Changes in the biomass production and total soluble protein spectra of nitrate-fed and nitrogen-fixing soybeans subjected to gradual water stress

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ABSTRACT

The effect of the sources of nitrogen nutrition (nitrogen fixation or nitrate assimilation) and a gradual water stress on the relative water content, total fresh and dry biomass production, leaf growth, and changes in the total soluble protein spectra were studied. The plants were cultivated as soil cultures in a naturally illuminated greenhouse. Comparative studies were carried out with respect to well-watered, control plants. Nitrogen-fixing control and drought plants had relatively smaller root development but better relative water content and large leaf area on the last sampling day than nitrate-fed soybean plants. Water deficit effects on plant biomass at the end of the period studied (21 days) were independent on the nitrogen source. There was no qualitative difference in the total soluble protein spectra of nitrate-fed and nitrogen-fixing soybean leaves neither with the progress of development nor under drought conditions. But there was a difference in response to drought in termostable proteins of nitrate-fed and nitrogen-fixing plants. The quantity of termostable proteins in inoculated control plants was lower in some degree compared to uninoculated ones. In inoculated plants the water stress caused an increase in the amount of soluble termostable proteins.

Keywords: nitrogen sources; *Glycine max* L.; water stress; biomass production; leaf area; relative water content; soluble protein spectra; termostable proteins

Nitrogen is required by plants in greater quantities than any other mineral element and support their growth and development. At the physiological level N can induce both the NO_3^- assimilatory pathway and reprogramming of C metabolism to provide reductants and C skeletons for this pathway (Crawford 1995, Stitt 1999). At the developmental level N is an important regulator of processes that include leaf expansion (McDonald and Davies 1996, Walch-Liu et al. 2000), root branching (Forde and Lorenzo 2001), and the allocation of resources between shoot and root growth (Scheible et al. 1997). The success of approaches in increasing crop production depends on the improved understanding of physiological and biochemical processes underlying environmental stresses and subsequent tolerance to these stresses (Yu and Rengel 1999). Frechilla et al. (2000) concluded that the nitrogen source is a major factor affecting pea responses to water stress, however, this fact was not due directly to the nitrogen assimilation process itself, but to interactions with stomatal conductance and photorespiration. Water deficit effects on plant biomass and transpiration rate were highly dependent on the nitrogen source but nitrogen assimilation declined during the drought period and was independent of nitrogen source (Frechilla et al. 2000). The same authors were reported that free amino acid content declined in leaves of pea plants grown under both nutrition regimes, reflecting the decrease in nitrogen assimilation.

Unfortunately, influence of different N-sources and water stress on the changes in the total soluble protein spectra was insufficiently investigated.

The aim of the present study was to determine some responses of nitrate-fed and nitrogen-fixing soybeans to progressive water stress, with special regard to changes in the biomass production and total soluble protein spectra.

MATERIAL AND METHODS

Plant material and growth conditions

Soybean seeds (*Glycine max* L. Merr. cv. Hodgson) were surfacely sterilized with 70% ethanol. The plants were cultivated as a soil cultures (in plastic vessels containing 4 kg soil) in a naturally illuminated greenhouse with photoperiod of 15-h and day/night temperatures were maintained at 28–30°C during the day and 22–24°C during the night. The plants were divided into two sets: (1) Uninoculated soybean plants grown in soil, which nitrate was controlled on constant level of 12 mg NO_3^-/100 g soil at the development stage 1th–8th trifoliate expanded leaves. (2) Inoculated soybean plants (soybean seeds were inoculated with bacterial suspension of *Bradyrhizobium japonicum* strain 273 at approximately 10^8 viable cells per cm^3) were grown in nitrogen deprived soil, which was supplied with 4 mg NO_3^-/100 g soil to stage V6 (5th trifoliate expanded leaf).

Water stress was applied for 21 days during the vegetative stages (stages 5th trifoliate expanded leaf, 6th trifoliate expanded leaf, 7th trifoliate expanded leaf, 8th trifoliate expanded leaf) by decreasing the amount of watering. The plants were divided into four treatments: (1) Uninoculated control soybean plants; (2) Uninoculated water deficiency soybean plants; (3) Inoculated control soybean plants; (4) Inoculated water deficiency soybean plants. From day 1 to day 7 (5th–6th trifoliate expanded leaf) stressed plants received the equivalent of 80% of the transpirational water loss measured on day 0; from day 8 to day 14 (6th–7th trifoliate expanded leaf), 70% and from day 15 to day 21 (7th–8th trifoliate expanded leaf), 50%. Plants were watered every day. Transpiration was determined by weighting the pots (Minguez and Sau 1989). The surface of each pot was covered with transparent plastic so loss of water by direct evaporation from soil was negligible.

Relative water content

Relative water content (RWC) was measured as described by Morgan (1986). The RWC values were calculated according to the formula:

$$\text{RWC (\%)} = (\text{initial fresh biomass} - \text{dry biomass})/(\text{full turgid biomass} - \text{dry biomass}) \times 100$$

The leaves for RWC were weighed immediately, cut in several sections and then weighed again (for turgid weight) after floating on water in a closed breaker for 20 h. A final dry biomass was determined after the leaf sections had dried at 105°C to constant weight.

Leaf area

Leaf area were calculated according to the formula:

$S = 100b/a$

where: a is weight of dm^2 from paper (rice paper), b – weight of the paper from copy of the leaf, S is leaf area in cm^2

Total protein analysis

The investigations of protein patterns were carried out with soybean leaf tissues. All steps of extraction were performed at 4°C. The fresh leaf tissues (3.0 g) was homogenized with 9 ml 0.1M Tris-HCl buffer pH 8.0. The extract was centrifuged at 12 000 g for 30 min and the supernatant was used as a crude enzyme extract. Protein content was determined after trichloroacetic acid precipitation by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The fraction of termostable soluble proteins was obtained by the procedure of Close et al. (1993). The supernatants with soluble proteins were boiled at 100°C for 10 min, kept on ice and then centrifuged at top speed (15 000 g) in a microcentrifuge for 15 min at 4°C. The supernatants contained termostable soluble proteins. Total and termostable soluble proteins were separated by native electrophoresis in 7.5% polyacrylamide gel (PAG) according to Davis (1964). Protein bands were stained with Coomassie Brilliant blue G 250. Protein spectra were scanned densitometrically (ERI-10, Germany). The differences and identity of the individual protein bands were appreciated by their number and values of the relative electrophoretic mobility. Relative mobility (Rm) = a/b, where a is a distance (in cm) of protein band from the start of gel to its place on gel; b is a distance (in cm) from the start of the gel to the front (marker dye – brom phenol blue). Equal amounts of protein (100 or 200 μg) were run on gels. Chemicals were purchased from Sigma Chemical Co. (St. Louis, USA). Data calculation and statistical analyses were conducted using *SYSTAT* software (SYSTAT, Inc., Davis, USA).

RESULTS AND DISCUSSION

Although the early stages of the interaction between soybean and *B. japonicum* have been well studied at the histological levels (Green and Emerich 1999), not too much is known about alteration in root metabolism after plant inoculation. The period of inoculation of soybean could play a key role in the experiment because nodule formation was needed from a long time – a month or more. In our experiments we inoculated soybean seeds and determine some responses of uninoculated (nitrate-fed) and inoculated (nitrogen-fixing) soybeans to progressive water stress during the vegetative stage 5th–8th trifoliate expanded leaf, when the nodules of inoculated plants was completely developed and nitrogen fixation was very effective.

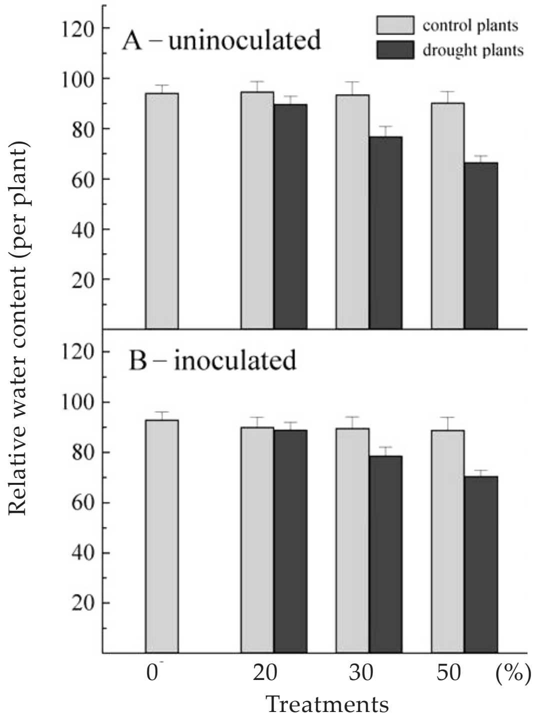

Figure 1. Relative water content (RWC) in uninoculated (A) and inoculated (B) soybean plants, grown at different (0, 20, 30 and 50%) water stress; values represent means ± *SD* (*n* = 6)

Relative water content (RWC)

Data for changes in RWC in leaves of soybean plants during the experiments are presented in Figure 1. The most significant change of RWC (23.75% reduction compared to the control) was observed in 50% water deficient nitrate-supplied plants (21 days after drough). Inoculated plants grown under the same conditions had higher RWC (reduction of only 18.44% compared to the control). This suggests that inoculated plants subjected to 50% water deficiency have better water status, which probably results in more intensive metabolic processes than these in nitrate supplied 50% water deficient soybean plants. Nitrogen-fixing plants were more conservative in terms of water use and initiated stomatal closure at higher leaf water potentiales and osmotic adjustment was delayed (Minguez and Sau 1989).

Fresh and dry biomass production

The effect of the sources of nitrogen nutrition (nitrogen fixation or nitrate assimilation) and water stress on fresh and dry biomass production of soybean plants is shown in Figure 2. The experiments had been carried out in soil, a situation not far from the natural condition. Control nitrate-fed plants produced more total fresh and dry biomass than nitrogen-fixing control plants with the exception of plants on the last sampling day when nitrogen-fixing control plants produced more total fresh and dry biomass. Water stress reduced fresh and dry biomass accumulation in both nitrogen treatments. At the end of the period of investigation, fresh biomass of nitrate-fed stressed plants represented 77.7% of that of nitrate-fed controls. For nitrogen-fixing plants this value was 69.6%. Total fresh and dry biomass of stressed nitrate-fed plants on the last sampling day were no significant difference compared to those of nitrogen-fixing stressed plants.

Root of control nitrate-fed plants produced more total fresh biomass than nitrogen-fixing control plants (32% more on the last sampling day – Figure 3). Water stress reduced root fresh biomass accumulation in both nitrogen treatments but drought nitrate-fed plants produced 11–21% more fresh biomass than nitrogen-fixing drought plants. Minguez and Sau (1989) reported that nitrate-fed stressed plants tend to adopt tolerance mechanisms related to root expansion and turgor maintenance through osmotic adjustment but turgor maintenance must have been effective in both nitrogen treatments.

Leaf growth

Leaf growth is one of the most sensitive to drought among all plants processes (Hsiao 1973, Bacon et

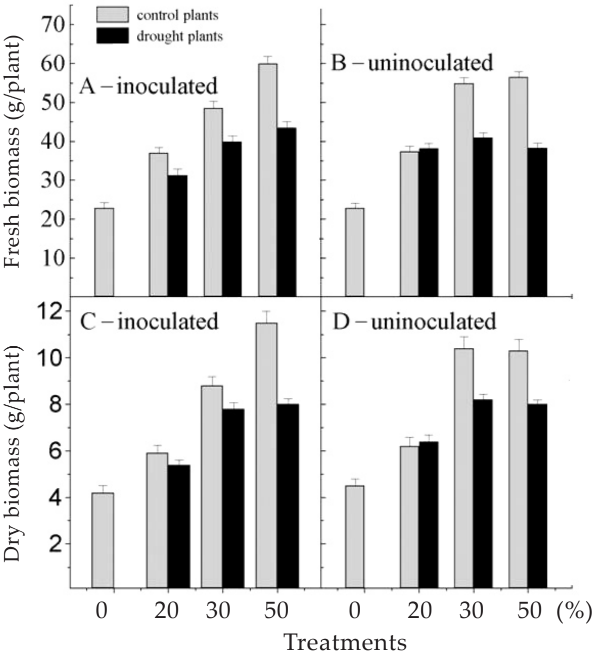

Figure 2. Total fresh and dry biomass of inoculated (A, C) and uninoculated (B, D) soybean plants, grown at different (0, 20, 30 and 50%) water stress; values represent means ± *SD* (*n* = 4)

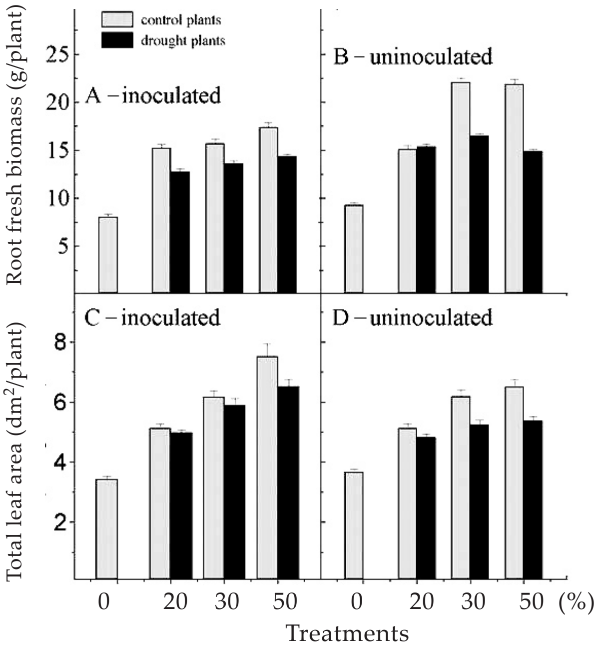

Figure 3. Root fresh biomass and total leaf area of inoculated (A, C) and uninoculated (B, D) soybean plants, grown at different (0, 20, 30 and 50%) water stress; values represent means ± *SD* ($n = 4$)

al. 1997). Plant growth and yield appears to be more sensitive to water stress conditions than to any other unfavourable factor within the environment (Kramer and Boyer 1997).

In our experiments a tight relationship has been detected between nitrogen nutrition and water stress on the one hand and the physiological status of leaves on the other. At stage 5th fully expanded leaf the total leaf area was larger in uninoculated plants compared to inoculated when plants were grown under optimal water supply (Figure 3C, D). Contrary, when plants were with eighth fully expanded leaf the total leaf area in the inoculated plants was greater and reached the highest values of all studied treatments. The reason for this observation was that nitrogen fixation of these plants assessed per plant was highest during this period. These results show the relationship between nitrogen sourses and leaf growth rate. At 20 and 30% water stress in the total leaf area of inoculated and uninoculated plants, there were no significant differences (Figure 3C, D). Leaf growth was stimulated when inoculated plants were grown under 50% water stress. Leaf area of these plants was increased with 1.15 dm^2 per plant compared to the uninoculated plants grown under drought conditions (Figure 3). Water stress resistence of the inoculated plants probably is due to the higher amount of nitrogen during this period as well as to the nitrogen fixation source and better water status (better RWC) than nitrate-fed plants. Sage and Pearcy (1987) found that there is a close relationship between photosynthetic capacity of leaves and their nitrogen content and that greater part of this nitrogen is utilized for the synthesis of the components of the photosynthetic apparatus. Our results are in agreement with the data published by these authors.

Protein spectra

Numerous genes expressed in response to limited water availability encode RAB (Response to ABA) proteins or dehydrins. Water stress also induces different classes of heat-shock protein genes (Almoguera and Jordano 1992), several enzymes involved in glycolysis (Velasco et al. 1994) and peroxidases (Botella et al. 1994). In maize (*Zea mays* L.) subjected to progressive water stress, seventy-eight proteins showed significant quantitative variation (Riccardi et al. 1998).

In the total soluble protein spectra of control soybean leaves one dominating slow migrating band (Rubisco) and two groups of moderate moving bands were detected (Figure 4). A slight increase in the amount of soluble protein was revealed with the progress of plant development. A decline was manifested for moderate and fast moving protein bands in leaves of plants subjected to moderate drought in comparison with plants at the same stage of development but grown under optimal water supply. A similarity between protein spectra of control of uninoculated and inoculated plants as well as the changes induced by plant development and water stress applied was established.

It seems reasonable to expect a different behavior in the changes in nitrogenous compounds in water stressed pea plants grown with different sources of N-nutrition (Frechilla et al. 2000). Our experiments with soybean did not completely support this assumption. For instance, there was no qualitative difference in the total soluble protein spectra of nitrate-fed and nitrogen-fixing soybean leaves neither with the progress of development nor under drought conditions (Figure 4).

Very small amounts of termostable proteins were detected in the leaves of control uninoculated plants at stage of 5th leaf (Figure 5). With the progress of plant development a drastic decline in the amount of termostable protein occurred. There were no changes in the termostable protein profiles of uninoculated plants subjected to moderate water stress but 50% drought led to an enhancement of their amount. The quantity of termostable proteins in inoculated control plants was lower in some degree compared to uninoculated one. There were no changes in termostable soluble protein spectra in the course of development of inoculated plants

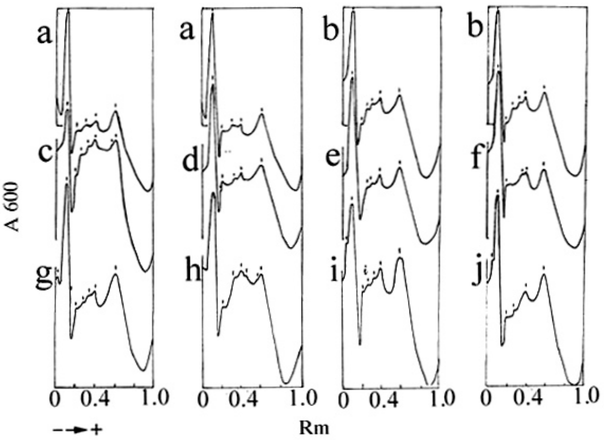

Figure 4. Densitometric scans of total soluble protein spectra (native PAGE) of soybean leaves; a = uninoculated control, optimal water supply, 5th leaf; b = inoculated control, optimal water supply, 5th leaf; c = uninoculated, optimal water supply, 7th leaf; d = uninoculated, 30% water reduction, 7th leaf; e = inoculated, optimal water supply, 7th leaf; f = inoculated, 30% water reduction, 7th leaf; g = uninoculated, optimal water supply, 8th leaf; h = uninoculatd, 50% water reduction, 8th leaf; i = inoculated, optimal water supply, 8th leaf; j = inoculated, 50% water reduction, 8th leaf; two hundred micrograms of total protein was loaded in each tube; total protein profile of samples was visualized on gels by staining with Coomasie Blue

grown at optimal water supply. The water reduction caused an increase in the amount of soluble termostable proteins and the severe drought brought about their higher extent of enhancement. In all cases termostable proteins were localized in a group of moderate moving bands. At stage 5th leaf bands with lower *Rm* values prevailed but in the course of development and with the progress of water stress the faster moving bands prevailed in the mentioned group. In the case of uninoculated plants this shift was caused only by plant development. In inoculated plants this shift was brought about by plant development as well as by increased of water stress.

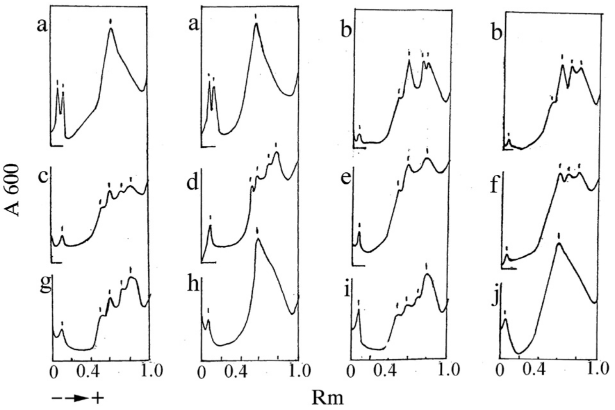

Figure 5. Densitometric scans of termostable soluble protein spectra (native PAGE) of soybean leaves; two hundred micrograms of total protein was loaded in each tube; total protein profile of samples was visualized on gels by staining with Coobiomassie Blue; for symbols see Figure 4

In conclusion, nitrogen source has a major effect on the response of soybean plants to water stress only in some cases. Nitrogen-fixing drought plants had relatively smaller root development but better relative water content and large leaf area on the last sampling day than nitrate-fed soybean plants. There was a difference in termostable proteins content of nitrate-fed and nitrogen-fixing plants. The quantity of termostable proteins in inoculated control plants was lower in some degree compared to uninoculated one. In inoculated plants the water stress caused an increase in the amount of soluble termostable proteins and the severe drought brought about their higher extent of enhancement.

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ABSTRAKT

Změny produkce biomasy a spekter celkových rozpustných bílkovin u rostlin sóji vystavených postupnému vodnímu deficitu při výživě nitrátem nebo fixovaným dusíkem

Byl studován vliv dusíkaté výživy (fixace vzdušného dusíku nebo příjem nitrátu) a postupného vodního stressu na relativní obsah vody, produkci celkové svěží hmotnosti a sušiny a na změny v celkovém obsahu bílkovin. Rostliny byly kultivovány v substrátu ve skleníku za přirozeného osvětlení. Za kontrolu sloužily rostliny pěstované bez vodního deficitu. Rostliny vyživované fixovaným vzdušným dusíkem a rostliny rostoucí za vodního deficitu měly relativně méně vyvinuté kořeny, avšak lepší relativní obsah vody a velkou listovou plochu v době posledního odběru než rostliny, u kterých byla dusíkatá výživa zajištěna nitrátem. Vodní deficit ani zdroj dusíku neměly vliv na produkci biomasy na konci pokusné doby (21 dní). Proteinová spektra zjištěná v listech sóji u rostlin pěstovaných při obou zdrojích dusíkaté výživy nevykazovala kvantitativní rozdíly v závislosti na vývinu ani na vodním deficitu. Vliv vodního deficitu mezi rostlinami s různou dusíkatou výživou se však projevil v obsahu termostabilních proteinů. Množství termostabilních proteinů v kontrolních rostlinách vyživovaných fixovaným dusíkem bylo do jisté míry nižší než u rostlin, u kterých byla výživa zajištěna nitrátem. U rostlin vyživovaných fixovaným dusíkem způsoboval vodní deficit zvýšení obsahu rozpustných termostabilních bílkovin.

Klíčová slova: zdroje dusíku; *Glycine max* L.; vodní stress; produkce biomasy; listová plocha; relativní obsah vody; spektra rozpustných bílkovin; termostabilní bílkoviny

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