	41.6 a
Pella		2.3 b	0.93 b	57.4 b	5.4 ab	35.4 b
W 82	W+B	2.8 a	1.22 a	63.2 a	5.1 b	44.6 a
Hutcheson		2.1 b	0.89 b	53.8 b	5.7 a	42.6 a
Forrest		2.9 a	0.94 b	59.7 b	5.2 b	46.5 a

[a] Soybean plants were grown at field capacity at -15 to -20 kPa according to Bellaloui et al., (2011). Soybeans were grown under greenhouse conditions similar to those previously reported [10]. Values within columns and within in each B treatment sharing a letter are not significantly different (P>0.05) using Fishers' test. W 82=Williams 82.

Table 6. Effect of foliar boron on soybean seed sugars in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under well watered conditions without boron (W-B) and with boron (W+B) under greenhouse conditions [a].

Variety	**Boron**	**Glucose (mg g^{-1})**	**Fructose (mg g^{-1})**	**Sucrose (mg g^{-1})**	**Raffinose (mg g^{-1})**	**Stachyose (mg g^{-1})**
Pella	WS-B	0.8 b	0.74 a	19.3 a	6.5 b	45.5 b
W 82		1.1 b	0.65 b	16.4 b	7.3 a	52.5 a
Hutcheson		1.5 a	0.42 c	21.3 a	7.4 a	47.3 b
Forrest		0.9 b	0.69 b	15.4 b	6.4 b	50.3 a
Pella		1.5 b	0.76 a	27.5 ab	7.1 a	41.6 b
W 82	WS+B	1.5 b	0.75 a	25.4 b	6.2 b	48.7 a
Hutcheson		1.9 a	0.64 b	31.2 a	7.4 a	50.3 a
Forrest		1.6 b	0.72 a	30.5 a	6.3 b	48.5 a

[a] Soybean plants were grown under water stress (WS) (-90 to -100 kPa soil water potential). Soybeans were grown under greenhouse conditions similar to those previously reported [10]. Values within columns and within each B treatment sharing a letter are not significantly different (P>0.05) using Fishers' test. W 82=Williams 82.

Table 7. Effect of foliar boron on soybean seed sugars in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under water stress conditions without boron (WS-B) and with boron (WS+B) under greenhouse conditions [a].

Variety	Boron	Glucose (mg g^{-1})	Fructose (mg g^{-1})	Sucrose (mg g^{-1})	Raffinose (mg g^{-1})	Stachyose (mg g^{-1})
Pella	SWS-B	0.65 a	0.54 a	17.5 a	8.5 b	73.5 a
W 82		0.53 b	0.43 b	14.3 b	9.5 a	65.4 b
Hutcheson		0.51 b	0.47 b	15.3 ab	8.3 b	73.7 a
Forrest		0.48 c	0.52 a	11.6 c	9.5 a	65.7 b
Pella		0.59 a	0.53 a	19.5 a	8.5 b	62.1 b
W 82	SWS+B	0.48 c	0.47 b	13.2 b	9.5 a	73.6 a
Hutcheson		0.52 b	0.51 a	17.6 a	8.7 b	68.5 ab
Forrest		0.51 b	0.48 b	14.2 b	7.2 c	68.5 ab

[a] Soybean plants were grown under severe water stress (soil water potential between –150 to –200 kPa). Soybeans were grown under greenhouse conditions similar to those previously reported [10]. Values within columns and within each B treatment sharing a letter are not significantly different (P>0.05) using Fishers' test. W 82=Williams 82.

Table 8. Effect of foliar boron on soybean seed sugars in genotypes of maturity group III (Pella and William 82) and MG V (Hutcheson and Forest) under severe water stress conditions without boron (SWS-B) and with boron (SWS+B) under greenhouse conditions [a].

3.4. Effect of B and water stress on $\delta^{15}N$ ($^{15}N/^{14}N$ ratio) and $\delta^{13}C$ ($^{13}C/^{12}C$ ratio)

Foliar B did not result in changes in $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratios, but significant differences in these ratios were observed between irrigated and non-irrigated soybean with or without foliar B (Figure 3). The alteration of $^{15}N/^{14}N$ by increasing ^{15}N (derived from soil nitrogen that is used for nitrate assimilation) and decreasing ^{14}N (derived from atmospheric nitrogen that is used for nitrogen fixation) indicated that the source of nitrogen use changed, and plants favored N from soil over atmospheric nitrogen, indicating that nitrogenase is more sensitive than nitrate reductase under water stress. The mechanisms of this shift are not understood, but one possible explanation is that the shift in $^{15}N/^{14}N$ may reflect a possible mechanism to compensate for the inhibition of nitrogen fixation under water stress conditions. Previous research indicated that $\delta^{15}N$ values in the xylem and plant tissues were associated with acquired N, and changed with N metabolism [59]. The increase in $\delta^{13}C$ or higher $^{13}C/^{12}C$ ratio (less negative) in seed under severe water stress conditions indicated that the source of carbon fixation used was shifted. Previous research reported that that the $\delta^{13}C$ value in plant tissues can be affected by water supply [60], plant physiology [61], and mycorrhizal infection [62]. The level of $\delta^{13}C$ was dependent on the environmental factors and their association with plant gas exchange, stomatal conductance, and CO_2 fixation [63]. It was found that drought stress leads to stomatal closure and ^{13}C fixation increase, resulting in less discrimination against $\delta^{13}C$ [64,65]. Previous research indicated that the the shift in $^{13}C/^{12}C$ ratio was a result of a shift in carbon fixation metabolism from ribulose bisphosphate (RuBP) carboxylase pathway to phosphoenolpyruvate carboxylase (PEP). This shift resulted in $\delta^{13}C$ enrichment [60]. It was found that in C3 species, to which soybean belongs, carbon isotope composition changes among and between

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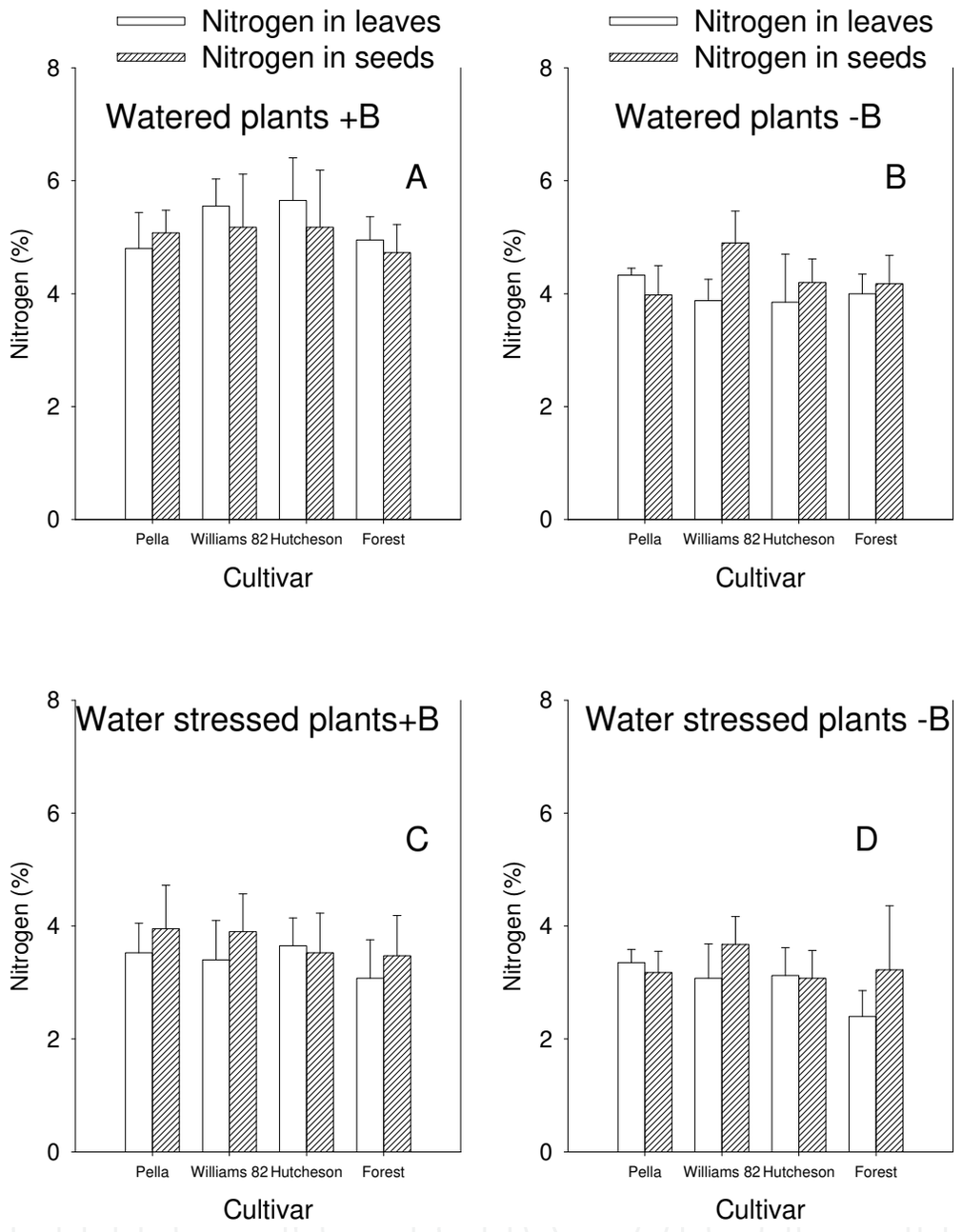

Figure 1. Effects of foliar boron application (1.1 kg B ha^{-1}) on boron concentration in leaves and seed in soybean genotypes in watered (A,B) and severe water stressed (C,D) soybean genotypes. Soybean plants were grown under severe water stress (soil water potential between –150 to –200 kPa). Soybeans were grown under greenhouse conditions similar to those previously reported [10].

genotypes correlated with water use efficiency, and the stable isotope ^{13}C would be discriminated against during photosynthesis, leading to a smaller ^{13}C to ^{12}C ratio [66]. The enrichment of ^{13}C may be due closure of stomatal conductance under severe water stress, leading to ^{13}C fixation increase and less ^{13}C discrimination [67,68]. Our current results are in agreement with previous reports that environmental stresses, including drought, can alter $\delta^{13}C$ due to the drought effects on the balance between stomatal conductance and carboxylation [67,68,69].

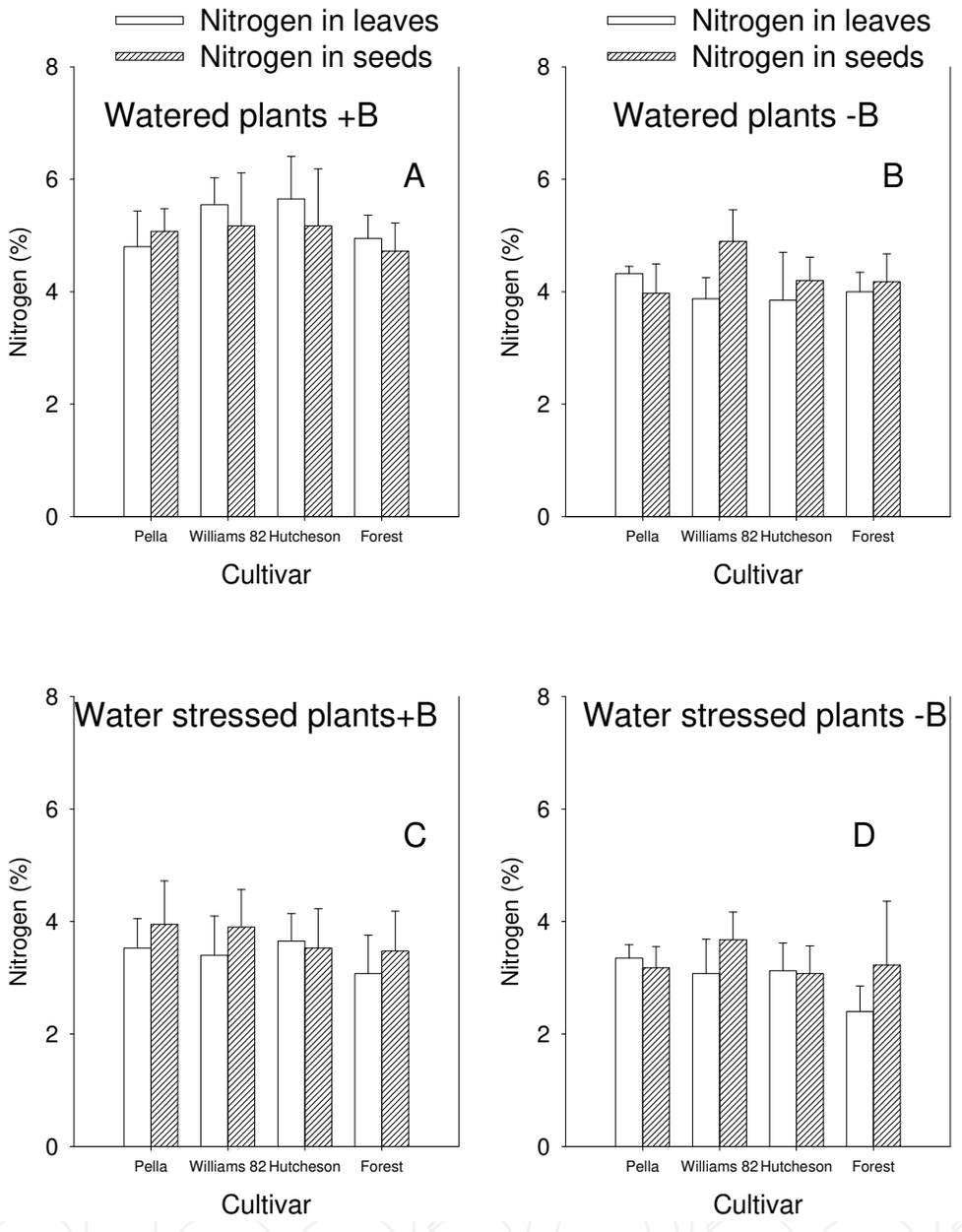

Figure 2. Effects of foliar boron application (1.1 kg B ha^{-1}) on nitrogen percentage in leaves and seed in soybean genotypes in watered (A,B) and severe water stressed (C,D) soybean genotypes. Soybean plants were grown under severe water stress (soil water potential between –150 to –200 kPa). Soybeans were grown under greenhouse conditions similar to those previously reported [10].

During carbon fixation by photosynthesis, the naturally occurring stable isotope ^{13}C is discriminated against, and plants would have a smaller ^{13}C to ^{12}C ratio than ^{13}C to ^{12}C ratio in fixed CO_2 of the air, suggesting a possible use of this technique to select for water use efficiency (Farquhar et al., 1989). Our results demonstrated that $\delta^{15}N$ and $\delta^{13}C$ values changed and enrichment occurred under water stress conditions, suggesting that both nitrogen and carbon metabolism pathways were affected during water stress, impacting seed production and seed quality.

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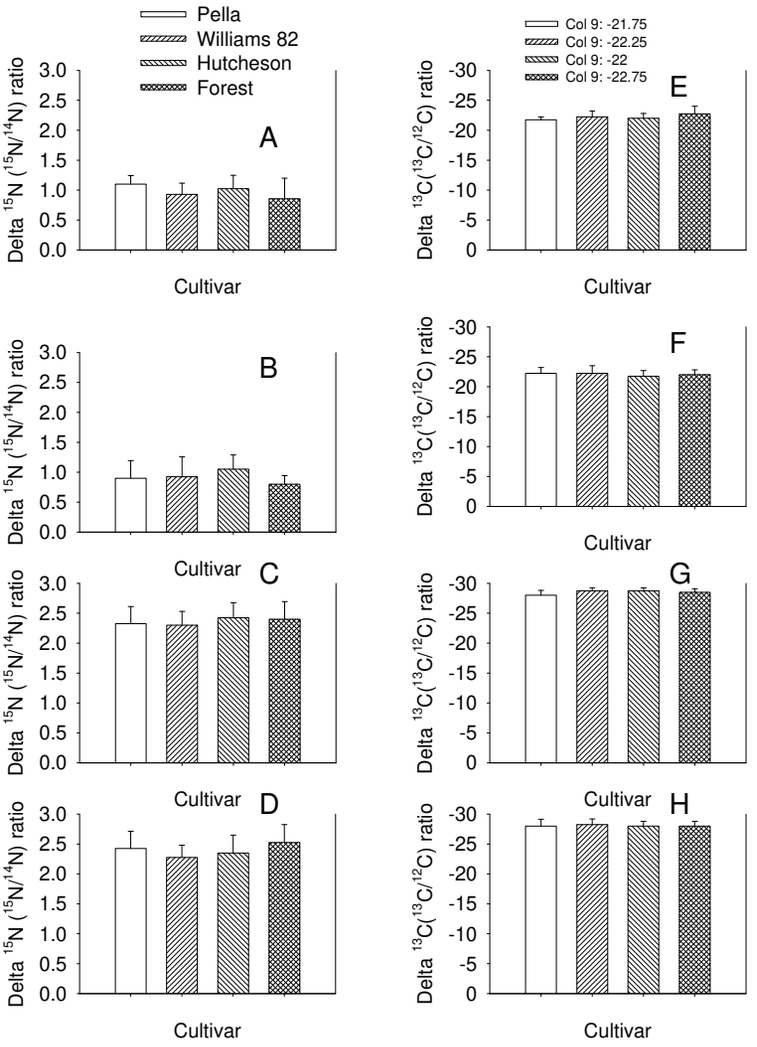

Figure 3. Effects of foliar boron application (1.1 kg B ha^{-1}) on seed $\delta^{15}N$ ($^{15}N/^{14}N$ Ratio) (A-C) and $\delta^{13}C$ ($^{13}C/^{12}C$ Ratio) (D-H) in soybean genotypes in watered plants with B (A) and without B (B); in water stressed plants with B (C) and without B (D); in soybean genotypes in watered plants with B (E) and without B (F); in water stressed plants with B (G) and without B (H); Soybean plants were grown under severe water stress conditions (soil water potential between –150 to –200 kPa). Soybeans were grown under greenhouse conditions similar to those previously reported [10].

4. Conclusions

Foliar boron application resulted in nodule growth by increasing the number and mass of nodules under well watered or moderate water stress conditions. Also, foliar boron resulted in higher nitrogen fixation and nitrogen assimilation under well watered or moderate water stress conditions. Foliar B application under severe water stress did not enhance nodule number or mass, nitrogen fixation and nitrogen assimilation. This is because severe water stress altered nitrogen and carbon fixation as indicated by changes in the values of ^{15}N and ^{13}C natural isotopes. Nitrogen fixation is more sensitive than nitrogen assimilation under

severe water stress conditions. Foliar B enhanced seed sugars under well watered conditions, but sever water stress resulted in redistribution of sugar fractions by increasing monosaccharides such as glucose and fructose, decreasing sucrose as a result, but increasing both raffinose and stachyose due to their possible roles in drought and the acquisition of desiccation tolerance during seed development and maturation. Increasing sugars by foliar B is desirable as high glucose, fructose, and sucrose contribute to soybean seed quality by improving the taste and flavor of soymeal based products such as tofu, soymilk, and natto.

Although our research showed that B has beneficial effects on nodules and nitrogen fixation and seed quality, further research is needed to test these findings under field and drought conditions in multi-year and multi-location experiments so that recommendations can be made.

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Author details

Nacer Bellaloui[1], Alemu Mengistu[2], My Abdelmajid Kassem[3], Craig A. Abel[4] and L.H.S. Zobiole[5]

1 USDA-ARS, Crop Genetics Research Unit, Stoneville, USA

2 USDA-ARS, Crop Genetics Research Unit, Jackson, TN, USA

3 Plant Genomics and Biotechnology Lab, Department of Biological Sciences, Fayetteville State University, Fayetteville, NC, USA

4 Corn Insects and Crop Genetics Research, Iowa State University, Ames, Iowa, USA

5 Crop Protection, R&D Dow AgroSciences – Brazil, Cascavel, Paraná, Brazil

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