Plant Physiol. (1996) 111: 109–113

Water Deficit-Induced Changes in Concentrations in Proline and Some Other Amino Acids in the Phloem Sap of Alfalfa

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Changes in amino acid composition of alfalfa (*Medicago sativa* L.) phloem sap were studied in response to a water deficit. Sap was collected by stylectomy. As the leaf water potential (Ψ) decreased from −0.4 to −2.0 MPa, there was a significant increase of the total amino acid concentration, due to that of some amino acids: proline, valine, isoleucine, leucine, glutamic acid, aspartic acid, and threonine. Asparagine concentration, which is the main amino acid assayed in the phloem sap of alfalfa (it accounts for 70% of the total content), did not vary with the plant water status. The other amino acid concentrations remained stable as Ψ varied; in particular, γ-amino butyric acid concentration remained unchanged, whereas it varied in response to wounding. The more striking change in the sieve tubes was the accumulation of proline, which was observed below a Ψ threshold value of about −0.9 MPa (concentration ×60 for a decrease of Ψ from −0.9 to −2.0 MPa). The role of such changes in phloem sap amino acid concentration in osmotic adjustment of growing tissues is discussed.

Physiological and metabolic responses of plants to water deficits have been well documented (Bradford and Hsiao, 1982; Hanson and Hitz, 1982, and refs. therein). In particular, changes in the concentration and the composition of the N-soluble fraction in response to water deficits have been demonstrated in a wide range of species, and accumulation of free Pro is evident in various tissues of plants grown under stress, such as intact plants (Singh et al., 1973), detached leaves (Stewart, 1972), mature leaves (Lawlor and Fock, 1977), growing leaves (Riazi et al., 1985), and roots (Navari-Izzo et al., 1990). However, the effect of a water deficit on the forms of transport of soluble N in the phloem sap is not well documented. Variations of phloem sap composition have been studied in response to fluctuations of various factors such as light (Hayashi and Chino, 1986) and season (Weibull, 1987). But only one work (as far as we are aware) deals with modifications of the phloem sap composition of water-stressed plants (Tully and Hanson, 1979). These authors demonstrated that the amino acid composition of phloem sap, collected from water-stressed barley leaves using the EDTA method, was not drastically modified by water deficits, although this stress led to an increase of Ser and the appearance of small amounts of Pro and Gaba. It was concluded that this low level of Pro supports the idea that "Pro does not assume a major role in N transport during water stress." In this paper, we report the impact of a water deficit on the free amino acid composition of alfalfa (*Medicago sativa* L.) phloem sap, using the stylectomy method, which is a more appropriate collecting technique for fine biochemical analysis (Girousse et al., 1991).

MATERIALS AND METHODS

Plant Material

Tip cuttings of alfalfa (*Medicago sativa* L.) were made from a clonal culture of a hybrid of Flemish origin, maintained in the nursery of the Institut National de la Recherche Agronomique (Lusignan, France). Fragments of stems were transplanted in sterilized sand in vats placed in a greenhouse. When the root system was well developed, cuttings were transferred into 1-L pots and filled with a mixture of sandy soil and compost (equal volumes). Until their use, plants were maintained in the greenhouse under natural light. A few weeks before the beginning of the experiments, 6-month-old plants were cut in order to homogenize regrowth, and were then transferred to an environmental chamber providing a 16-h photoperiod, PPFD of 200 μmol m^{-2} s^{-1} (Cool White and Grolux fluorescent tubes [Sylvania]), 20/18°C day/night temperatures, and 50 to 70% RH. Plants were used at a vegetative stage (defined by five to eight internodes in the stem) and were nitrogen-fixing at this time. Half of the plants were watered daily ad libitum with tap water, without nutrient solution; the overflow was removed. The other half was subjected to a progressive watering reduced by half each day, until total withholding after 5 d. Sap was collected from nonstressed plants and from plants subjected to various durations of water deficit (see legend for Fig. 1).

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Abbreviations: Gaba, γ-amino butyric acid; Ψ, leaf water potential.

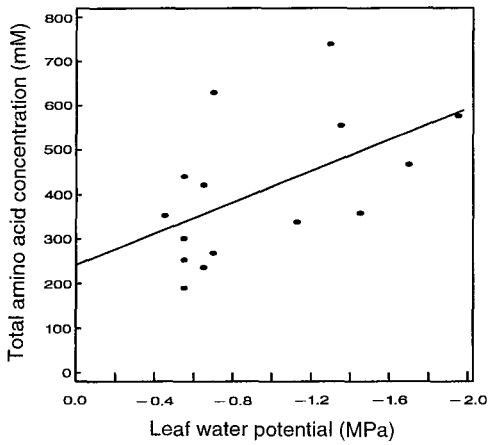

Figure 1. Total amino acid concentration of alfalfa phloem sap in relation to Ψ. Each point represents an individual sample and the full line represents the linear regression equation fitted to the plotted points ($F = 5.27$; $P > F = 0.039$; $df = 14$; $r = 0.54$). Ψ values of about −0.5 MPa were measured on daily-watered plants. Ψ values equal or less than −0.6 MPa were measured on plants submitted to various durations of water deficit (between 1st and 5th d, watering was reduced by one-half each day; between 6th and 15th d, total withholding), but Ψ values were not tightly correlated with the duration of the water deficit ($r = -0.54$).

Insects for Stylectomy

The stock culture was derived from an alfalfa field population by the pea aphid *Acyrthosiphon pisum* Harris, green form, from Lusignan, France. To decrease aphid variability, a clone of *A. pisum* was isolated from the stock culture. It was maintained on alfalfa (cv Milfeuil) in ventilated cages in a controlled chamber (20°C, 50% RH, 16-h light/8-h dark cycles). Apterous adults were preferentially taken from the clone culture for stylectomy (supplemented by fourth instar larvae only when necessary).

Sap Collection

Sap was collected by stylectomy using radio frequency microcautery (Unwin, 1978). The stylectomy apparatus is described elsewhere (Girousse et al., 1991). About 10 aphids per plant were allowed to settle in the upper part of the stems a few hours before the beginning of the collection in order to let the aphids choose their feeding site. The conditions of sap collection are detailed by Girousse et al. (1991). Sap collection always took place at the same time (between the 4th and 9th h of the beginning of the photoperiod) to minimize the variability linked to diurnal fluctuations of sap composition. Experiments were conducted in an environmental chamber (20°C; RH was maintained as high as possible to minimize evaporation and was ≥97%). Phloem exudates were collected as quickly as possible in microcapillaries. The volume of sap was evaluated by weighing, and the sap was then frozen before biochemical analysis.

Ψ Measurements

If stylectomy was successful and phloem sap was collected, the Ψ of expanded leaves taken from the nearest point of sap collection was measured with a pressure chamber (model 650, PMS Instruments, Corvallis, OR) (Scholander et al., 1965). If stylectomy was a failure, plants were put into the experimental device again and submitted to the same water treatment as before. The amino acid composition of phloem sap was analyzed over a range of Ψ from −0.4 MPa (nonstressed plants) to −2.0 MPa (highly stressed plants).

Amino Acid Analysis

Free amino acids were assayed on an autoanalyzer (ion-exchange ninhydrin) (Liquimat III, Kontron, Montigny le Bretonneux, France). The free amino acid concentrations were measured with norvaline as internal standard.

Statistical Analysis

Taking into account the variability of the water status of the plants used for sap collection, our results were treated by linear regression analysis using the Proc Glm procedure of SAS/STAT software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Stylectomy is a reliable technique for fine biochemical analysis of phloem sap composition, but it is a meticulous and fastidious technique to use. During this experiment, of 196 aimed aphid stylets (stylets for which the electrical signal of the microcautery device has been pulsed), 119 were successfully severed and only 59 were exuding stylets. To minimize the error arising from weighing, we kept only those sap samples with a volume above 50 nL. Thus, only 15 samples were available for amino acid analysis. The reasons for such difficulties are explained elsewhere (Girousse et al., 1991).

The total amino acid concentration of the phloem sap varied by a factor of 3 even for the same or almost the same value of Ψ. Such a variability is not due to evaporation that could occur during exudation. First, RH was maintained as high as possible (RH ≥ 97%). Second, there is no relation between total amino acid concentration and exudation rate of the phloem sap. This variability is probably related, at least in part, to structural features. The conducting system of the young internodes where aphids were settled is composed of bundles connected with donor leaves from various levels and more or less able to supply the upper growing organs. In spite of this variability, it appeared that the amino acid concentration increased (×1.8) significantly at a 5% level when Ψ decreased from −0.5 to −2.0 MPa ($F = 5.27$, $P > F = 0.039$) (Fig. 1). This result is similar to that observed in various plant organs, and the increase in total amino acid content is of the order of magnitude (×2) of that measured in tomato cell cultures with 20% PEG (Ψ= −1.6 MPa) (Handa et al., 1983).

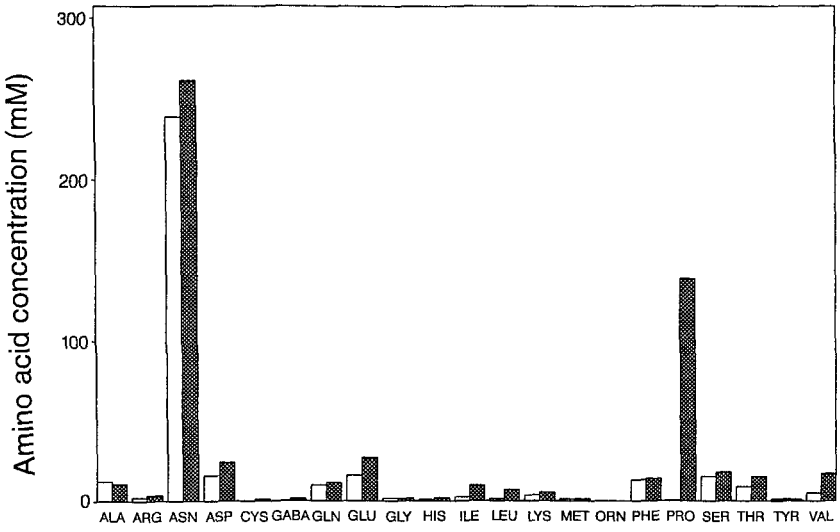

Figure 2. The amino acid composition of alfalfa phloem sap and their concentrations in a non-stressed plant (Ψ = −0.5 MPa, empty vertical bars) and a water-stressed plant (Ψ = −2.0 MPa, cross-hatched vertical bars).

The response of each amino acid to the variations of Ψ was different. An example of amino acid composition collected from two plants with extreme Ψ is given in Figure 2. Pro, which is in very low amounts in the phloem sap of the daily-watered plants, is a major amino acid, just after Asn, in the phloem sap of water-stressed plants. Two groups of amino acids, however, could be distinguished (Table I). In the first group (Pro, Val, Ile, Leu, Glu, Asp, and Thr in descending order), the amino acid concentration increased as Ψ decreased; in the second group (Ala, Arg, Asn, Gaba, Gln, Gly, His, Lys, Met, Orn, Phe, and Tyr), the amino acid concentration remained stable. It must be emphasized that the concentration of Asn, which is the major amino acid present in the phloem sap of alfalfa (it accounts for 70% of the total content [Girousse et al., 1991]), was not affected by a water deficit (Fig. 3A). These results are in accordance with those obtained in tomato cell cultures containing 25% PEG (Ψ= −2.2 MPa), where a noticeable increase of concentration in Pro, Leu, Val, Thr, and Ile (in descending order) was observed (Rhodes et al., 1986).

Table I. *Linear regression estimates of the analysis between the various amino acid concentrations and* Ψ

F, Value of *F* test; P > F, probability of observing a linear function of the parameters significantly different from 0; r^2, determination coefficient.

Amino Acid	*F*	P > *F*	r^2
First group P < 0.05			
Pro	107.24	0.000	0.89
Val	26.95	0.000	0.67
Ile	25.25	0.000	0.66
Leu	18.46	0.000	0.59
Glu	6.89	0.021	0.35
Asp	4.90	0.045	0.27
Thr	4.75	0.048	0.27
Second group P > 0.05			
Asn	0.39	0.544	0.29
Ser	2.03	0.178	0.14
Gln	0.10	0.753	0.01
Gly	0.02	0.884	0.00
Ala	0.07	0.802	0.01
Gaba	2.68	0.126	0.17
Met	0.04	0.845	0.00
Tyr	0.50	0.490	0.04
Phe	3.69	0.077	0.22
Orn	0.32	0.580	0.02
Lys	1.31	0.273	0.09
His	1.00	0.337	0.07
Arg	0.30	0.594	0.02

In the phloem sap of alfalfa under our conditions, no change in Gaba concentration occurred, in contrast to some water-stressed tissues where Gaba content increased in response to water deficit, such as cotton leaves (Hanower and Brzozowska, 1975), apex and leaves of wheat (Munns et al., 1979), and water-stressed barley leaves (Tully and Hanson, 1979). Yet, it has been demonstrated that Gaba concentration increased (×7) in the phloem sap collected from watered, excised leaves of alfalfa plants (Girousse et al., 1991). Consequently, additional experiments were made to study the effect of wounding on the phloem sap composition, not from isolated organs but from the plant itself. The wounding consisted of apex decapitation. Gaba concentration of alfalfa phloem sap increased from 0.66 ± 0.51 to 4.6 ± 2.7 mM (mean ± SE, n = 6) within 24 h after the excision of the shoot apex, whereas the Pro concentration remained unchanged (0.82 ± 0.91 for controls, 0.71 ± 0.80 mM for wounded plants; mean ± SE, n = 6). Thus, we suggest that an increase of Gaba concentration in the phloem sap is rather specific of wounding in alfalfa.

The most striking response to a water deficit was that of Pro. At the most negative Ψ value (−2.0 MPa), the Pro concentration of the phloem sap reached about 60 times its level in non-water-stressed plants. The curve given by a nonlinear segmented model relating Pro concentration to Ψ indicated a threshold value of about −0.9 MPa (Fig. 3B). A similar threshold value is obtained when considering the Pro:Asn ratio (a way to reduce residual variability) as a function of Ψ (Fig. 3C). It is interesting that the threshold value is similar to that measured in tomato cell cultures (−1.1 MPa) below which a marked increase of the concentration in Pro was observed (Handa et al., 1986). It has been

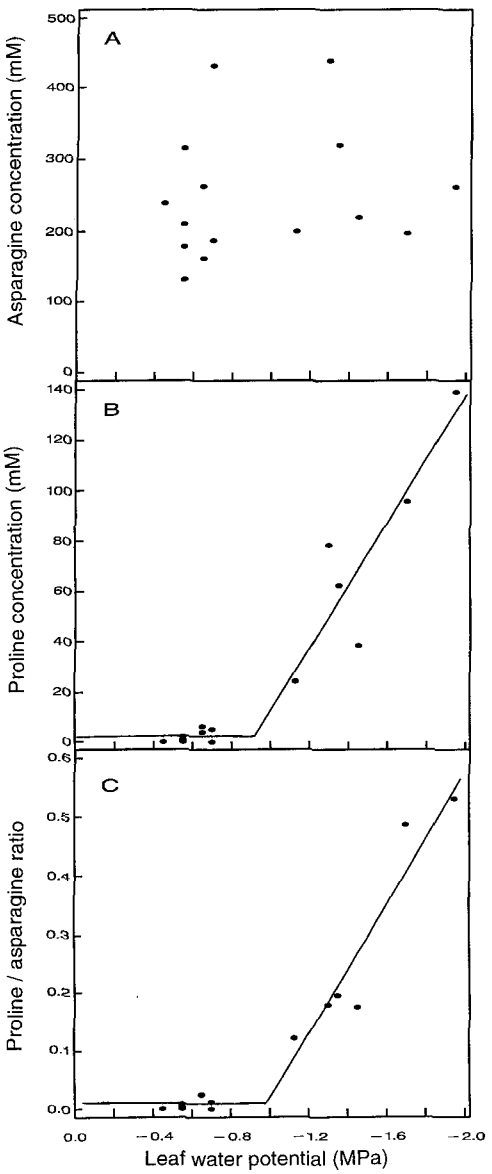

Figure 3. Relationship between individual amino acid concentration of alfalfa phloem sap and leaf water potential. A, Asn concentration; B, Pro concentration; C, Pro:Asn ratio. Each point represents an individual sample. The full line represents a nonlinear-segmented adjustment of the plotted point.

known for a long time that Pro accumulates in the detached dehydrated leaves of grass and leguminous species as a consequence of a profound disturbance of amino acid and protein metabolism (Barnett and Naylor, 1966; Routley, 1966). The increase in Pro concentration in response to a water deficit has been well documented in various tissues or organs (Stewart, 1972; Singh et al., 1973; Riazi et al., 1985; Handa et al., 1986), but until now not in the phloem. This lack of information also concerns the xylem sap. In this respect, it has been demonstrated that water stress induces a strong increase of Pro content both in leaves (Routley, 1966; Raggi, 1994) and nodules (Kohl et al., 1991; Irigoyen et al., 1992) in legumes. Moreover, it is well known that most of the N-organic compounds translocated from nodules to the leaves via the xylem sap are then allocated through the phloem to the growing organs (Pate, 1986, and refs. therein). Thus, it would be of particular interest to study the Pro content of xylem sap during water stress to determine the respective part of nodule and leaf metabolisms in the striking increase of Pro concentration in phloem sap.

In terms of functional significance of Pro accumulation, the most common hypothesis considers Pro as an osmoticum and a protective agent for cytosolic enzymes and membrane structures (Lahrer et al., 1993). Pro, especially, seems to be a major solute involved in the osmotic adjustment in meristematic tissues in soybean seedlings (Meyer and Boyer, 1981) and in maize primary root (Voetberg and Sharp, 1991). It has been suggested that in growing organs osmoregulation depends entirely on the import of osmotically active solutes (Munns et al., 1979; Sharp et al., 1990). In this regard, our results suggest that Pro could be loaded in leaves and transported to meristematic tissues in order to contribute to osmotic adjustments in growing tissues.

In conclusion, the major change in the forms of N transport in phloem sap of alfalfa in response to a decrease of water potential from −1.0 to −2.0 MPa is a dramatic increase of Pro concentration. Although this increase is a common observation in mature leaf tissues or growing tissues, to our knowledge it is the first time that it has been measured in the sieve tubes. Can our results be generalized to other plant species? Tully and Hanson (1979) obtained contrasting results concerning both Pro and Gaba in barley phloem sap, but they used the EDTA collecting method that may induce some artifacts (Girousse et al., 1991). Our discussion stresses the necessity to investigate this point with a larger number of plant species and to extend the analysis to xylem sap.

ACKNOWLEDGMENT

We are grateful to Dr. J. Dainty for critical reading of the manuscript.

Received October 27, 1995; accepted January 30, 1996.

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