

EFFECT OF TRANSIENT SHORT-TERM BORON DEPRIVATION ON THE GROWTH, NODULATION AND N₂-FIXATION OF SOYBEAN PLANTS

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Summary. Exposure to transient 10-days boron (B) deficiency stress created during different periods of growth cycle of nodulated soybean plants grown in a liquid culture was found to inhibit the nodule number and to increase the nodule dry weight. Nodule fresh weight was decreased in the stressed plants especially in the older ones. The loss of tissue water in the B-deficient nodules was accompanied by some increase of soluble sugar content and a decrease of nitrogen fixation. Acetylene reduction rate of stressed nodules was lower than in controls. The mechanism of action of the short term B-deficiency stress on the nodule growth and functions was explained by the participation of boron ions in development of the root and nodule cell walls and cell membrane permeability.

Key words: Boron deficiency, soybean *Glycine max* L. Merr, nitrogen fixation, nodulation rate, acetylene reduction rate.

Abbreviations: DAP – days after planting; FW/DW – fresh and dry weight of plants; AR – acetylene reduction, RGR – relative growth rate – mg DW/g DW/day; B – boron ion; PPP – phenyl-propanoid pathway; YEM – yeast extract – mannitol medium for laboratory cultivation of *Rhizobium* spp.

Introduction

Boron is an essential mineral element for vascular plants (Marschner, 1987). The function of boron is primarily extracellular and related to cell lignification and membrane

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permeability (Lewis, 1980). The mechanism of interactions of boric anion (BO_3^{3-})(B) with plant cells is based on its properties to form stable mono- and diesters with compounds having *cis*-diol configuration of its polyhydroxyl groups (Loomis and Durst, 1992). Such compounds include a number of sugars, phenols and their derivatives. A substantial quantity of boron (from 3 to 30 $\mu\text{g/g DW}$) is found bound to cell wall constituents pectin and hemicellulose (Marschner, 1987). The firmly attached to cell wall B is highly unavailable for utilization during growth. In their study on the compartmentation of B in sunflower plants, Dannel et al. (1998) distinguished between two B pools of different water solubility in the roots and shoots: B in the water insoluble residue (WIR), which represented B closely bound to cell wall structures, and B in cell sap, which is a mixture of cytozol, vacuole and apoplastic fluid, mainly soluble B in the symplasm (cytozol and vacuole). Li et al. (2001) demonstrated how the B concentration in these two pools responded to B starvation. Yamagushi Yamamoto, (1994) showed how long term B deficiency influenced mineral nutrition efficiency and nodulation rate of soybean plants. However, there is no information available how the host and nodules of soybean respond to transient short term B deficiency during different periods of plant and nodule development. The development of soybean root nodules is a sequence of events, terminated in time and strongly connected with host plant growth. In most cases these stages are indicated as periods of nodule formation which include cell division and differentiation, and states of nodule functioning characterized as states of high metabolic activity (host shoot carbon delivery and nodule fixed nitrogen export and, finally, declination of growth and nodule death (Brewin, 1991). The rate of nodule growth differs greatly from the growth rate of the host plant. All these growth alterations are accompanied by dramatic changes of nodule cell wall growth and differentiation which is a B-dependent process (Brewin, 1991, Blevins and Lukaszewski, 1998). The growth of cell wall is usually terminated with lignification which also depends on the availability of B in the growth medium (Lovatt, 1984). Lignin is a product of the phenyl-propanoid metabolic pathway (PPP) in plants, but in legumes, some flavonoid derivatives of PPP have recently been found to play an important role in plant-rhizobia interactions (Denarie et al., 1992, Dixon et al., 1994). Lee and Aronoff (1967) put forward the hypothesis about the participation of boron in the regulation of phenyl-propanoid metabolism, thus predicting its role in the legume-*Rhizobium* interactions. The long-term absence of B was found to increase total soluble phenol content in root apoplast exudates (Zehirov and Georgiev, 2000). This processes was accompanied by some decrease of nodule number per plant. The *in vitro* experiments with the B-deficient plant root extract added to the laboratory cultivated *ex planta* Bradyrhizobium japonicum 639 on YEM medium, showed an inhibition of cell growth in comparison with control cells (data not shown). These results give reason to suggest that B nutrition possibly also influenced the processes connected with the initiation of soybean nodules.

Based on these achievements this study was conducted to describe the effects of transient short-term B starvation induced during the growth cycle of infected by

Bradyrhizobium japonicum soybean plants, on nodule number and size, rate of acetylene reduction, fresh and dry weight of nodules, as well as their relation to the host plant growth.

Material and methods

Soybean seeds (*Glycine max* L. Merr. cv Beeson) were surface sterilized with 4% sodium hypochlorite, soaked for 1h in sterile, distilled water and then germinated under controlled conditions at 25 °C on wet filter paper in glass pans. After 3 days seedlings were inoculated with bacterial suspension of 10⁸ cells/ml of *Bradyrhizobium japonicum* strain 639 grown on liquid YEM medium (Georgiev et al., 1996) and transferred to 1.2 L plastic pots containing mineral nutrient medium free of nitrogen, established for growth of N₂-fixing legumes (Zehirov and Georgiev, 2000). Boron was 44 mg B/L for control plants supplied as H₃BO₃. In the experimental treatments boron (B) was omitted from the medium for 10 days of the growth period in the following manner: Control – 44 mg B/L during the whole growth period; Experiment 1-B was omitted from the medium in the course of 10 days after germination up to 10 days after planting (DAP); Experiment 2 – B was omitted between 10 and 20 DAP; Experiment 3 – B was omitted between 20 and 30 DAP. Plants were grown in a naturally lit and heated green house (day – 30 °C, night – 20 °C). Nutrient solutions were aerated daily and renewed every 3 days. at the end of stress period control and experimental plants were harvested, dried in an oven at 105 °C and fresh and dry weights were determined. Nodule number and weight were determined after every harvest period. Acetylene reduction rate of the detached nodules was measured immediately after every harvest period by the method of Hardy et al. (1968) with some modifications (Georgiev et al., 1996). The freshly harvested nodules detached from 3 plants were incubated in the sealed 30 ml bottles containing mixture of air plus 10% acetylene. 1 ml of gas samples from the sealed bottles was collected after 30 min of incubation and was analyzed for ethylene with the gas-chromatograph “Perkin-Elmer” 104 supplied with the flame-ionization detector. Soluble sugars in plant samples were determined in the recovered from the 80% ethanol extracts water solutions colorimetrically at 450 nm (Dubois et al., 1956). The specific nodulation and relative growth rates of nodules and roots were also calculated (Georgiev et al., 1996). Significant differences of the means of 3–6 replicates were calculated (Dosphehov, 1985).

Results and Discussion

The fresh and dry matter contents of nodulated soybean plants showed different changes after the periods of transient 10 days B deprivation, applied during the growth

Table 1. Fresh and dry matter accumulation in leaves of N₂-fixing soybean plants subjected to transient 10 days boron deficiency

Treatments		Fresh and dry weight of leaves, g/plant		
		Duration of boron deprivation		
		from 0 to 10 DAP	from 10 to 20 DAP	from 20 to 30 DAP
+B	FW	0.410±0.03	1.375±0.12	2.470±0.24
(control)	DW	0.066±0.01	0.250±0.01	0.549±0.03
- B	FW	0.410±0.02	1.250±0.03	2.480±0.03
(stress)	DW	0.067±0.03	0.240±0.05	0.725±0.12

Data are means from 5 replicates ±SD

Table 2. Fresh and dry matter accumulation in roots of N₂-fixing soybean plants subjected to transient 10 days boron deficiency

Treatments		Fresh and dry weight of soybean roots, g/plant		
		Duration of boron deprivation		
		from 0 to 10 DAP	from 10 to 20 DAP	from 20 to 30 DAP
+B	FW	0.975±0.053	2.800±0.211	4.810±0.350
(control)	DW	0.037±0.003	0.217±0.003	0.490±0.015
- B	FW	1.100±0.032	2.440±0.050	3.860±0.042
(stress)	DW	0.046±0.001	0.200±0.060	0.510±0.002

starting from 0 and terminated at 30 DAP (Table 1, 2). A more evident decrease of fresh weight of leaves and roots was detected during the stress period imposed between 20 and 30 days of growth. These changes, however, were accompanied by some increase of the plant root and leaves dry weight. The B starvation brought also to some increase of shoot/root DW ratio of plants (120% of control 30 DAP), however, when expressed on a FW basis this ratio appeared to decrease (89,5% of control). These changes indicate that the short term B starvation inhibits the above ground part of plants to a greater extent and, at the same time, decreases tissue water content. Another apparent effect of the applied short term B-deficiency was the inhibition of total leaf area of plants (Fig. 1). These changes were accompanied with the elevation of specific leaf mass (SLM – mg DW/dm² leaf area), which indicated that certain alterations in leaf morphology might occur even after the period of 10 days B deficiency stress.

The stressed plants showed also significant changes in tissue water content of their organs. The loss of water from plant nodules was more apparent only when B deprivation was imposed between 20th and 30th days of growth of (Fig. 2). The results obtained after B deprivation (0–10 DAP and 10–20 DAP), however, showed

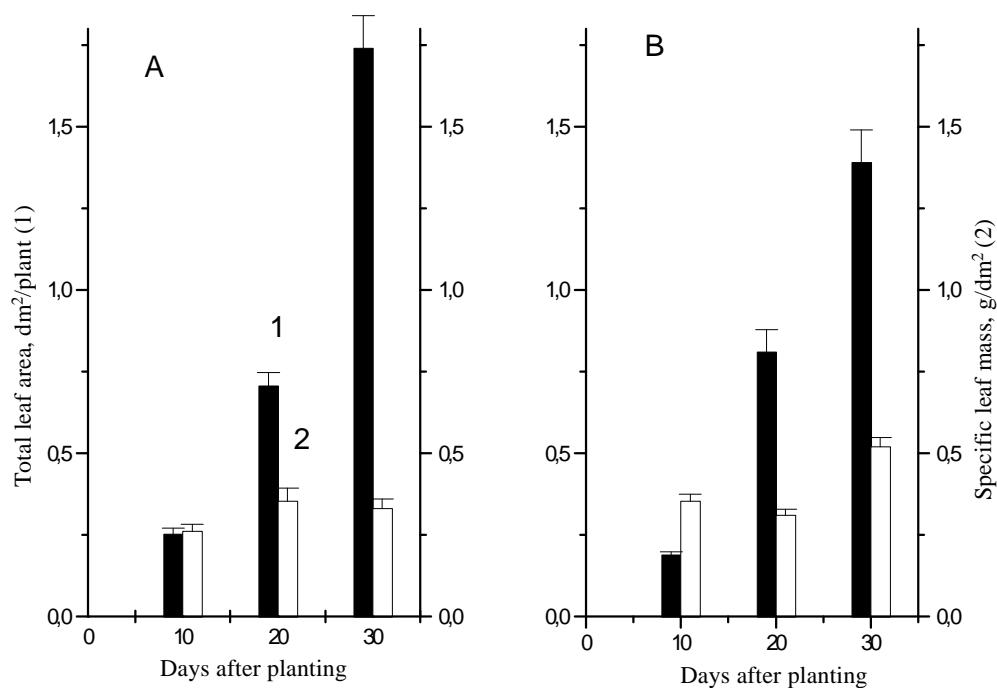


Fig 1. Total leaf area (TLA) (black column(1) and specific leaf mass (SLM) (white column(2) of control (A) and subjected to B deficiency for 10 days (B) during growth soybean plants

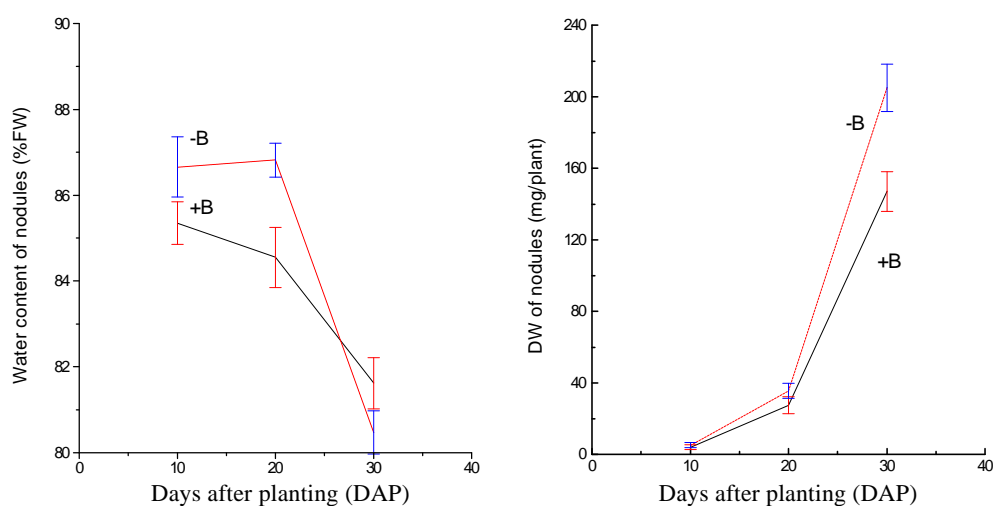


Fig 2. Dry weight (DW) and water content in nodules of control (+B, solid line) and deprived for 10 days during growth period (-B, dashed line) soybean plants.

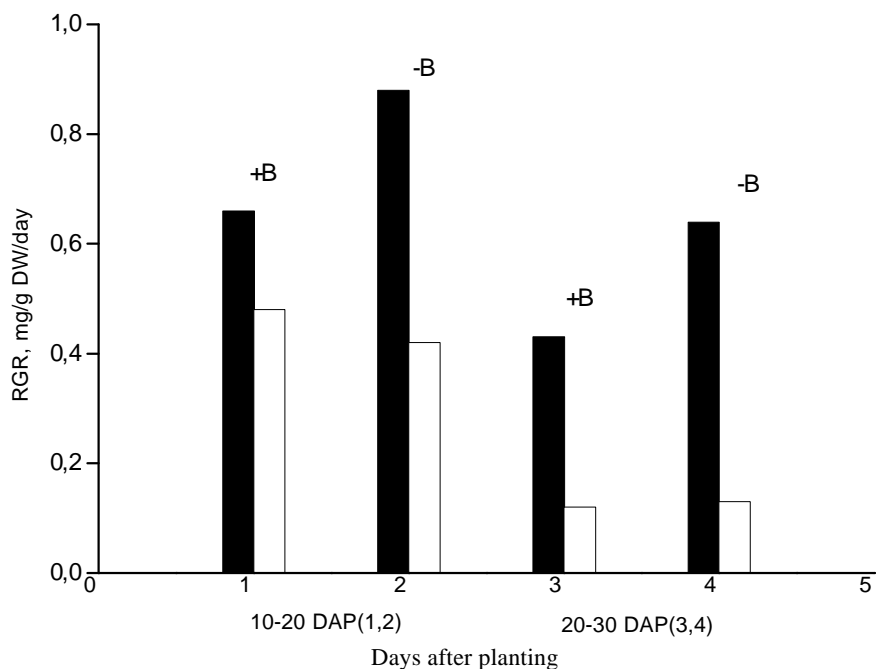


Fig 3. Relative growth rate (RGR) between 10th and 20th (1,2) and between 20th and 30th (3, 4) days of growth of soybean nodules (black column) and roots (white column) of control (+B) and subjected to 10 days B deficiency (-B) during the growth period soybean plants.

some increase of relative growth rate of plant organs (Fig. 3). This was in contrast to the decreased RGR of the stressed roots. These results indicate that, probably, higher sink activity of stressed by B starvation nodules is the reason for their increased growth rate. This process was accompanied by some increase of the concentration of solutes extracted from the nodules which possibly, could be the driving force for the increased growth rate (Fig. 3, Table 3).

Table 3. Effect of induced for 10 days B deprivation during different periods of the vegetative growth of N_2 -fixing soybean plants on the specific nodulation and acetylene reduction rates

Treatment	Specific nodulation rate, number of nodules/g DW root				Acetylene reduction rate, $\mu\text{mol C}_2\text{H}_4/\text{g FW nodules/h}$			
	from 10 to 20 DAP		from 20 to 30 DAP		from 10 to 20 DAP		from 20 to 30 DAP	
	nodules/g DW	%	nodules/g DW	%	$\mu\text{M/g/h}$	%	$\mu\text{M/g/h}$	%
+B	260±11	100	240±12	100	10.4±1	100	11.4±1	100
-B	216±9	83.0	131±20	54.5	7.4±2	70.6	5.8±1	50.6

Data are means of 6 replicates ± SD

Transient short-term B deficiency influenced strongly the water relations of plant organs, which was another reason for the altered growth rate as well (Fig. 3). Based on the results of disturbed water relations, certain acceleration of the senescence processes of soybean plants can be assumed. As a result of all these alterations a decrease of N_2 fixation efficiency of plants occurred. Another reason for the growth changes in N_2 fixing soybean plants can be the altered cell permeability for solutes in the roots found when B deficiency stress was applied in the course of 10 days (Zehirov and Georgiev, 2001). These alterations contributed to the disturbance of the mechanism of assimilate exchange between nodules and roots leading to metabolite starvation of nodules (Zehirov and Georgiev, 2001). Albeit, the cells of stressed nodules contained more sugars and free amino acids, the water content of such nodules declined less rapidly than roots. As a result, the observed inhibition of nodule functioning, was less pronounced than in the roots. There is evidence that it can be a consequence of cell osmotic potential adjustment (Cosgrove, 1987). Nevertheless, the increased solute concentration of the affected by B deficiency soybean plant organs can provide enough turgor pressure for proceeding the cell growth even under unfavorable water relations (Pfeffer et al., 1998). The depositions of non-cellulose carbohydrates in the cell wall found to occur under B deficiency, can be also the reason for the changed rate of water exchange among the cells (Cosgrove 1987; Blevins and Lukaszewski, 1998). The impaired carbohydrate metabolism considered as the main result of the growing B-deficiency, can be one of the factors contributing to the increase of soluble sugars in the cells of nodules (Raven et al., 1989). The sucrose content in nodule cytozol was increased significantly (230% in the stressed 20 old nodules), which contributed to the increase of osmotic potential and the uptake of water in these cells (Table 3). In fact, the water content of these nodules was found also to increase (120% of control) (Fig. 2). The increased sink strength for metabolites of the stressed 20 days old nodules can contribute also to some decrease of uptake rate for water and solutes of the subsidiary root cells competing not successfully enough for it. This can be the probable reason for decreased root growth in comparison with the attached to them nodules. The altered apoplast/ symplast compartmentation of electrolytes of stressed roots and nodules can be used to support such conclusion (Zehirov and Georgiev, 2001, Li et al., 2001).

The increased content of sucrose in nodule cytozol was accompanied with a significant decrease of acetylene reduction rate, thus indicating an inhibition of nitrogenase activity (Table 3). The decline in the quantity of fixed atmospheric N_2 , however, was in contrast to the increased growth of stressed nodules (Fig. 3, 5). This discrepancy can be explained by the probable effect of the altered water relations on the mechanism of diffusion of oxygen through the nodule cortex (Witty et al, 1986). When the supply with oxygen from the air through the nodule cortex to the infected with bacteroids central zone of nodules was disturbed, as a consequence always the inhibition of respiration efficiency and nitrogenase activity were detected (Tjepkema and

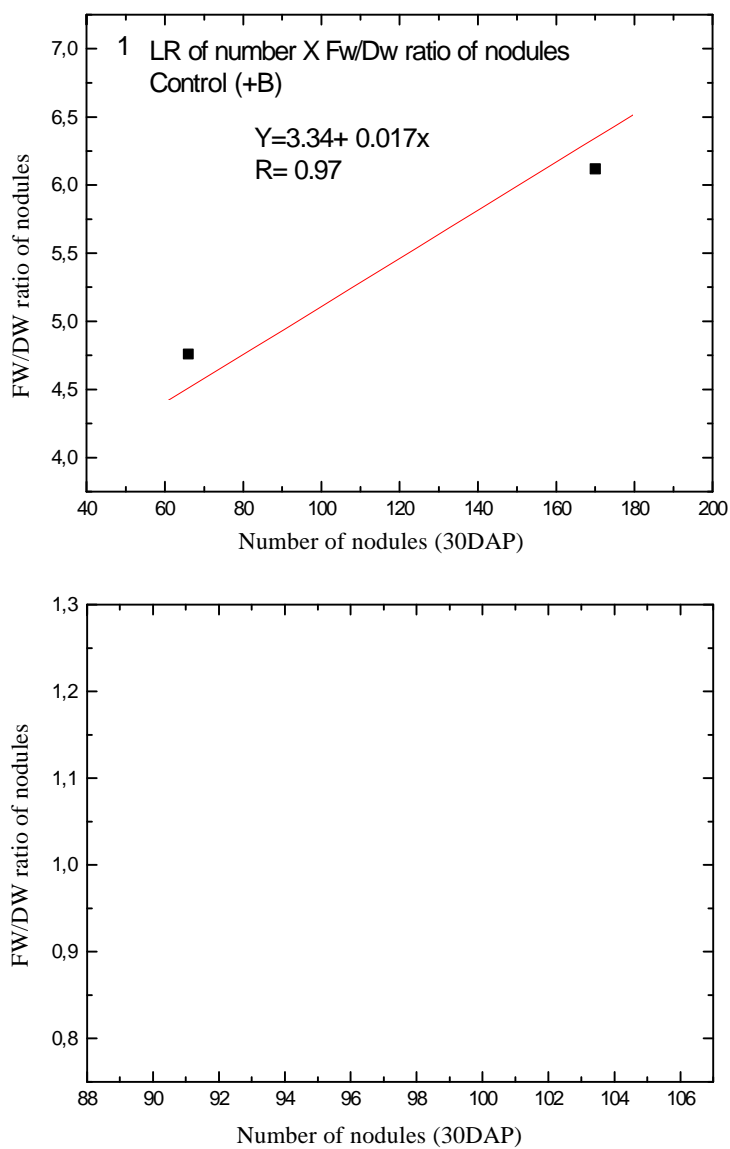


Fig 4. Linear regression between nodule number per plant and water content (FW/DW ratio) of control (1) and B-deprived for 10 days (2) (20th to 30th DAP) soybean plants

Yocum, 1974; Witty et al., 1986). As the water filled intercellular spaces of nodules determine the main route of resistance of oxygen diffusion through the nodules, the negative effect of disturbed water relations on the nitrogenase activity can be suggested.

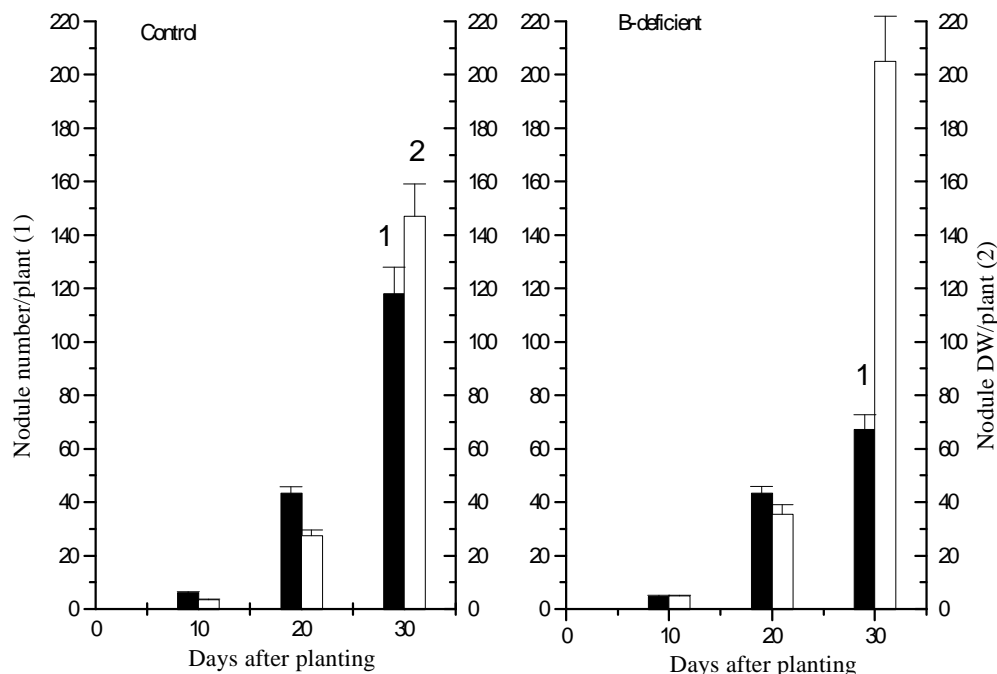


Fig 5. Nodule number per plant (black column) and dry matter content (white column) of control (+B) and subjected to B deficiency for 10 days (-B) soybean plants

Applied B deficiency stress exerted a stronger negative effect on the nodule setting than on the nodule dry matter accumulation. The results showed that the inhibition of the number of nodules was more sensitive than their growth (Fig. 3, 5). Specific nodulation rate was also decreased in the stressed nodules, albeit some time dependent differences were found during the B deficiency revelation (Table 3). The correlation coefficient between the number of nodules and water content (calculated as FW/DW ratio) was highly positive for the control plants but changed drastically for the B deficient nodules (Fig. 4). These calculations can be accepted as another evidence for the important role of water relations on the nodulation processes especially when the short-term B-deficiency was applied (Fig. 4, 5). The discrepancy between dry matter accumulation and nodule number per plant found after application of short term B starvation can be explained by the existence of some auto regulatory mechanism adjusting the relations between host and nodules (Brewin, 1991; Stougaard, 2000). This mechanism is considered to play a leading role in the regulation of the relations between number of nodules and their dry matter especially when growth conditions are inhibitory (Denarie et al., 1992). The short term B deficiency effects differed greatly from these of long-term stress (Zehirov and Georgiev, 2000, Yamagushi et al., 1994). As the process of nodule setting is considered to be the most sensitive process, it can be suggested, that the short term B-deficiency stress affects, pre-

Table 4. Content of sucrose in root and nodules of soybean plants subjected to 10 days B deprivation

Treatment of plants	Sucrose content, $\mu\text{g/g}$ FW					
	from 0 to 10 DAP		from 10 to 20 DAP		from 20 to 30 DAP	
	root	nodule	root	nodule	root	nodule
Control	3.7 \pm 0.3	3.5 \pm 0.2	2.4 \pm 0.2	3.6 \pm 0.5	4.5 \pm 0.4	10.0 \pm 1
- B	2.1 \pm 0.1	2.4 \pm 0.1	3.5 \pm 0.3	8.4 \pm 0.8	5.4 \pm 0.1	9.6 \pm 0.3

sumably, the pre-infection–infection interactions between rhizobia and plant host (Denarie, 1992). As the B is known to play a regulatory role in phenol metabolism, some effect of B absence on the structure and content of produced by PPP specific flavonoids, known to take part in the recognition processes, can not be excluded, despite the fact that no direct evidence has been found yet. The lowered number of nodules of subjected to transient B starvation plants can be regarded also as a result of low efficiency exchange of signal molecules between symbiotic organisms (Phillips et al., 1991, Stougaard, 2000).

The growth of nodules depends strongly on the mechanism of sink-source relationships and relates to the carbohydrate and nitrogen assimilate partitioning between symbiotic partners (Brevin, 1991, Georgiev et al., 1996). The higher growth rate of non-aging B deficient nodules is more probably due to the effect of altered cell permeability affecting the accumulation and conversion of metabolites in the cells. On the other hand, this effect can be a result of the influence of some phytohormone disorders, like auxins accumulation, which occur under B deficiency. This process can drive cells to one extraordinary extension (Lewis, 1980). Another reason for the increased growth of the nodule cells can be searched in the altered lignification rate of root and nodule tissues (Skok, 1958). The less lignified cell walls possess higher extensibility, thus allowing cells to extend more, especially when accumulation of solutes occurs inside. Our previous data have shown that B deficient nodules contain less quantities of two major lignin fractions, insoluble “Klason” and soluble “acetyl bromide”, indicating some changes in cell wall lignification under B deficiency (Zehirov and Georgiev, 2000).

Independently from the growth reaction of nodules, the mechanism of N_2 -fixation has been always disturbed, indicating that this mechanism is more sensitive to B deficiency than nodule growth. The rate of AR in the disturbed nodules was inhibited to a different extent during the whole period studied.

Conclusion

In this paper, we report that transient 10-days B starvation exerts diverse effects on the growth and nodule number of soybean plants. The process of nodule setting is

found to be the most negatively affected by the absence of B during the stress period from 20th to 30th DAP. The accumulation of dry weight of stressed nodules was, however, positively changed. The effect of 10 days B deficiency is dependent on the nodule and plant age and is more strongly expressed in the older plants. On the other hand, acetylene reduction rate of stressed nodules is negatively affected and depends on nodule and host plant age. The action of short-term B deficiency on the nodule cells is accompanied by plant and nodule water relations disorders accepted as manifestation of an accelerated senescence process and connected mainly with modifications of cell wall structure and membrane permeability. All these changes lead to assimilate and water starvation of nodules and decrease N₂-fixing efficiency of soybean plants.

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