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Waterlogging effect on xylem sap glutamine of nodulated soybean

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Abstract

Waterlogging of soybean plants (*Glycine max* L.) led to impaired symbiotic N_2 fixation and a marked decline in glutamine (Gln) concentration in xylem bleeding sap. Xylem Gln concentration increased during the growth cycle of the plant and was correlated with nodule formation. Treatments known to impair N_2 fixation, such as exposing the root system to pure N_2 gas or a mixture of Ar and O_2 (80:20; v/v), led to specific declines in xylem sap Gln. The decrease in Gln observed during waterlogging was also seen on transfer of nodulated plants to aerated hydroponics, where the decline was highly correlated with ureide content in the xylem sap. Upon flooding the nodulated root system, the specific decline in xylem sap Gln could be detected within 10 min and reached a minimum within 60 min, indicating that waterlogging has an immediate effect on N_2 fixation. It is concluded that xylem Gln arises directly from N_2-fixation and is a useful indicator of N_2 fixation activity of symbiotic soybean plants.

Aditional key words: *Glycine max*, hypoxia, nitrogen fixation, nitrogen transport.

Introduction

Plant responses to stress often involve changes in amino acid metabolism (Rai 2002, Nayyar and Walia 2003, Tsai *et al.* 2003). Waterlogging has a profound effect on root metabolism, mainly through the deficiency of oxygen (Drew 1997) and amino acids such as alanine may accumulate (Ricard *et al.* 1994). Nitrogen fixation of symbiotic legumes is regarded as being particularly sensitive to waterlogging, probably in view of the oxygen requirement of this process (Minchin and Pate 1975, Minchin *et al.* 1978), since nitrogenase appears to be limited by oxygen availability (Layzell and Hunt 1990, Layzell *et al.* 1990). Long-term studies of waterlogging and N_2 fixation in soybean have revealed severely decreased N accumulation and growth suggesting that N_2-fixation is strongly diminished during the stress (Becanamwo and Purcell 1999). Exposure of the plants to low concentrations of oxygen produced a similar response which supports the idea that oxygen deficiency underlies the effect of waterlogging. Moreover, long-term oxygen deficiency led to large decreases in both nodulation and specific nitrogenase activity (Becanamwo and Purcell 1999).

Our previous short-term studies with waterlogged soybean revealed marked changes in the amino acid composition of the xylem (Puiatti and Sodek 1999, De Sousa and Sodek 2003) where the increase in alanine is substantial. Glutamine (Gln) content is high in soybean dependent on N_2 fixation compared to plants dependent on nitrate assimilation (Puiatti and Sodek 1999) but declines from over 20 % of total amino acids to about 5 % after 5 d of flooding. This phenomenon is also observed in plants transferred to hydroponics without aeration. Transfer to aerated hydroponics, which prevents hypoxia of the roots, does not abolish the effect but does attenuate the rate of decline. Since xylem sap ureides behave in a manner similar to Gln (Puiatti and Sodek 1999) and ureides exhibit a close relationship with N_2 fixation (McClure *et al.* 1980, McNeil and LaRue 1984, Herridge and Peoples 1990, Herridge *et al.* 1990), it would appear that impaired N_2 fixation due to waterlogging underlies the decline in Gln contents in the xylem.

In the present study we attempted to prove this hypothesis. Since technical difficulties prevent the *in vivo* measurement of nitrogenase activity of submerged nodules (Minchin and Pate 1975), our objective was to show that changes in xylem sap Gln are related to N_2 fixation using other experimental systems where nitrogenase is known to exhibit a clear change in activity, as well as carrying out a detailed time-course study of the waterlogging effect itself.

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Abbreviations: Ala - alanine; Asn - asparagine; Asp - aspartic acid; Gaba - γ-amino butyric acid; Gln - glutamine; Ser - serine.

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Materials and methods

Soybean plants [*Glycine max* (L) Merr. cv. IAC-17] nodulated with strain SMS-463 (SEMIA 5019) of *Bradyrhizobium elkanii* were grown in pots with vermiculite (except where stated) and N-free nutrient solution in a greenhouse under natural light and temperature conditions, as described previously (Lima and Sodek 2003). Treatments were initiated with plants at stage R2 (for stage definitions, see Fehr and collaborators (1971); R2 = flowering (early reproductive stage), under our growth conditions normally reached at the vegetative stage V7 where 7 nodes bearing fully expanded leaves are present).

For the aerated hydroponics treatment, plants were removed from pots and the root system carefully washed in tap water to remove the vermiculite before transfer to 3 dm^3 pots (3 plants per pot) containing N-free nutrient solution at one-third of normal strength and aerated continuously with compressed air. The whole root system (including the nodules) was maintained submersed in the nutrient solution. Plants maintained in vermiculite were used as controls.

For the waterlogging treatments, plants growing in pots of vermiculite were placed inside a second pot of similar size and filled with N-free nutrient solution at one-third of normal strength sufficient to bring its level just above the vermiculite.

In experiments where the nodulated root system was exposed to specific gases, plants were grown in special pots (1 dm^3) (one plant per pot, 3 replicate pots per treatment) filled with gravel instead of vermiculite. In order to maintain the humidity of the gravel, the surface was covered with moist cotton wool and N-free nutrient solution supplied at frequent intervals. When treatments were initiated, the cotton wool was removed and the pots sealed with tight-fitting lids and the orifice around the plant stem sealed with modelling clay. The single opening at the base of the pot was connected to a small air-pump and the appropriate gas drawn from a large plastic bag and flushed through the pot and out via a small outlet in the lid. The flow rate was adjusted to 500 cm^3 min^{-1} for the first 2 min and then maintained at 200 cm^3 min^{-1} (gaseous volume in pot was *ca.* 200 cm^3). The gases used were pure N_2 or a mixture of argon:oxygen (80:20; v/v). The duration of the experiment was 2 h and sap was collected for 1 h during the second hour of gas treatment.

Root (xylem) bleeding sap was collected according to McClure and Israel (1979), after cutting the stem just below the cotyledonary node.

Immediately after harvest of the root system, nodules were detached and rinsed in cold distilled water, blotted, weighed and ground in a *Polytron PT10/35* ultrasonic homogeniser (*Kinematica,* Lucern, Switzerland) with methanol:chloroform:water (12:5:3; v/v/v) (adapted from Bieleski and Turner 1996) using 10 cm^3 per 1 g tissue. The nodule-free roots were prepared similarly, except for initial grinding with liquid N_2 in a mortar before extraction. The aqueous phase (containing the amino acids and other soluble nitrogenous components) was recovered following phase separation on standing after addition of chloroform and water to the supernatant (1:1.5:4; v/v/v). The aqueous phase was then reduced to a known volume by evaporation at 38 °C and kept frozen until analysis.

Amino acids were analysed by reverse-phase HPLC of their *o*-phthaldialdehyde (OPA) derivatives, as described previously (Puiatti and Sodek 1999). Total amino acids (Yemm and Cocking 1955) and ureides (Vogels and Van Der Drift 1970) were determined colorimetrically using leucine and allantoin as standards, respectively.

Except where stated, experiments were carried out with 3 replicates using a randomized complete block design, each replicate being composed of 1 pot with 3 plants (material pooled for each replicate). Standard errors are shown where appropriate. Other details of statistical analysis are given with the data.

Results and discussion

Xylem sap Gln and nodule development: It is well established that nitrogenase activity is highly correlated with nodule mass during nodulation of soybean (Schubert 1981, Reynolds *et al.* 1982). The measurement of xylem sap Gln concentration during the growth cycle of the plant revealed an increase that correlated well with nodule formation (Fig. 1). At early stages of growth (V1/V2), before nodule formation, the concentration of Gln was relatively low in the xylem sap but rose rapidly as nodules started developing (after V2). The highest values were obtained after nodule fresh mass had reached its maximum at around R4/R5 (pods fully expanded/ initial pod-fill). Gln represented only 3 % or less of the total amino acids in the xylem sap at early stages rising rapidly to a maximum of 23 % during nodule growth (data not shown).

Xylem sap Gln and impaired N_2 fixation: In order to obtain further evidence for the association between xylem Gln and N_2 fixation, the nodulated root system was exposed to a mixture of $Ar:O_2$ (80:20) or pure N_2, treatments which are known to seriously impair nitrogenase activity (Atkins *et al.* 1984, Layzell *et al.* 1990). When the root system was flushed for 2 h with pure N_2 a large decline in Gln was observed relative to the control flushed with air or the untreated plant (Table 1). Gln content in the xylem was typically around 20 % of the total amino acids before treatment but declined to less than 1 % after treatment with pure N_2 gas. Conspicuous decreases in aspartic acid (Asp) and increases in alanine (Ala), γ-amino butyric acid (Gaba) and serine (Ser) were also observed in this treatment, whereas asparagine (Asn), the most abundant amino acid, presented no change. A sharp decline in Gln was also observed in the treatment with an argon/oxygen mixture but, in contrast to the treatment with pure N_2, other amino acids changed very little except for Asp which increased somewhat. Nevertheless, both treatments (absence of oxygen and absence of N_2) led to the expected sharp declines in xylem Gln levels. Ureide content of the xylem sap was also strongly reduced by these treatments. Besides the distinct amino acid composition of the roots and nodules compared with xylem sap, the only noteworthy changes were the increases in Ala and Gaba (and Ser in nodules) in the treatment with pure N_2. The increases in these amino acids were the only ones to parallel the changes seen in the xylem sap.

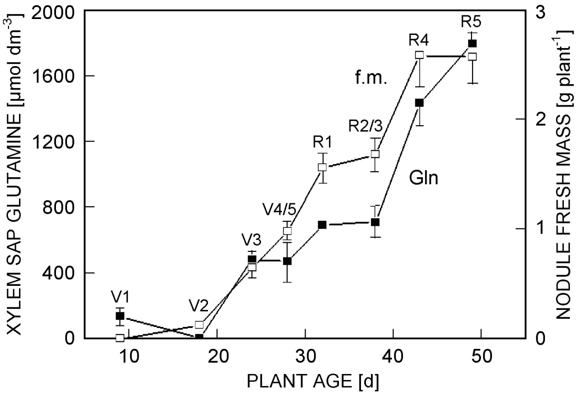

Fig. 1. Glutamine (Gln) concentration in xylem sap (*closed symbols*) and nodule fresh mass (*open symbols*) during the growth of soybean plants. V1 ... R5 - developmental stages as defined by Fehr *et al.* (1971). Means ± SE of 3 replicates. Correlation coefficient, $r = 0.95$ for Gln × nodule fresh mass.

Table 1. Amino acid content of the xylem sap [μmol dm^{-3}], roots and nodules [μmol kg^{-1}(f.m.)] of soybean plants after submitting the root system to different gas mixtures (air, 100 % N_2, Ar/O_2 80:20). Xylem sap was collected for 1 h during the second hour of treatment. Means followed by different letters are significantly different by Duncan´s multiple range test ($P < 5$ %, $n = 3$). Amino acid composition [% total amino acids] is given in parenthesis.

		No treatment	Air	N_2	Ar:O_2
Xylem sap	Asp	327 (8.8)	334 (9.8)	41 (1.1)	515 (15.1)
	Glu	93 (2.5)	78 (2.3)	143 (3.8)	113 (3.3)
	Asn	1529 (41.1)	1494 (43.8)	1525 (40.6)	1432 (42.0)
	Ser	78 (2.1)	82 (2.4)	316 (8.4)	99 (2.9)
	Gln	740 (19.9)	658 (19.3)	26 (0.7)	147 (4.3)
	Ala	33 (0.9)	48 (1.4)	568 (15.1)	55 (1.6)
	Gaba	100 (2.7)	82 (2.4)	248 (6.6)	119 (3.5)
	others	818 (22.0)	634 (18.6)	891 (23.7)	931 (27.3)
	total aa	3718 a	3410 a	3758 a	3411 a
	ureides	11080 a	9440 a	4440 b	4570 b
Roots	Asp	163 (8.9)	125 (8.6)	157 (10.9)	159 (12.7)
	Glu	176 (9.6)	129 (8.9)	134 (9.3)	159 (12.7)
	Asn	143 (7.8)	116 (8.0)	89 (6.2)	80 (6.4)
	Ser	172 (9.4)	129 (8.9)	127 (8.8)	109 (8.7)
	Gln	75 (4.1)	59 (4.1)	36 (2.5)	50 (4.0)
	Ala	88 (4.8)	58 (4.0)	144 (10.0)	46 (3.7)
	Gaba	128 (7.0)	94 (6.5)	181 (12.6)	65 (5.2)
	others	888 (48.5)	744 (51.2)	572 (39.7)	585 (46.7)
	total aa	1833 a	1454 a	1440 a	1253 a
	ureides	750 a	940 a	700 a	1140 a
Nodules	Asp	2454 (10.0)	2284 (8.7)	1225 (6.3)	2225 (8.2)
	Glu	4123 (16.8)	4541 (17.3)	4026 (20.7)	5050 (18.6)
	Asn	4883 (19.9)	3728 (14.2)	2683 (13.8)	4289 (15.8)
	Ser	1325 (5.4)	1680 (6.4)	1983 (10.2)	1656 (6.1)
	Gln	172 (0.7)	105 (0.4)	175 (0.9)	190 (0.7)
	Ala	2453 (10.0)	2573 (9.8)	4065 (20.9)	2986 (11.0)
	Gaba	2429 (9.9)	3203 (12.2)	3326 (17.1)	2442 (9.0)
	others	6699 (27.3)	8138 (31.0)	1983 (10.2)	8335 (30.7)
	total AA	24538 a	26252 a	19463 a	27173 a
	ureides	2370 a	2950 a	2000 a	1820 a

Xylem sap Gln after transfer to hydroponics: A more detailed study of our original observation that the transfer of nodulated soybean plants growing in vermiculite to aerated hydroponics (with the whole root system submerged) leads to a sharp decline in xylem Gln (Puiatti and Sodek 1999, Lima and Sodek 2003), not only confirmed the observation but revealed the time-course of the decline (Fig. 2). Initially, Gln represented 21 % of the amino acids (575 μmol dm^{-3}) but declined exponentially to 3.4 % (28 μmol dm^{-3}), reaching the lowest values within 24 h of treatment. The greater decline in absolute amounts compared to percent values reflects a decline in the total amino acid concentration of the sap of plants transferred to hydroponics, especially at 24 and 48 h where the concentration was only about one-third of the initial value (data not shown). The decline in Gln was paralleled very closely by a decrease in ureide content (correlation coefficient of 0.977). The Gln content in plants maintained in vermiculite over the same experimental period remained fairly constant (582 ± 18 μmol dm^{-3}), as did the ureide content. Besides Gln, only Asp and Asn revealed marked changes over the same period (Fig. 2*B*). Here, the data revealed an inverse relationship where Asp content increased as Asn decreased, a phenomenon reported earlier (Lima and Sodek 2003), though on a broader time scale. The most marked changes occurred between 14 and 48 h, well after most of the decrease in Gln. Asp rose sharply to nearly 60 % of the total amino acids while Asn declined to 18 %.

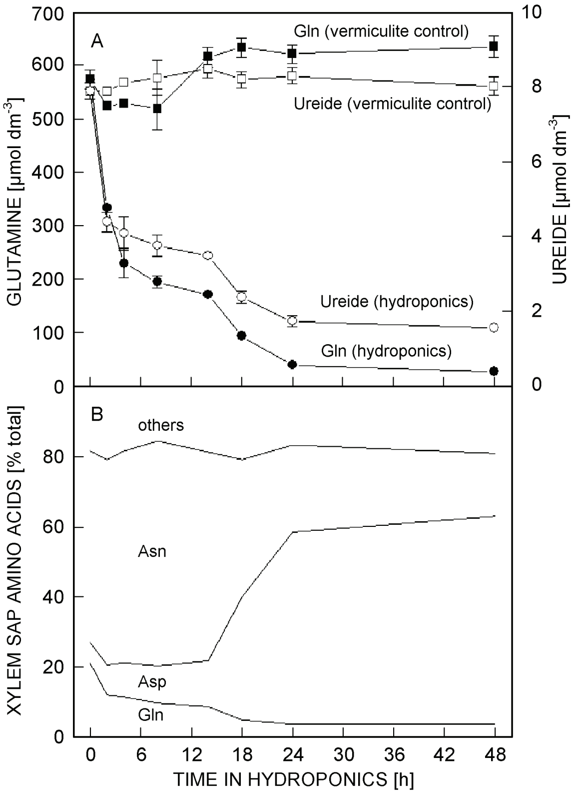

Fig. 2. *A* - Changes in xylem sap ureide (*open symbols*) and glutamine (*closed symbols*) concentration after transfer of nodulated soybean plants to hydroponics (*circles*) or maintained in vermiculite (*squares*). Means of 3 replicates; *bars* represent SE. *B* - Changes in xylem sap amino acid composition of the plants transferred to hydroponics. Plants were cultivated in vermiculite with N-free nutrient solution before transfer to aerated hydroponics (whole root system submerged in N-free nutrient solution) at stage R2 for 48 h.

Xylem Gln after flooding: A similar experiment was conducted whereby the plants in vermiculite were simply flooded by standing them in nutrient solution to the base of the stem. This treatment is similar in many respects to the previous where plants were transferred to hydroponics, the main difference being the absence of aeration plus the fact the plants were not disturbed. In contrast to the previous experiment, the concentration of oxygen in the solution will decrease and become critical even for root respiration within hours (De Sousa and Sodek 2003). For this reason, the usefulness of this treatment for specifically inhibiting N_2 fixation is limited to short-term studies. In this experiment, the time course of changes in xylem sap amino acid composition was followed by collecting sap sequentially in lots of 0.05 cm^3 over recorded time intervals beginning immediately on flooding (Fig. 3). This was compared with other plants flooded at the same time but where the stems were severed for sap collection at 30 and 60 min after initiating the flooding treatment (Fig. 3). The data reveal a rapid decline in Gln beginning minutes after initiating the flooding treatment. Within 20 min (or 0.40 cm^3 accumulated sap) Gln declined from 23.3 % (265 μmol dm^{-3}) to 9.6 % (157 μmol dm^{-3}) and subsequently to less than 2 % (46 μmol dm^{-3}) by 60 min

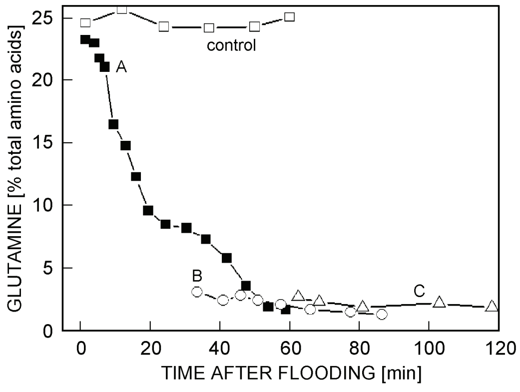

Fig. 3. Time course of changes in relative glutamine content in xylem sap of nodulated soybean plants after flooding of the root system. Plants were cultivated in vermiculite with N-free nutrient solution to stage R2. Root system flooded at 0 min (*curves A*, *B* and *C*) and stem cut for xylem sap collection at 0 min (*curve A*), at 30 min (*curve B*) and at 60 min (*curve C*). Sap collected in lots of 0.050 cm^3 at timed intervals (accumulated volume = number of data points × 0.05 cm^3). Data points are plotted at the mid-point of the time interval. Non-flooded control to *curve A*: only some of the 0.05 cm^3 samples were analyzed. *Curves A*, *B* and *C* each obtained from single plants, but similar results were obtained from 3 replicate experiments.

($0.75\ cm^3$ total accumulated sap). Although not shown, the other amino acids presented an increase proportional to the decrease in Gln. The control presented constant values for Gln (24.4 ± 0.24 %). The fact that Gln did not decline in the control, and that curves B and C revealed similar low values to those seen towards the end of curve A, eliminates any possibility that the phenomenon is an artefact related to the cutting of the stem. Although insufficient material was available for total amino acid analysis, estimates from the recovery of amino acids after HPLC analysis revealed a gradual rise in concentration over the experimental period (that could, at least in part, be explained by the diminished flow rate after 20 min).

General considerations: Both waterlogging and transfer of nodulated soybean to aerated hydroponics lead to a rapid and specific decline in xylem Gln. That this decrease in Gln is related to impaired N_2 fixation is supported by data presented here where similar changes in Gln of the xylem bleeding sap were observed under other conditions where clear changes in nitrogenase activity are known to occur. Furthermore, the time-course of the decline in xylem Gln in plants transferred to hydroponics correlated very closely with xylem ureide content, which correlated with nitrogenase activity (McClure *et al.* 1980, McNeil and LaRue 1984, Herridge and Peoples 1990, Herridge *et al.* 1990). Other evidence for the association between xylem Gln and N_2 fixation may be found in the literature. Higher ratios of Gln/Asn were recorded for symbiotic compared to non-symbiotic plants of a number of amide- (Peoples *et al.* 1987) and ureide-exporting legumes (soybean included) (Pate *et al.* 1980). Reduced nodulation and ureide export after exposure of soybean to aluminium solution was also associated with lower xylem Gln (Silva and Sodek 1997). The work of Atkins *et al.* (1984) with nodulated cowpea plants exposed to $Ar:O_2$ for 3 d revealed a transient increase in xylem Gln following the return of the plants to air. This was attributed to the immediate recovery of Gln formation in view of the stability of nitrogenase and glutamine synthetase activities of the nodule during the 3-d treatment, in conjunction with lower activities of unstable Gln-utilizing enzymes. A decline in xylem Gln of nodulated cowpea was observed following treatment with allopurinol (Atkins *et al.* 1988) which, besides its known inhibitory effect on ureide synthesis, was found to reduce nitrogenase activity, partly through an oxygen limitation. There is, therefore, a large body of evidence that xylem Gln is closely related to N_2 fixation in symbiotic legumes. This supports the conclusion that the decline in xylem Gln in waterlogged nodulated soybean is indeed related to impaired N_2 fixation.

That oxygen deficiency underlies the waterlogging effect on N_2 fixation is supported by the data of the experiment with pure N_2, as well as by other studies in the literature (Minchin and Pate 1975, Minchin *et al.* 1978, Becanamwo and Purcell 1999), although in these cases the effects were long-term and involved a reduction in enzyme levels. In our short-term study, where the nodulated root system was exposed to pure N_2, xylem Gln presented the usual sharp decline but in this case variations in Ala, Gaba, Asp and Ser were also observed. The changes in these amino acids can be attributed to the anoxic conditions which would affect root respiration as well as that of the nodule. Previous work with soybean has revealed that under oxygen deficiency induced by waterlogging, large quantities of Ala together with some Gaba and Ser are produced by the roots and transported in the xylem, apparently formed, at least in part, from Asp (De Sousa and Sodek 2003). Similarly, the changes in Ala, Gaba and Ser seen here in the xylem of plants exposed to N_2 were accompanied by corresponding changes in the root and/or nodule tissue.

Nevertheless, it is clear that in the case of Gln the decline in the xylem is not accompanied by such a change in either the root or nodule tissue. A similar situation exists for the large changes in xylem Asp and Asn seen under certain conditions (Lima and Sodek 2003). It appears therefore that the xylem sap amino acid composition can reflect dynamic aspects of some of the metabolic processes taking place in the root system that do not lead to detectable changes in amino acid composition in the tissues of origin.

The timing of the decrease in xylem Gln following waterlogging was extremely rapid, being detectable within minutes of the simultaneous cutting of the stem for sap collection and initiating the flooding treatment. This fact itself suggests that most of the xylem Gln arises directly from N_2 fixation, rather than by some recycling phenomenon involving the shoot. The removal of the shoot at the same time that the root system was flooded rules out such a possibility. This is an important point in view of the potential contribution to xylem transport of amino acids supplied via phloem from the shoot and recycled through the root (Pate *et al.* 1981, Parsons and Baker 1996).

In conclusion, our data suggest that xylem Gln of nodulated soybean arises directly from N_2 fixation and is a useful indicator of N_2 fixation activity in symbiotic plants. On this basis, waterlogging appears to impose an immediate restriction of N_2 fixation activity, apparently due to oxygen limitation.

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