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REGULAR ARTICLE

Nitrogen metabolism and seed composition as influenced by foliar boron application in soybean

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Abstract The physiological effects of foliar boron application (FB) on nitrogen metabolism and seed composition have not been well established in soybean [(*Glycine max*(L.)Merr.)]. Therefore, the effect of FB on nitrogen metabolism and seed composition was investigated. Nitrate assimilation was evaluated by measuring nitrate reductase activity (NRA) and nitrogen fixation was evaluated by measuring nitrogenase activity and natural abundance of $^{15}N/^{14}N$. NRA were significantly ($P \leq 0.05$) higher in plants that received FB than the control plants. Higher rate of FB (One application of four times of commercial rate) inhibited nitrogen fixation as measured by natural abundance of $^{15}N/^{14}N$ ratio, but increased NRA. The higher activities of NR and nitrogenase by FB were accompanied with a higher B concentration in leaves. The significant ($P<0.0001$) enrichment of $^{15}N/^{14}N$, accompanied with a higher rate of FB, suggested a possible mechanism where nitrate assimilation may compensate for the decrease in nitrogen fixation. FB increased seed protein by 13.7% and oleic acid by 30.9% compared to the control plants. This alteration was accompanied by a higher B concentration in leaves and seed. The results suggest that FB affects nitrogen metabolism and alters seed compositions, especially protein and unsaturated fatty acids.

Keywords Boron · Nitrate reductase · Nitrogenase · Nitrogen assimilation · Nitrogen fixation · Seed composition

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Introduction

Boron (B) is an essential micronutrient and plays a major role for plant growth and development (Pilbeam and Kirkby 1983; Marschner 1995) and crop quality (Dordas 2006, Dordas et al. 2007). It was demonstrated that foliar boron (FB) improved seed set, seed yield, and seed quality of alfalfa (Dordas 2006) and sugar beet (Dordas et al. 2007) and increased fruit set and yield in sunflower (Asad et

al. 2003). It was reported that floral and fruiting organs are sensitive to B deficiency (Gauch and Duggar 1954; Dell et al. 2002) and higher requirement for B during flowering and seed set was shown even where the B levels in leaves are in the adequate range (Dordas 2006).

In spite of the well documented literature on the structural and metabolic role of B (Pilbeam and Kirkby 1983; Marschner 1995; Brown et al. 2002), the beneficial effects of B on soybean yield is still controversial (Reinbott and Blevins 1995; Ross et al. 2006) and the physiological effects of B on the nitrogen metabolism and seed composition in soybean have not been well understood. Foliar B fertilizer at 0, 0.28, 0.56, 1.12, and 2.24 kg B ha^{-1} near the V2 (plants have two fully developed trifoliolate leaf nodes) or R2 (full flowering) growth stages had no significant effect on soybean yield at one site but increased seed yields from 4 to 130% at three sites. B application at V2 increased yields by 13% compared with applications at R2 (Ross et al. 2006). However, at a site where leaf B concentrations were sufficient, B applied at the R2 significantly increased seed yields by 5% compared with V2 B applications. Trifoliate leaf B concentrations at the R2 stage increased as B rate increased. Seed B concentrations also increased as B rate increased. Ross et al. (2006) concluded that application of 0.28 to 1.12 kg B ha^{-1} during early vegetative or reproductive growth was sufficient to produce near maximal yields. Touchton and Boswell (1975) reported that soybean responded positively to B fertilization at three of nine site-years. The inconsistency of B effect on yield could be due to differences in environmental conditions and soil chemical and physical properties that influence B availability to plants (Moraghan and Mascagni 1991). Foliar B resulted in a significant increase in the number of pods/branch, and split application, twice during flowering showed that a total of 0.56 kg ha^{-1} was optimal for increasing pods/branch (Schon and Blevins 1990). Also, Schon and Blevins (1990) showed that foliar B applications resulted in an increase of leaf B concentration above 60 mg B kg^{-1} that was previously accepted as the upper level of tolerance for soybeans, and optimal branching and per plant yield parameters were achieved by plants with B leaf concentrations greater than 160 mg B kg^{-1}.

Researchers reported that B is an essential micronutrient for the development of nitrogen-fixing root nodules in pea (*Pisum sativum*) (Bolanos et al. 1996). In plants grown in B-deficient medium, lower infection of the host plants with *Rhizobium* was noticed compared to plants supplied with adequate B (Bolanos et al. 1996). Bolafios et al. (1994) reported that nodules showed little or no ability to fix N_2 under B-deficient plants, leading to N deficiency and necrosis of nodulated pea plants. It was shown that nitrogen fixation in peas and soybean were sensitive to B deficiency and toxicity (Bolafios et al. 1994; Carpena et al. 2000) and B deficiency resulted in the reduction in early nodullin protein (ENOD2) in nodule parenchyma cells and malfunction of oxygen diffusion barrier (Bonilla et al. 1997). In spite of the above B research, there is no convincing evidence that there is a direct effect of B on nitrogen metabolism (Shelp 1993; Marschner 1995; Bonilla et al. 1997), nor is there well established information on the relationship between nitrogen fixation and nitrogen assimilation under FB in soybean.

Nitrogen metabolism in legumes is a result of both symbiotic N_2 fixation and mineral N assimilation processes. Atmospheric N_2 is fixed by the enzyme nitrogenase in the bacteroids of nodules (Kanayama et al. 1999), and nitrate reduction (assimilation) is catalyzed by the enzyme nitrate reductase (NR). Both NR and nitrogenase enzymes coexist in nodules competing for reductant (Caba et al. 1995). So far, there is limited information on the effects of FB on nitrogen assimilation and fixation and their impact on seed protein and fatty acids in soybean. Therefore, the objective of this study was to investigate the physiological effects of FB on nitrogen metabolism and seed composition.

Materials and methods

Experiment 1

A repeated greenhouse experiment was set up to investigate the effect of FB application on nitrogen metabolism and seed composition in soybean. Soybean seeds were germinated in flat trays in vermiculite. Four uniform size seedlings at about V1 stage (Fehr et al. 1971) were transplanted into 9.45 L pots filled with field soil (sandy loam, fine-loamy, mixed thermic Molic Hapludalfs) 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 0.72 mg kg^{-1}, and it contained an abundant native population of *B. japonicum*. To avoid water stress effect, soil water was daily monitored by Soil Moisture Meter (WaterMark Company, Inc., Wisconsin, U.S.A.) using soil water potential sensors. Soil water potential was kept between −15 and −20 kPa (this was considered the field capacity value in our soil conditions). No fertilizers were added to irrigation water. Boron, as boric acid, of a rate of 0.45 kg ha^{-1} was foliar applied using hand sprayer. Measures to avoid boron drift to the control plants were taken. Boron applications were made at either V 5 only (T1), R2 only (T2), or both V5 and R2 (V5+R2, T3). Samples were taken 5 days after each FB for NRA, nitrogenase, and leaf B. For NRA measurements only V5 and R2 FB were used, but not V5+R2 FB. For the control plants, only water was sprayed to plants. Four replicates were used for each treatment and each sampling time for each experiment. Mature grain weight was obtained from R8 stage. Plants were considered mature (reached R8 stage) according to Fehr et al. (1971). Nodules were gently detached from roots for fresh weight determination. Each pot is considered one replicate and each pot had four individual plants. Temperature was about 32±7°C during the day and about 20±5°C at night with a photosynthetic photon flux density (PPFD) of about 800–1,500 µmol m^{-2} s^{-1}, measured by Quantum Meter (Spectrum Technology, Inc., Illinois, U.S.A.). The range of light intensity reflects a cloudy or bright/sunny day, respectively. The source of lighting in the greenhouse was a mixture of natural light, bulb light (60 W), cool white (250 W).

Experiment 2

To test possible B mobility from mature leaves to young tissue, plants were grown under the same growth conditions as above in Experiment 1, except that at R2, one group of plants was transferred to half-strength of Hoagland solution (Hoagland and Arnon 1950) with 10 mg BL^{-1} concentration. The second group was grown in 0 B, but their fully expanded mature lower leaves were foliar-applied with 300 mg BL^{-1} (lower leaves were immersed in B solution twice for about 30 s once every two days during one week). The third group was grown in 0 B in the medium with 0 FB. Three weeks after treatments, the concentration of B in young leaves, mature leaves, and R6 immature seed was determined to observe if there is any B disappearance in mature leaves or B appearance in young leaves and immature seed. Measures to prevent drift to the growth medium or control plants during FB were taken.

To test the effects of higher FB on nitrogen fixation using Delta (δ) ^{15}N using natural abundance method, four times of FB commercial rate (1.8 kg B ha^{-1}) for soybean was applied.

In vivo nitrate reductase assay

In all experiments, unless indicated otherwise, samples were taken 5 days after each FB. Four replicates were used from each treatment. Nitrate reductase activity (NRA) was measured in leaves (fully expanded leaf), stems, roots, and nodules (nodules were gently and carefully separated from roots and placed in the buffer solution for in vivo NRA assay) based on the method of Klepper and Hageman (1969) and as described for soybean in detail by Bellaloui et al. (2006). A root sample from four plants (one pot of four plants was considered one replicate) was excised, mixed, and random, combined sample was used for analysis.

To determine potential NRA (PNRA) under conditions when nitrate concentration could not be a limiting factor, exogenous nitrate was added to the incubation solution at a concentration of 10 mM. To determine the effect of B on NRA in leaves, stems, roots, and nodules, B was added to the buffer solution at a concentration of 0.5 mM as boric acid. Nitrate reductase activity per part was calculated by multiplying the concentration of NRA of that part by its weight. Total NRA per plant was calculated by adding NRA $part^{-1}$ for leaves, stems, and roots.

Acetylene reduction assay

In all experiments, unless indicated otherwise, samples were taken 5 days after each FB. Four replicates were used from each treatment. Plants with roots and shoot were carefully removed from pots and immediately transported to the laboratory, and assayed for nitrogenase activity. Nitrogenase activity was assayed using the acetylene reduction assay as described elsewhere (Hardy et al. 1968; Zablotowicz et al. 1981; Bellaloui et al. 2008). Roots with nodules intact were excised and incubated in 1 L Mason jars. Four

roots were placed in the Mason jars and sealed. A 10% volume of acetylene was added to the jars. 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. An Agilent HP6960 (Agilent Technologies, Wilmington, DE) gas chromatograph was equipped with manual injector, injector loop, sample splitter, flame ionization detector (FID), and thermal conductivity detector (TCD) was 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 developed for each day of analysis and used to determine ethylene and carbon dioxide evolved. Samples having <9% acetylene were not used in the analysis. Following the incubation, roots were washed, the nodules were removed from the roots, and the dry weight of nodules and roots was determined following oven-drying at 60°C for 4–5 days.

Delta (δ) ^{15}N using natural abundance method

Delta ^{15}N abundance was evaluated from nitrogen isotope $^{15}N/^{14}N$ ratio according to (Shearer and Kohl 1986; Bellaloui et al. 2008) using about 0.9 mg of ground seeds. A Thermo FinniGlyn Delta Plus Advantage Mass Spectrometer with a FinniGlyn ConFlo III, and Isomass Elemental Analyzer (Bremen, Germany) was used for isotopic analysis. Delta values were obtained using Isodat software version 2.38. The elemental combustion system was Costech ECS 4010 with an autosampler (Bremen, Germany).

Boron determination

Boron was determined in leaves and seed with the Azomethine-H method (Lohse 1982). Calcium carbonate powder was added to seed samples before ashing to prevent losses of volatile B compounds. Briefly, one g of dry samples was placed in a porcelain crucible for ashing at 500°C for 8 h. After ashing, samples were extracted with 20 ml of 2 M HCl at 90°C for 10 min, and after filtration the samples were transferred to plastic vials. Then 2 ml of the solution was added to 4 ml of buffer solution (containing 25% ammonium acetate, 1.5% EDTA, and 12.5% acetic acid) and 4 ml of azomethine-H solution containing 0.45% azomethine-H and 1% of ascorbic acid prepared right before the analysis (John et al. 1975). The color was left to develop for at least 45 min, and the amount of B was determined using a Beckman Coulter DU 800 spectrophotometer (Fullerton, California) at 420 nm.

Cell wall B determination was based on those of Hu and Brown (1994). Fresh samples of the fully expanded leaves were taken from plants grown in 10 mg B L^{-1} in the medium with 0 FB, 0 B in the medium with 300 mg L^{-1} FB, and 0 B in the medium with 0 FB (Control). Samples were homogenized with an ice cold mortar and pestle in cold water. The homogenate was then centrifuged at 1,000 g for 10 minutes. The residue was washed three times with 10 vol of 80% ethanol and once with 10 vol of methanol:chloroform mixture (1:1, *v*/*v*). Finally, the precipitate was washed with 10 vol of acetone. The samples were then dried and ashed for cell wall B determination using the method described above.

To determine B in soil, four replicates of soil samples were analyzed at the Soil, Plant, and Water Laboratory, University of Georgia, Athens, GA using inductively coupled plasma spectrometry (ICP) using Thermo Elemental, Thermo Jarrell-Ash model 61E ICP, USA.

Protein, oil, and fatty acids analyses

Seed from each treatment were analyzed for protein, oil, and fatty acids, using near-infrared (NIR) reflectance (Wilcox and Shibles 2001; Bellaloui et al. 2008), diode array feed analyzer, Perten. Calibrations were developed by Perten using Thermo Galactic Grams PLS IQ. The calibration curve has been updated by University of Wisconsin, USA, for unique samples using AOAC (1990a) and AOAC (1990b) methods. The analysis was performed on the basis of percent dry matter (Wilcox and Shibles 2001; Boydak et al. 2002).

Statistical and experimental design

Treatments were arranged in a randomized complete block design with four replications. The experiment

was repeated twice. The data were subjected to analysis of variance using Proc GLM (SAS 2001). Means were separated by Fisher's least significant difference test at the 5% level of probability.

Results

Grain weight and nodule mass

Foliar B (FB) at V5+R2 combined resulted in a significant ($P<0.0001$) increase in grain weight per plant (7.4 g plant^{-1}) compared to the control plants (5.8 g plant^{-1}) (Table 1). Also, FB at V5 alone or R2 alone resulted in a significant ($P\leq0.05$) increase in grain yield compared to the control plants (Table 1). Nodule mass under FB was 83 mg nodules fwt plant^{-1} compared with 71 mg nodules fwt plant^{-1} in control plants (Table 1).

Boron concentrations in leaves and seed

B concentration in leaves (Fig. 1a,b) and seeds (Fig. 2) of FB-treated plants was significantly ($P\leq$ 0.0001) higher than those of the control plants. Seed B concentration increased with FB comparing to C, but differences between at R2 and V5+R2 not significant (Fig. 2). B concentration in mature leaves and R6-seed of FB or B in the growth medium was significantly ($P\leq0.0001$) higher than those of the control plants (Table 1).

Boron concentration of 10 mg L^{-1} in the medium or FB of 300 mg L^{-1} increased B in young and mature leaves, and R6-seed compared with 0B in the medium. Boron concentration in mature leaves or in R6-seed did not significantly differ when B concentration of 10 mg L^{-1} in the growth medium or 300 mg L^{-1} foliar B was used (Table 1). Cell wall B was higher in leaves of plants grown in 0B in the medium. However, cell wall B was lower in both 10 mgL^{-1} in the medium or FB 300 mgL^{-1} compared with leaves from those grown in 0B in the medium (Table 1). It must be noticed here that no toxicity symptoms were observed in plants grown in 10 mg B L^{-1}.

Nitrate reductase activity in leaves, stems, roots, and nodules

Foliar B significantly ($P<0.0001$) increased the rate of NRA in leaves and roots at FB at V5 (Fig. 3a). Foliar B did not result in any significant differences in NRA in stems (Fig. 3a). Foliar B at R2 resulted in an increase in leaves NRA, but no changes were observed in either roots or stems (Fig. 4a). The same general trend of FB effect on NRA in leaves was noticed on NRA Part^{-1} ($P\leq0.0001$) at V5 or R2 (Figs. 3b and 4b). Foliar B application at either V5 or R2 increased total NRA Plant^{-1} significantly ($P=$ 0.0125) (Fig. 5a,b). NRA in nodule showed a different pattern in that FB at V5 did not result in significant change compared to the control (Fig. 6a,b). However, FB at R2 significantly increased NRA in nodules and this pattern was observed either when NRA was expressed as µmol nitrite g^{-1} nodule h^{-1} ($P=0.0038$) or as µmol nitrite plant nodule^{-1}h^{-1} ($P=$ 0.0079) (Fig. 6c,d).

Table 1 Effect of foliar B (FB) application at V5 (five fully developed trifoliolate leaf node), R2 (full flowering), and at V5+R2 stages on nodule mass and grain weight, and effect of foliar B and B in the medium on B cell wall percentage in the fully expanded leaf, B in young leaves (YL), mature leaves (ML), and R6 seed in soybean. Control (C) received 0 FB

Boron			Boron				
Foliar Application	Nodule mass (mg fwt plant^{-1})	Grain weight (g plant^{-1})	Foliar and in the medium	B in cell wall (%)	B in YL	B in ML	B in R6-seed
C	71±2.63 b	5.8±0.19 c	0	89±7.0 a	18.4±1.9 c	34.9±2.2 b	19.3±2.9 b
V5	83±2.99 a	6.4±0.10 b	10 mgl^{-1}	57±4.0 c	46.8±1.5 a	49.1±1.0 a	44.9±1.3 a
R2	86±2.58 a	6.7±0.10 b	FB 300 mg l^{-1}	69±4.3 b	32.2±2.9 b	46.3±2.0 a	47.0±1.3 a
V5+R2	87±2.45 a	7.4±0.29 a					

Means within a column followed by the same letter are not significantly different at the 5% level as determined by Fishers' LSD test. Values are means ± SD

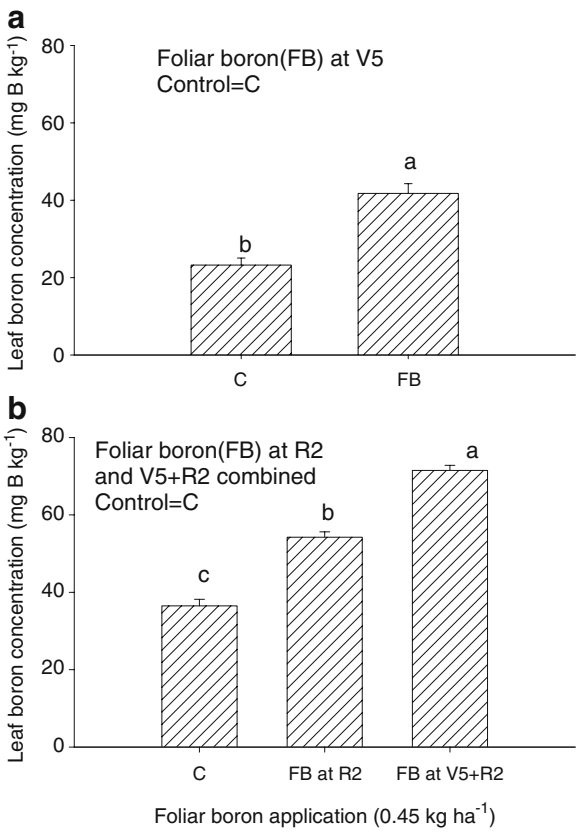

Fig. 1 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node) (**a**), R2 (full-bloom), and V5+R2 (**b**) stages on B concentration in leaves in soybean. Control = C (0FB). Bar values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

Acetylene reduction activity

Foliar B resulted in a significant increase ($P \leq 0.0001$) in nitrogenase activity compared with those of the control (Fig. 7a, b). The highest nitrogenase activity was recorded when FB was applied at R2 or at V5+R2 combined (Fig. 7a). No significant differences in nitrogenase between R2-FB and V5+R2 combined FB treatments (Fig. 7b).

Seed protein, oil, and fatty acids

Comparing with the control, FB resulted in higher seed protein, with FB at R2 and FB at V5+R2 combined having the highest protein percentage (Table 2). The trend for oil percentage was the opposite to those of protein (Table 2). Oleic acid percentage was higher in FB at R2 or FB at V5+R2 combined treatments than the control or FB at V5 treatment. The opposite trend occurred for linolenic acid percentage compared to oleic acid (Table 2).

Nitrogen fixation using natural ^{15}N abundance method

Acetylene reduction assay method is an instantaneous measure that allows the comparison of different treatments at a given time, but any extrapolation to estimate nitrogen fixation over a growing season is delicate because environmental conditions differ within and between days (Amarger et al. 1979. Therefore, nitrogen fixation using ^{15}N natural abundance method is more applicable for estimating nitrogen fixation, especially during a full growing period (Sprent et al. 1996). The method of ^{15}N is based on the discrimination of the soil nitrogen (^{15}N) and atmospheric nitrogen (^{14}N), and we used it to compare $^{15}N/^{14}N$ ratio between boron treatments. Four times applications (higher rate) of B significantly ($P \leq 0.0001$) altered the abundance of δ ^{15}N compared to ^{14}N, resulting in a higher $^{15}N/^{14}N$ ratio in FB-treated plants compared to the control (Fig. 7c).

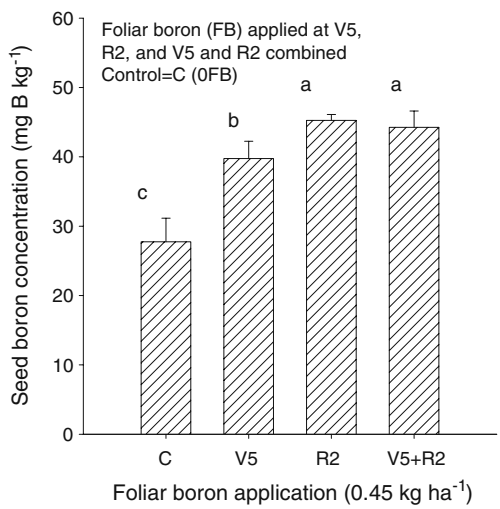

Fig. 2 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node), R2 (full flowering), and V5+R2 stages on B concentration in seed in soybean. Control = C (0FB). Bar values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

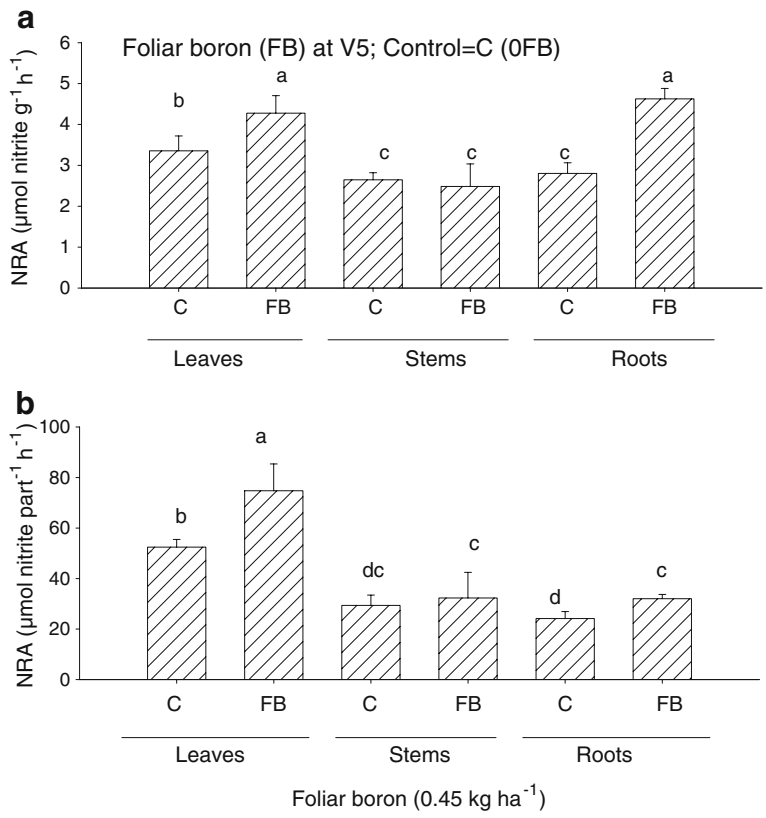

Fig. 3 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node) on nitrate reductase activity (NRA) per g fwt (µmol nitrite g fwt^{-1} h^{-1}) in leaves, stems, roots (**a**), and on NRA per part (µmol nitrite $part^{-1}$) (**b**). Control = C (0FB). Bar Values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

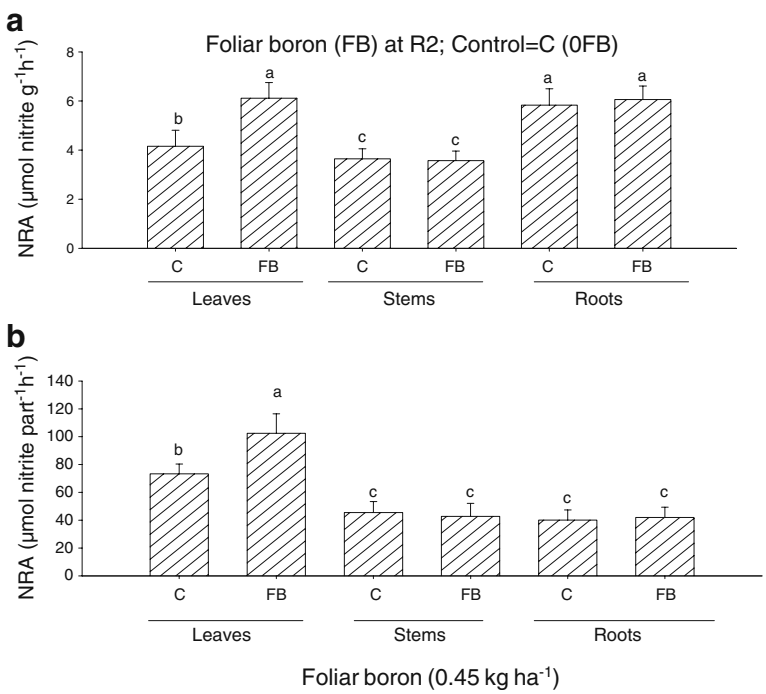

Fig. 4 Effect of foliar boron application (FB) at R2 (full flowering) on nitrate reductase activity (NRA) per g fwt (µmol nitrite g fwt^{-1} h^{-1}) in leaves, stems, roots (**a**), and on NRA per part (µmol nitrite $part^{-1}$) (**b**). Control = C (0FB). Bar Values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

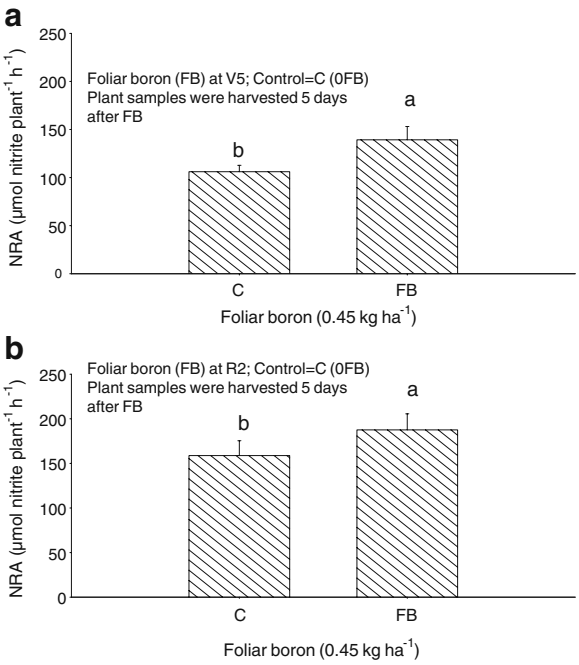

Fig. 5 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node) (**a**) and at R2 (full flowering) (**b**) on nitrate reductase activity (NRA) per the whole plant (µmol nitrite plant^{-1}). Control = C (0FB). Plant samples were harvested 5 days after FB application. Bar Values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

Discussion

Grain yield and boron concentration in leaves and seeds

The increase of grain yield by FB, especially when FB was applied at V5 and R2 combined indicates that B resulted in higher grain yield. It must be noted here that although grain yield of FB at V5 and R2 were not significantly different, grain yield of FB at V5 and R2 were significantly higher than the control. Our results are in agreement with those of (Reinbott and Blevins 1995), who found that B application increased soybean seed yield. Generally, negative, positive, or no yield responses from direct applications of B fertilizer have been reported by other researchers (Reinbott and Blevins 1995; Ross et al. 2006). Timing of B application for most uptake and utilization of B is still unclear in soybean (Schon and Blevins 1990).

It is clear from our results, however, that FB at V5 or R2 increased B in leaves compared with their corresponding controls. Foliar B increased B in seed, and the increase was highly significant when FB had been made at the reproductive stage (R2 only or at V5

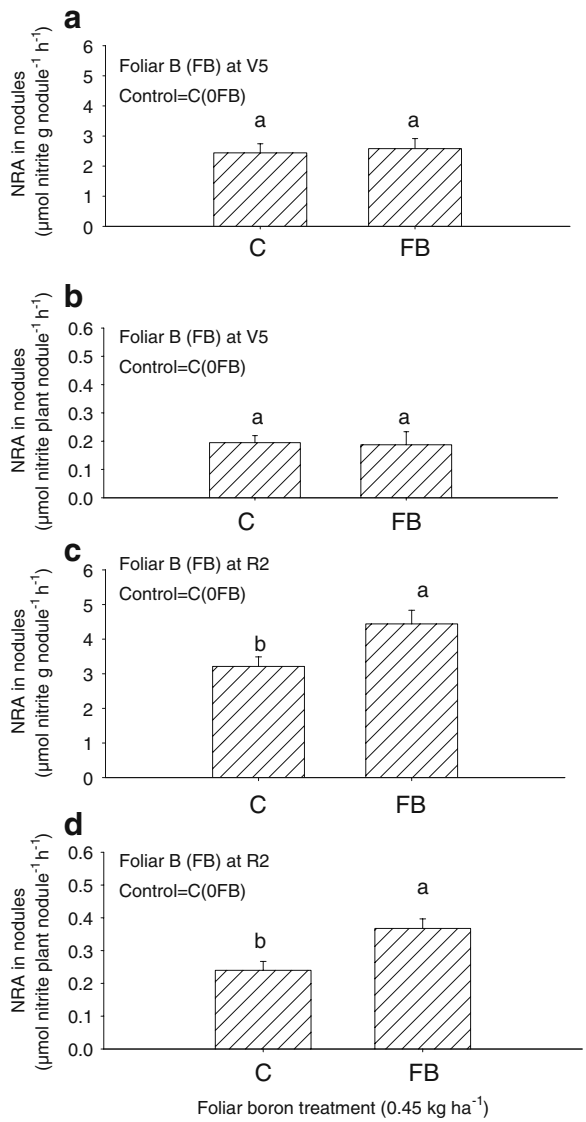

Fig. 6 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node) (**a**, **b**) and at R2 (full flowering) (**c**, **d**) on nitrate reductase activity (NRA) per g nodule^{-1} (µmol nitrite g nodule^{-1} h^{-1}) and on NRA per plant-nodule (µmol nitrite plant nodule^{-1} h^{-1}. Control = C (0FB). Bar Values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

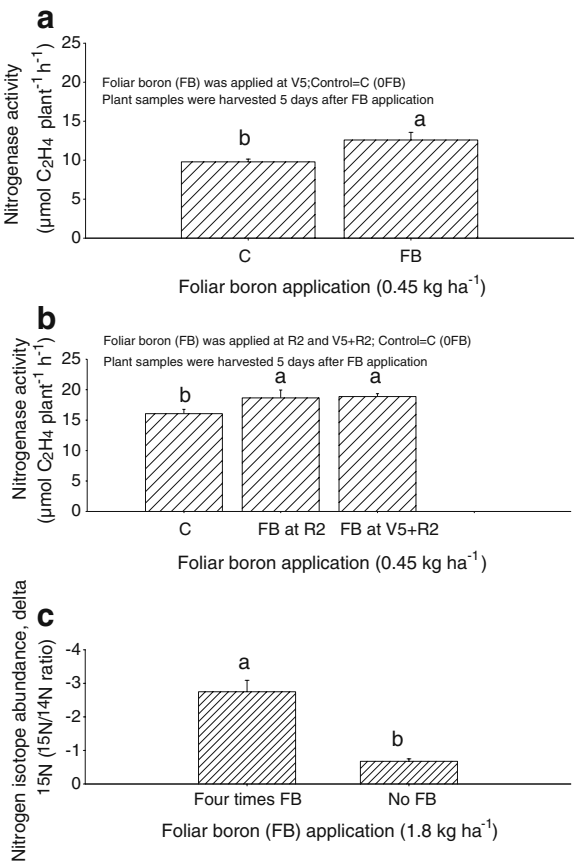

Fig. 7 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node) (**a**), at R2 (full flowering) and at V5+R2 stages (**b**) on nitrogenase activity (µmol C_2H_2 $plant^{-1}$ h^{-1}); plant samples were harvested 5 days after FB application. Effect of four times of the commercial dose of foliar B application (FB) on natural ^{15}N abundance, delta ^{15}N ($^{15}N/^{14}N$ ratio) (**c**). Bar values are means ± SE and same letters are not significantly different at the 5% level as determined by Fishers' LSD test

and R2 combined). Since leaf samples were taken at different stages, it cannot be claimed that FB resulted in higher B across treatments and stages because B accumulates more as the plant ages. Two applications, at V5+R2, resulted in higher B concentrations in seed than one application, indicating that B concentration in seed may increase with more than one application as long as FB rate does not reach a toxic level. Schon and Blevins (1990) indicated that FB resulted in significant increases in leaf B concentration far above the 60 mg kg^{-1} that was previously accepted as the upper level of tolerance for soybeans.

Foliar B application with 300 mg B L^{-1} to the lower fully expanded mature leaves of plants grown with 0 B in the medium resulted in a significant ($P \leq 0.001$) increase of B in young leaves and in R6- seed compared with those grown in 0 B in the medium and without FB (Table 1). The higher B concentration in plants with FB of 300 mg B L^{-1} compared with the control may indicate that the possibility of B mobility from fully expanded mature leaves to younger leaves and seed in FB-applied plants. It must be noticed here that the current study cannot prove that the increase in B concentration in younger leaves in FB-applied plants was due to only the decrease/disappearance of B in mature leaves. Our results support the well established reports that B mobility depends on species, as boron is mobile in species containing sugar alcohols (Brown and Hu 1996; Bellaloui et al. 2003). The relationship between carbohydrate, especially sugar alcohol, and B mobility in soybean needs further investigation. The higher (89%) contribution of cell wall B in the fully expanded leaf to the total B in plants grown in 0 B in the medium without FB compared with 57% and 69%, respectively for plants

Table 2 Effect of foliar boron application (FB) at V5 (five fully developed trifoliolate leaf node), R2 (full flowering), and at V5+R2) stages on soybean seed composition constituents. Control (C) received 0 FB

Boron treatment	Protein (%)	Oil (%)	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Linolenic (%)
C	39.72±1.6 d	21.16±1.5 a	12.03±0.46 a	3.03±0.48 a	22.40±1.8 b	55.00±1.5 a	13.96±1.7 a
FB at V5	41.41±1.5 c	18.87±1.1 b	11.68±0.40 a	3.14±0.51 a	21.68±2.3 b	53.30±1.4 a	13.00±2.3 a
FB at R2	43.36±2.2 b	18.66±1.9 b	11.71±0.59 a	2.99±0.46 a	28.0±1.8 a	54.30±1.4 a	10.87±1.4 b
FB at V5 and R2	45.18±0.2 a	18.11±2.2 b	12.04±0.46 a	3.26±0.32 a	29.21±3.3 a	53.51±1.5 a	9.32±0.77 c

Means within a column for each water treatment separately followed by the same letter are not significantly different at the 5% level as determined by Fishers' LSD test

grown in 10 mg B L^{-1} in the medium or 300 B mg L^{-1} FB. This supports the well established previous finding (Hu and Brown 1994). Therefore, under B deficiency conditions, most of the B absorbed is accumulated in the cell wall, and does not translocate to other developing tissues and seed unless B requirements by plants are fulfilled.

Nitrate reductase activity in leaves, stems, roots, and nodules

The increase of NRA by FB may be due to an indirect effect of B on nitrate uptake and assimilation (Marschner 1995). It appears that B may directly or indirectly induce nitrate assimilation, possibly by inducing NRA, stimulating de novo synthesis, or making nitrate (NR substrate) available for NR. This hypothesis could be supported by the increase of NRA when 0.5 mM B was added to the incubation solution as boric acid. The 0.5 mM B concentration increased NRA by 30, 25, and 35%, respectively in nodules, roots, and leaves compared to NRA under B-free buffer solution (data not shown). Based on this experiment, B may affect the NR itself (its activity or its de novo synthesis), or facilitate nitrate availability in the cytoplasm to NR for reduction or induce nitrate translocation from the vacuoles to the cytoplasm, leading to higher NRA activity. The effect of B on ion uptake was reported previously (Marschner 1995; Goldbach 1985) and suggested to be mediated by direct or indirect effects of B on the plasma membrane-bount H^+ ATPase (plasmalemma H^+-ATPase activity) (Schon et al. 1990; Camacho-Cristóbal and González-Fontes 2007), cell wall structure and membrane integrity (Marschner 1995; Schon et al. 1990). Our results support those of Marschner (1995) in that B may have an indirect effect on nitrate uptake and assimilation, and induces NRA by increasing substrate (nitrate) availability. It was hypothesized that the increase in NRA and nitrate assimilation at adequate B level may be due to the increase of de novo synthesis of the protein involved in the metabolic process or as a result of facilitating nitrate absorption (Ruiz et al. 1998). Bonilla et al. (1980) studied the effect of B on nitrogen metabolism in sugar beet by measuring NRA in leaves and nitrate in sap, and found that at adequate level of B increased NRA and decreased nitrate in xylem sap comparing to B toxicity or deficiency level. Bonilla et al. (1980) reported that B has a possible role in nitrogen metabolism through its effect on NR enzyme protein synthesis and nitrate metabolism. This was supported by Ruiz et al. (1998), working on tobacco plants (*Nicotiana tabacum* cv. Sevilla), who observed a positive effect of B on B concentration in leaves and on nitrate assimilation under greenhouse conditions, and reported that this changes may be due to an activation of the enzymatic proteins involved in nitrate assimilation.

Nitrogen fixation

The increase in nitrogen fixation with FB could be due to the positive effect of B on size and number of nodules or on nitrogen fixation capacity to fix N_2 more efficiently, or both. Nodule mass under FB was 83 mg nodules fwt $plant^{-1}$ compared with 71 mg nodules fwt $plant^{-1}$ in control plants (Table 1). Our results are in agreement with other reports that there may be an indirect effect of B on nitrogen metabolism via effecting sink demand activities, leading to more nitrogen demand (Shelp 1993), enhancing nodule development, and increasing nitrogen fixation capacity (Bolafios et al. 1994; Bolanos et al. 1996). Also, it was reported that rhisobia inside nodules showed little or no ability to fix N_2 under B-deficient plants, leading to N deficiency and necrosis of nodulated pea plants (Bolafios et al. 1994). Our results are in agreement with those of Bolafios et al. (1994) and Bolanos et al. (1996) in that FB increased nitrogen fixation and nodule development, and in our case increasing nodule mass. It was hypothesized that B protects nitrogenase against oxygen damage by affecting membrane integrity and function (Bonilla et al. 1997) and may interact with polyhydroxy components of the membrane such as glycoproteins and glycolipids to maintain the proper conformation in nitrogen-fixing cells (Brown et al. 2002).

Results from nitrogen fixation using ^{15}N natural abundance ($\delta\ ^{15}N$) are in agreement with those reported by Sprent et al. (1996) in that plants which fix some or all of their nitrogen will have lower $\delta\ ^{15}N$ values than plants which obtain all their nitrogen in combined form from the soil. Four times of commercial FB rate (1.8 kg B ha^{-1}) significantly altered $^{15}N/^{14}N$ ratio by increasing $\delta\ ^{15}N$, indicating inhibition of nitrogen fixation. The alteration of $^{15}N/^{14}N$ ratio by higher rate of FB may suggest a possible mechanism associated with the relationship between nitrogen

fixation and nitrogen assimilation. Four times of the commercial FB decreased nitrogen fixation in seed (Fig. 7c), but increased NRA in roots, leaves, and nodules (data not shown). This indicates that nitrogen fixation can be inhibited by higher rate of FB. Bearing this in mind, four times or even six times of commercial rates FB did not result in any visual B toxicity symptoms. The interrelationships between $^{15}N/^{14}N$ under environmental or chemical stresses and how these stresses affect the $^{15}N/^{14}N$ ratio are not yet understood (Bellaloui et al. 2008; Bellaloui and Mengistu 2008). Mechanisms controlling the relationships between nitrogen fixation and assimilation for better nitrogen use efficiency are still unclear and need further investigation.

Seed composition

Foliar B application resulted in higher protein and higher oleic acid percentages and lower oil and lower linolenic acid percentages (Table 2). The alteration in seed composition was accompanied by an increase in B concentration in leaves and seed. The increase of protein could be due to higher rates nitrogen fixation and nitrogen assimilation resulted from FB. Since there is an inverse relationship between protein and oil percentages in seed (Burton 1985; Bellaloui et al. 2009a), protein and oil showed an opposite trend (Table 3). The increase in oleic acid and decrease in linolenic acid by FB could be due to the effect of B on the activity of the fatty acid desaturases that control the accumulation and conversion of unsaturated fatty acids (oleic, linoleic, and linolenic acids). The possible mechanism of how B affects oleic and linolenic acids is not known and needs further investigation. Our results showed that accumulation of B in leaves and seed was accompanied with higher accumulation of protein and oleic acid and lower accumulation of oil and linolenic acid. These results are supported by previous work indicating that higher seed protein and oleic acid were accompanied by higher seed B and soil B (Bellaloui et al. (2009b). Recently Bellaoui et al. (2009c) found that FB increased seed protein and oleic acid and seed B. It must be noticed here that research is still showing inconsistent results on the effect of fertilization on seed composition, but often increased total oil and protein production (Haq and Mallarino 2005). Therefore, further research in this area is needed.

Conclusions

The increase in nitrogenase and NR activities in leaves and nodules resulted from FB indicated the significant role of B in nitrogen metabolism. Although there is still no evidence for a direct effect of B on the enzyme nitrate reductase, B may induce NRA and facilitate nitrate translocation and availability for reduction. Under low B concentration in leaves, cell wall B is accounted for a major fraction of the total B, and under these conditions B may not be translocated from mature tissues to young tissues or seed. The increase of protein and oleic fatty acids occurred at the expense of oil and linolenic fatty acid as both oil and linolenic percentages decreased with FB. This suggest that B may play a role in protein:oil ratio, and this may be associated with unsaturated fatty acid desaturases and N:C ratio. The hypothesis that B is associated with fatty acid desaturases needs further investigation.

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