

## Effect of Water Deficit on $^{15}\text{N}$ Shoot Allocation During Seed Filling in Soybeans

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### Abstract

Drought strongly limits crop productivity in semi-arid and many subhumid areas. Since soybean plants are sensitive to limited water supply at the beginning of the seed filling period, we studied effects of water deficits on protein synthesis in different organs of different ages during seed filling in two genotypes under well watered conditions, water deficit and after stress release. We used a flexible stem injection technique of  $^{15}\text{N}$  application in order to quantify the importance of the internal cycling of N to support new protein. Restricted irrigation reduced seed mass accumulation in PI 416937, but not in Hutcheson. Leaves were susceptible to mild water deficits with regard to daily incorporation of  $^{15}\text{N}$  into protein. During the rewatering period, this capacity remained on a low level, this was most pronounced in Hutcheson. Seed protein synthesis rate was only partially affected by water deficit. These findings suggest that the two genotypes differ with respect to source-sink relationships in response to a water restriction and following rewatering period.

**Key words:**  $^{15}\text{N}$ -incorporation rate, *Glycine max. (L.)*, protein, drought, seed filling

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### INTRODUCTION

Soybean is an important source of protein and oil. Soybeans are grown in many areas where rainfall is marginal or where drought stress is intermittent. Plant water deficits occur when the rate of transpiration exceeds water uptake. Such deficits are to a certain degree a normal component of development, particularly during seed development. Cellular water deficit can result in changes of water potential gradients, a concentration of solutes, loss of turgor, changes in cell volume and membrane shape, and denaturation of proteins (Bray 1997; Pineiro and Chaves 2011). Drought strongly limits crop productivity in semi-arid and many subhumid areas. Continuous drought stress during the seed filling phase accelerates senescence and reduces yield (Boyer and Westgate 2004; De Souza et al. 1997; King and Purcell 2005). Thus, in most countries where soybeans are produced, improvement of drought resistance is an important research and breeding objective. In practice, resistance to drought is

difficult to define because it is always related to plant species, the severity and duration of the stress (Burton 1997). Soybean plants are sensitive to limited soil water at the beginning of the seed filling period (R5) (Fehr and Caviness 1989), when the pods have fully lengthened and seeds have begun to fill. Growth rate of seeds and whole plant up to the following growth stage 'full seed' (R6) is still very rapid. This growth stage corresponds to a time when large quantities of carbon and nitrogen assimilates are being remobilised from source tissues to the developing seeds. The synthesis of storage protein in seeds is regulated at different levels. The most important are availability and partitioning of assimilates and nitrogen compounds and the genetic properties of cultivars (Riera et al. 2005; Rolletschek et al. 2005; Schiltz et al. 2005). A precocious decrease in N level and the breakdown of proteins in leaves under suboptimal conditions cause a decrease in physiological activity and ultimately leaf

senescence, thus possibly restricting seed fill and yield.

In many humid environments where soybean is grown, especially those with shallow soils that have low water-holding capacity, short periods of drought stress may be more likely than long periods of continuous stress. Short periods of drought stress during seed filling are not well-documented (Brevedan and Egli 2003; Sinclair et al. 2003) and can have larger effects on seed size and yield than expected. Rewatering soybean plants after a relatively short stress period (3-5 days) did not completely eliminate the effects of drought stress on the senescence process (Brevedan and Egli 2003). Moreover, little is known about the effects of drought stress on growth of branches, their seed yield and their contribution to the over-all yield (Frederick et al. 2001). The research of Norsworthy and Shipe (2005) illustrates substantial differences in mainstem and branch yield among genotypes. Use of this knowledge in selecting genotypes for wide or narrow rows may improve yield, but there is some uncertainty about the stability of branch yield relative to mainstem yield during periods of moisture stress.

Understanding of the N distribution and utilization within a stand is relevant for the analysis of behaviour under limited growth conditions. Providing an enriched  $^{15}\text{N}$  label to plants and following its fate can be an effective way to differentiate between external and internal plant sources and to quantify the importance of the internal cycling of N to support new growth (Dawson et al. 2002). The purpose of this  $^{15}\text{N}$  tracer study was to evaluate the response of protein synthesis in different plant tissues of two soybean genotypes with differences in phenotype. Therefore, in this study we used a stem injection technique of  $^{15}\text{N}$  application (Götz and Herzog 2000) in order to evaluate N incorporation into proteins of different organs of different ages during seed filling in plants

under well watered conditions, water deficit and after stress release.

## MATERIAL AND METHODS

The experiment, designed as a three-factorial trial (genotypes x water supply x plant fraction) with 4 replications, and was conducted under controlled conditions. Two determinate soybean cultivars, of maturity group V, Plant Introduction (PI) 416937 and Hutcheson, were raised in two (replicated) growth-chambers at 26/22°C (light/dark), at 70% relative humidity. Plastic pots (diameter: 20.3 cm; height: 20.0 cm) were filled with 4.37 kg coarse gravel and peatlite (2:1) (Thomas and Down 1991).

During the 9-h day period, a combination of cool-white fluorescent and incandescent lamps provided a photosynthetic photon flux density of 670 to 735  $\mu\text{E m}^{-2} \text{sec}^{-1}$ . Ambient  $\text{CO}_2$  within growth chambers was monitored by infrared gas analysis and maintained at 350 to 400  $\mu\text{L L}^{-1}$ .

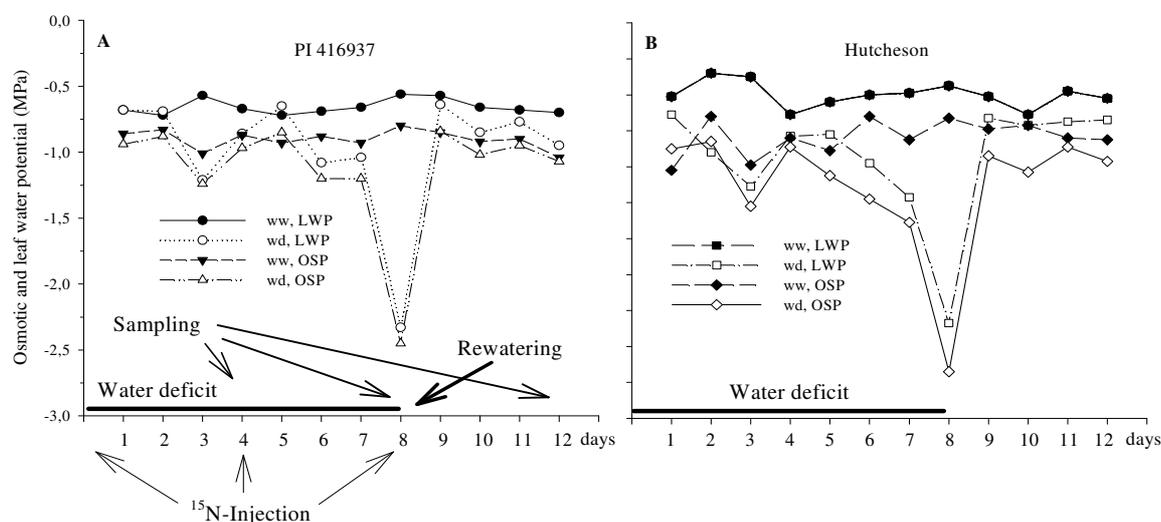
Soil moisture (water and nutrient solution) (Thomas and Down 1991) was maintained at field capacity (well watered, ww), but the water supply was reduced for the water deficit (wd) treatment (Table 1, Fig. 1) during an interval of 8 days (day 0-8, 56-64 DAS). Water deficit plants were then rewatered (day 8, 64 DAS). Plants of both treatments were irrigated 3 times a day at 8.00, 12.30 and 16.30.

At day 0, 4 and 8 (Fig. 1A) a single dose of 50  $\mu\text{l}$  with 2 mg  $^{15}\text{N}$  per plant ( $^{15}\text{NH}_4\text{Cl}$ , 95 atom%, CAMPRO Scientific, Berlin, Germany) was laterally injected into the stele at the stem basis, using a microliter syringe (needle diameter: 330  $\mu\text{m}$ ) and sampling of the whole plants was done 4 days after injections (Fig. 1A).

**Table 1** Daily supply of nutrient solution (mL) and water (mL) for PI 416937 and Hutcheson to the well watered (ww) and water deficit (wd) plants.

DAS†	Day	well watered (ww)			water deficit (wd)		
		Nutrient solution	H <sub>2</sub> O	Amount	Nutrient solution	H <sub>2</sub> O	Amount
		mL					
56	0	225	375	600	225	0	225
57	1	225	375	600	225	0	225
58	2	375	225	600	375	0	375
59	3	300	300	600	300	0	300
60	4	300	300	600	300	0	300
61	5	275	225	500	275	100	375
62	6	250	225	475	250	0	250
63	7	75	325	400	75	0	75
64	8	200	saturation		200	saturation	

†DAS, days after sowing



**Figure 1** Osmotic (OSP) and leaf water potential (LWP) of PI 416937 (1A) and Hutcheson (1B) under well watered (ww) and water deficit (wd) conditions, and during the rewatering period.

The plants were sampled, cut into mainstem plant fraction and branches. Plants were separated into the organs leaves, stems, pod walls and seeds. The samples were then dried for 2 days at 60 °C and ground to pass through a 0.5 mm sieve and prepared for the Kjeldahl-N analysis, for the determination of the trichloroacetic acid (TCA) precipitable N fraction (TCA-insoluble proteins) and the  $^{15}\text{N}$  determination. The TCA-insoluble proteins were extracted with 4 ml of 20% TCA for 30 minutes. Then the samples were centrifuged at 5000 g for 10 minutes and washed twice with 4 ml of 7% TCA. For the determination of the atom %  $^{15}\text{N}$ , the remaining solution of  $\text{NH}_4\text{Cl}$  following titration (Kjeldahl-N analysis) was evaporated, adjusted to a N concentration of about 500  $\mu\text{g ml}^{-1}$  and introduced into an emission spectrometer (Isonitromat 5200, Statron, Fürstenwalde, Germany (Götz and Herzog 2000)).

Double junction isopiestic thermocouple psychrometry, according the procedure of Sanchez-Diaz and Kramer 1971, was used to monitor changes in leaf water potential, throughout the drying and recovery cycle. The leaf disks were then frozen, thawed, and used to measure the osmotic potential (Patterson and Hudak 1996). The leaf area where measured with an integrating automatic leaf area meter (LiCor 3000). Data were analysed using statistical software from SPSS, version 17.0 (univariate model, Student's *t*-test).

## RESULTS AND DISCUSSION

To quantify the stress level, water status in the plants was examined during the period of water deficit (Fig. 1). Leaf

water potential (LWP) of well watered plants remained approximately constant during the experiment amounting to  $-0.66 \pm 0.02$  MPa in PI 416937 and slightly higher in Hutcheson ( $-0.63 \pm 0.03$  MPa,  $P < 0.05$ ). Water deficit (wd) lowered LWP of both genotypes to about  $-1$  MPa within six days. A further restriction of water supply (Table 1) carried out at day 6 and 7 because Hutcheson showed no leaf wilting, led to a stronger decrease of leaf water potential of  $-2.33$  MPa in PI 416937 and  $-1.87$  MPa in Hutcheson until stress end. Rewatering of the substrate normalised LWP within one day. The osmotic potential (OSP) of well watered PI 416937 plants was  $-0.90 \pm 0.02$  MPa and a little higher in Hutcheson ( $-0.84 \pm 0.03$  MPa, ( $P < 0.05$ )). The osmotic potential of the leaf tissues decreased and remained lower than the total water potential as water deficit increased to a certain value. This indicated the existence of a positive turgor in the leaf tissues during the experiment. The maintenance of a certain turgor is evidence for some osmotic adjustment in both cultivars (Bouchereau et al. 1996; Sionit and Cramer 1977).

During the water deficit period, total plant dry matter (Tab. 2A,B) of both genotypes was not affected by water restriction. This was evident at the onset and at the point of maximum stress (day 4 and 8) for all tissues of both genotypes. However, a slight dry matter reduction of leaves, pod walls and seeds of PI 416937 by restricted irrigation was indicated at the end of stress (day 8) and obviously augmented considerably later on, but was not detected in Hutcheson. Genotypic performance of leaf production under water deficit seemed to be related to different components: while Hutcheson produced smaller leaves, but more of them

due to its stronger capacity of branching than PI 416937.

Following four days of recovery from stress (Table 2C), the two genotypes responded quite differently. Wd-plants of PI 416937 accumulated less total plant dry matter (26%) than did the corresponding ww- plants, whereas wd- and ww-plants of Hutcheson continued to accumulate similar amounts of shoot mass. Partly, this was due a lowered DM-accumulation in leaves and pod walls of stressed compared to well watered plants of PI 416937 (33% and 26%, resp.), whereas these tissues of Hutcheson were unaffected by restricted irrigation. Moreover, restricted irrigation significantly reduced accumulation of seed mass of PI 416937 by 25%, but did not suppress Hutcheson's seed development. This differential performance led to a greater seed mass of stressed plants of Hutcheson than of PI 416937 four days following stress relief implying that rate of dry matter translocation to the seeds between day 8 and 12 has been slowed down only in PI 416937 as an after-effect of the restricted water supply before. Additionally, the amount of total protein produced per plant at day 12 was decreased in PI 416937 from 3.7 g (ww) to 2.9 g, but not in Hutcheson. Similar effects were observed by Sinclair et al. 2003. Therefore, under these circumstances Hutcheson is probably more capable of maintaining the C-assimilation under and after mild water deficit. Stress relief of soybean plants at 3 or 5 days after the onset of the water deficit stress (Brevedan and Egli 2003) restored carbon exchange rate to levels near or equal to those of the control plants. But the response to relief at 6 or 13 days after stress initiation was much smaller, and plants matured sooner, produced up to 23% significantly lower yields and 9 to 17% smaller seeds than control plants. In our experiment, the leaf area per plant one day after stress relief (day 9, data not shown) was not affected by restricted irrigation for either genotype. Although Hutcheson produced smaller leaflets, its stronger capacity for branching compared to that for PI 416937 ( $8.2 \pm 0.2$  vs.  $5.5 \pm 0.2$  branches plant<sup>-1</sup>,  $P < 0.05$ ) enabled Hutcheson to produce significantly more photosynthetic surface per plant than PI 416937 ( $39.3 \pm 1.2$  vs.  $31.2 \pm 1.3$  dm<sup>2</sup>,  $P < 0.05$ ). A close correlation between branch vegetative growth and branch seed yield indicates that good branch vegetative growth is essential for high seed yield in determinate soybean cultivars (Frederick et al. 2001). A greater leaf area expansion could increase the photosynthetic activity of the plant that provides larger quantities of C compounds to roots for supporting their leaf N uptake activity and increases the capacity of plants to store organic N in leaves, for example as Rubisco. Results obtained on individual plants with a dense canopy also support the hypothesis that leaf growth determines the dynamics of shoot N accumulation (Lamaire et al., 2005). On the other hand, the specific leaf weight, while unaffected by restricted irrigation (data not shown), was greater for PI 416937 than for

Hutcheson ( $0.278 \pm 0.001$  vs.  $0.222 \pm 0.0008$  g dry matter dm<sup>2</sup>,  $P < 0.05$ ).

**Table 2** Accumulation of dry matter (g plant<sup>-1</sup>, means) in plants and in different organs of PI 416937 and Hutcheson in different plant organs 4 days (A) and 8 days (B) after onset of water deficit and 4 days after rewatering (C).

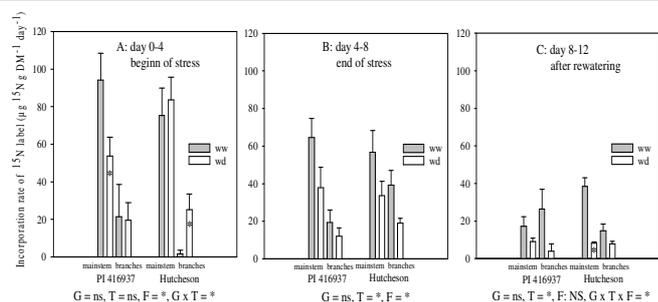
	T	Plants	Leaves	Stems	Pod walls	Seeds
(A) day 4						
PI 416937	ww	†	8.2a	4.7a	3.6a	2.6a
	wd	††	8.6a	4.7a	4.0a	2.7a
Hutcheson	ww		9.7a	6.3a	5.4a	4.5a
	wd		7.2a	5.4a	4.1a	3.0a
(B) day 8						
PI 416937	ww		11.5a	6.1a	5.7a	6.2a
	wd		9.1a	5.6a	4.1a	3.9a
Hutcheson	ww		8.6a	5.6a	4.8a	6.4a
	wd		8.0a	6.1a	5.1a	6.2a
(C) day 12						
PI 416937	ww	34.0a	11.7a	6.6a	6.2a	9.5a
	wd	25.0b	7.8b	5.5a	4.6b	7.1b
Hutcheson	ww	31.1a	9.4a	6.1a	5.5a	10.1a
	wd	31.0a	7.7a	6.1a	5.9a	11.3a

Treatment, T; † ww, well watered, †† wd, water deficit.

a,b indicate significant difference between treatment according to Student's *t*-test,  $P < 0.05$ .

Ammonium ions entering the shoot via stem injection are immediately assimilated. In higher plants, glutamine synthetase (GS) is a key enzyme involved in the assimilation of inorganic nitrogen into organic forms. Two groups of isoenzymes, plastidic (GS2) and cytosolic (GS1) have been identified, but the relative activity of both varies between tissues of shoots and between species (Brugiére et al. 1999).

The purpose of this <sup>15</sup>N tracer study was to evaluate the response of protein synthesis in the different plant tissues of PI 416937 and Hutcheson to restricted water supply (Fig. 2-5). The rates of synthesis are represented by daily incorporation of <sup>15</sup>N label into protein per gram dry matter of the two plant fractions, mainstems and branches, during 3 intervals.



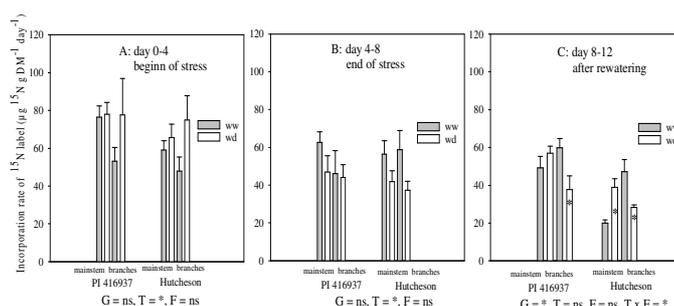
**Figure 2** Incorporation rate of  $^{15}\text{N}$  label ( $\mu\text{g } ^{15}\text{N g dry matter}^{-1} \text{ day}^{-1}$ ) into leaf proteins of mainstem and branches (Fraction, F) of two genotypes (G) under well watered (ww) and water deficit (wd) conditions (Treatment, T) (Means + SE) of four replicates; uni- or multivariate are non-/significant (ns, \*). Asterisk indicates significant differences between treatments (ww, wd) within a fraction according to Student's *t* test,  $P < 0.05$ .

Comparison of the three periods showed a general decrease of  $^{15}\text{N}$  incorporation into leaf proteins. Using the univariate model the incorporation rate of  $^{15}\text{N}$  (Fig. 2A,B,C) did not show any statistical difference among genotypes throughout the experiment. Likewise, restricted irrigation did not affect leaf protein synthesis during the first 4 days (Fig. 2A, main effect of T), but lowered it in the mainstem of PI 416937 and increased it in branches of Hutcheson. However, the impact of water deficit on both plant fractions increased through the experiment. During the interval 4 to 8 days (Fig. 2B), restricted irrigation suppressed synthesis by about 40% of the ww-plants in both, PI 416937 and Hutcheson. Upon completion of the water deficit during rehydration (Fig. 2C, interval 8 to 12 days) both genotypes displayed a clear decrease in the incorporation rate of  $^{15}\text{N}$  label of 70% of the control plants. Obviously, leaf senescence, once initiated and progressed far enough after 8 days of stress, was not reversible and could even not be stopped by rewatering the plants. Bredvan and Egli 2003 reported that the period of irreversible leaf senescence initiation ranges between 3 and 5 days. The senescence process, involving both a loss of photosynthetic capacity and cellular disassembly is highly coordinated, so it is not surprising that incorporation of  $^{15}\text{N}$  into proteins responded to the imposition and relief of water deficit. The relative quantity of 23 maize leaf proteins was found to decrease to 50% up to 80% of the control in stressed plants, this could be due to the repression of the synthesis, but also to differential turnover (Riccardi et al. 1998).

Drought may restrict the ability of plants to reduce and assimilate nitrogen, as a result of the inhibition of the activities of enzymes, e. g. nitrate reductase the activity of which has been shown to decline in drought stressed leaves of several species (Correia et al. 2005). Transport proteins, such as ion channels and aquaporins can also play an important role in water deficit avoidance (Tyermann et al. 2002), by controlling cellular water status, respectively. During drought

some proteins are irreversibly damaged and degraded by proteases (Campalans et al. 1999). Proteases play important regulatory roles, controlling various metabolic pathways via the rapid turnover of key or rate-limiting enzymes, and influencing developmental programs (Clarke 2005).

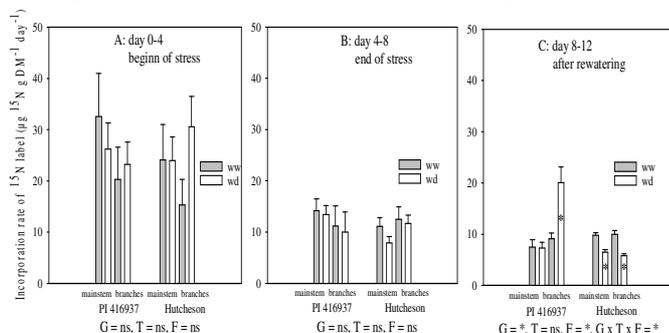
The accumulation and transient storage of N in stems seems to be particularly important in terms of yield, since it can be considered - at least in part - as an excess of over the actual demand for growing seeds and leaves. The highest yields were obtained when the growing stem had high N concentration and the senescing stem had low N concentration (Sinclair et al. 2003). The  $^{15}\text{N}$  incorporation into the stem proteins (Fig. 3A,B,C; univariate model) was neither different among genotypes nor between main stem and branches during the water deficit period. However, treatment effects were significant consisting in slightly increased rates of  $^{15}\text{N}$  incorporation due to water deficits during interval 0-4 days, but in reduced rates during interval 4-8 days. After stress release a reduced  $^{15}\text{N}$  incorporation by water deficit was still evident in the branches while the opposite was true for main stems.



**Figure 3** Incorporation rate of  $^{15}\text{N}$  label ( $\mu\text{g } ^{15}\text{N g dry matter}^{-1} \text{ day}^{-1}$ ) into stem proteins of mainstem and branches (Fraction, F) of two genotypes (G) under well watered (ww) and water deficit (wd) conditions (Treatment, T) (Means + SE) of four replicates; uni- or multivariate are non-/significant (ns, \*). Asterisk indicates significant differences between treatments (ww, wd) within a fraction according to Student's *t* test,  $P < 0.05$ .

The  $^{15}\text{N}$  incorporation into the pod wall proteins was not influenced by genotypes, treatments and plant fractions during the water deficit period (main effects, Fig. 4A,B). During the rewatering interval, PI 416937 out-yielded Hutcheson, but this was obviously due to differential after-effects of water deficit; in PI 416937 rates in mainstem and branches matched or considerably exceeded those of well watered plants, respectively, while in Hutcheson rates were decreased in both fractions. The twofold higher utilization of  $^{15}\text{N}$  in wd-PI 416937 pod walls might be an effect of a reduction of pod growth during the stress (comp. Table 2) by which less, but more active pods remained on branches after stress than at Hutcheson. Furbank et al. 2004 pointed out, that chlorophyll concentrations, closely related to the N concentration, are of the same order for pod wall, embryo,

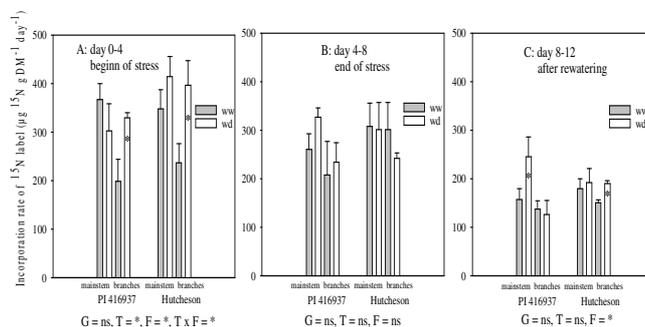
and seed coat tissues in younger chickpea pods, but decline differentially later on. Therefore, the ability of pods to compete for assimilates is probably a function of complex spatial and temporal interactions between plant organs (Dracup and Kirby 1996), especially under drought.



**Figure 4** Incorporation rate of  $^{15}\text{N}$  label ( $\mu\text{g } ^{15}\text{N g dry matter}^{-1} \text{ day}^{-1}$ ) into pod wall proteins of mainstem and branches (Fraction, F) of two genotypes (G) under well watered (ww) and water deficit (wd) conditions (Treatment, T) (Means + SE) of four replicates; uni- or multivariate are non-/significant (ns, \*). Asterisk indicates significant differences between treatments (ww, wd) within a fraction according to Student's *t* test,  $P < 0.05$ .

Legume seeds accumulate large amounts of proteins in the cotyledonary parenchyma cells, particularly stored protein bodies which survive desiccation on during seed maturation and undergo hydrolysis at germination. About 70% of the protein in soybean seeds is present as storage proteins glycinin and  $\beta$ -conglycinin which are also termed 11S and 7S proteins, respectively. The nutritional environment during seed filling is an important factor affecting relative abundance of these proteins. Besides storage proteins, soybean seeds contain several comparatively minor proteins including trypsin inhibitors, lectins, lipoxygenase and urease, which are relevant to the nutritional quality of seed (Krishnan et al. 2000; Sexton et al. 1998).

The incorporation rate of  $^{15}\text{N}$  label into seed proteins during the whole experiment did not show statistical difference between genotypes (univariate model, Fig. 5A,B,C). Water deficit affected seed protein synthesis only during the first 4 days, being more or less unaffected in mainstems, but even stimulated in branches. The impacts of water deficit were imperceptible in the second interval (Fig. 5B). During the rewating interval (Fig. 5C) rates were generally lower than before, however there were some differential after-effects of water deficit with enhanced rates in the seeds of mainstems in PI 416937, but of branches in Hutcheson. No differences were also noticeable between two soybean cultivars (Blanuša et al. 2000) classified as drought tolerant and drought susceptible, in the synthesis of  $\alpha$ - and  $\alpha'$ -subunits of the 7S protein.



**Figure 5** Incorporation rate of  $^{15}\text{N}$  label ( $\mu\text{g } ^{15}\text{N g dry matter}^{-1} \text{ day}^{-1}$ ) into seed proteins of mainstem and branches (Fraction, F) of two genotypes (G) under well watered (ww) and water deficit (wd) conditions (Treatment, T) (Means + SE) of four replicates; uni- or multivariate are non-/significant (ns, \*). Asterisk indicates significant differences between treatments (ww, wd) within a fraction according to Student's *t* test,  $P < 0.05$ .

Our results were obtained under a mild water stress of 8 days during seed filling if nitrogen is available under stress ( $^{15}\text{N}$  injection into the stem base) demonstrating that water deficits affected the capacity of protein synthesis in different plant fractions (mainstem/branches). Principally, protein synthesis capacity in seeds was reduced by mild water deficits in both genotypes, which on the one hand was achieved by a general reduction of protein synthesis in leaves during and even shortly after the stress period. On the other hand, synthesis in stems was reduced during the second half of the stress period, and only after stress certain differential stress-effects were observed among genotypes with regard mainstem or branches being preferentially enhanced. Thus, the low-dose stem injection of  $^{15}\text{N}$  allows a detailed quantitative internal assessment of N utilization with regard to different organs, treatments and plant fractions.

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