Proline Accumulation in Water-stressed Barley Leaves in Relation to Translocation and the Nitrogen Budget

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ABSTRACT

Mobilization of N from leaves of barley (Hordeum vulgare L.) during water stress, and the role of proline as a mobilized species, were examined in plants at the three-leaf stage. The plants responded to water stress by withdrawing about 25% of the total reduced N from the leaf blades via phloem translocation. Most of this N loss was during the first 2 days while translocation of 14C-photosynthate out of the stressed blade still remained active. Free proline accumulation in the blade was initially slow, and became more rapid during the 2nd day of stress. Although a major free amino acid, proline accounted for only about 5% of the total N (soluble + insoluble) retained in severely stressed blades. When the translocation pathway in water-stressed leaves was interrupted just below the blade by a heat girdle, a cold jacket, or by blade excision, N loss from the blade was prevented and proline began to accumulate rapidly on 1st day of stress. Little free proline accumulated in the blades until the ability to translocate was lost. Proline was, however, probably not a major species of N translocated during stress, because proline N accumulation in heat-girdled stressed leaves was five times slower than the rate of total N export from intact blades.

The onset of water deficit alters the over-all N budget of crops through effects on both N transport and N assimilation. Nitrate delivery via the xylem to the shoot is depressed (17), and NO3− reduction in the leaves declines (12, 17). Although phloem transport of assimilated N to N sinks is eventually reduced (4, 25), it is probable that phloem transport is sufficiently resistant to water stress to permit appreciable salvage of both N and carbon from wilted leaves that are dying as a consequence of desiccation (3, 6, 25).

Within the leaves of many plants subjected to moderate or severe water stress, one striking change in N metabolism is the accumulation of free proline as a result of net de novo synthesis from glutamic acid (e.g. 1, 2). It has been proposed that this accumulation is a metabolic adaptation which confers survival value, perhaps acting as a (phloem-mobile) reserve of N accessible for use upon stress relief (e.g. 1, 18, 21). This possibility cannot be evaluated without knowing: (a) how much N is exported from stressed leaves to other parts of the plant; and (b) whether proline accumulation and export are significant terms in the total N budget of a leaf during stress. Much of the published work on proline accumulation during water stress has been with detached leaves, which clearly cannot export N. Proline accumulation has also been investigated in intact plants. In only one study was the question of translocation addressed: Singh et al. (19, 20) suggested, on the basis of surgical experiments, that in wilted barley seedlings some of the proline synthesized in the leaves is translocated to the roots. Their data did not consider proline accumulation in leaves in the more general context of the over-all N status of stressed plants, and do not allow estimates of the relative rates of proline export and accumulation during stress.

In this paper we report on the mobilization of N from water-stressed barley leaves in relation to free proline accumulation, and assess the possible contribution of proline to N translocation during stress. We used two barley cultivars: Proctor, a drought-sensitive two-row cultivar; and Excelsior, a more drought-resistant six-row type. The effect of water stress on proline accumulation in these two cultivars has been described in detail (7).

MATERIALS AND METHODS

Plant Material and Growth Conditions. Seeds of Proctor CII11806 and Excelsior CII11509 barley (Hordeum vulgare L.) were obtained from the USDA small grains collection, Beltsville, Md., and were multiplied in spring field plots at Michigan State University. A sample of Proctor seeds (company reference no. 2174) was also provided by RHM Agriculture, High Wycombe, U.K.

Plants were grown in Perlite in plastic pots (7 × 12 cm) for 17 to 19 days after sowing (to the three-leaf stage) in a growth chamber under a regime of 16-h days, day/night temperature 22/16 C; day/night RH 70/85%, total irradiance 3.8 mw cm−2 (80% from fluorescent lamps, 20% from incandescent lamps), and were watered on alternate days with half-strength Hoagland solution. Prior to the experiments, the plants were thinned to four per pot.

Water Stress and Leaf Water Potential. Plants were osmotically stressed with PEG solutions as previously described (7). Pots were flooded with three successive 100-ml doses of a −19 bar PEG (Union Carbide) solution (400 g PEG 6000 plus 1,000 ml half-strength Hoagland) on the 1st day of stress, and with additional 100-ml doses on subsequent days.

Leaf samples for Ψsat measurement1 were 13-mm lengths sliced with a sharp razor from the midpoint of the second leaf blade. A Wescor model HR-33[T] dew point microvoltmeter equipped with model C-52 sample chambers was used to estimate Ψsat, as described by Nelsen et al. (14), using a 2-h equilibration period.

Dry Weight, Kjeldahl N, and Nitrate N. Plants were divided into second leaf blades, first and third leaf blades, leaf sheaths with enclosed shoot apex and expanding leaves (referred to as “culm” in the text), and roots. These parts were frozen in liquid N2, dried overnight at 60 C, and weighed. Kjeldahl N was determined by the nonreductive technique described by Steyermark (22), using a digestion mix of 1.3 g K2SO4, 32 mg HgO, and 2 ml H2SO4 per sample. After boiling for 4 h, the digests were brought to 100 ml with H2O. A 200- or 500-ml aliquot of each diluted digest was brought to 3.0 ml with 0.2 M NaOH; 100 μl of Nessler’s reagent was added, and the A550 was measured after 10 min.

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2 Abbreviations: Ψsat: leaf water potential; MCW: methanol-chloroform-water.
assay was calibrated with NH₄Cl, glycine, and a standardized preparation of wheat seed protein.

Even though a specific reductive step was not used, it was found that some fraction of the endogenous NO₃⁻ in the tissue samples was being recovered as NH₃ after digestion. To determine the significance of this effect in leaf tissue, samples of dry leaf powder were treated with various amounts of KNO₃ and digested. The endogenous NO₃⁻ level in the untreated leaf sample was determined by the method of Lowe and Hamilton (11). The results are shown in Figure 1, along with the curve obtained from digesting the KNO₃ standards alone with a reductive technique (22). Note that the curve for leaf digests plus KNO₃ has a slope of 0.45 that of the reductive method with KNO₃ alone, which means that 45% of the NO₃⁻ in a leaf sample was recovered as NH₃ in a standard digest. The bar in Figure 1 indicates the extreme range of endogenous NO₃⁻ concentrations encountered in this study. A trend was found toward a slight increase in NO₃⁻ as stress continued (Table 1), which would tend to increase the recovery of NH₃ from the digests. Because the effect is small compared to the larger changes caused by translocation (see later), no correction was attempted.

**Tissue Extraction and Proline and Amino Acid Determinations.** In some experiments, tissue was extracted by the simplified CMC technique described by Hanson et al. (7). In others, tissue was extracted by placing it in 5.0 ml H₂O in sealed tubes and heating at 100 C for 30 min. Proline was estimated by the acid ninhydrin method of Troll and Lindsley (23), using toluene instead of benzene for the color extraction (7) and omitting the Permutit treatment. Amino acids were estimated with ninhydrin according to Rosen (16), using a glycine standard.

Ammonia was recovered from extracts by vacuum distillation at 55 C, and amide N by steam distillation at 100 C, as described by Varner et al. (24). The NH₄⁺ in the boric acid trap was assayed with Nessler's reagent.

**Heat-girdling, Cold-jacketing, and Excisions.** The sheaths of barley second leaves were heat-girdled by gently separating the sheaths from the expanding third and fourth leaves, and directing a stream of hot air to a 2-cm zone at the top of the sheaths, just below the ligules. Culms were cold-jacketed by placing them in close contact with a length of brass tubing (6.5 × 0.7 cm) through which refrigerated water was passed. The temperature of the culms as measured with a thermocouple was 6 C. Cooling was begun 5 h after the initial PEG application.

Plants to be used in surgical experiments were first treated with PEG and placed in the illuminated growth chamber for 5 h to cause wilting. Various organs were separated and kept at constant water status by placing on moist paper toweling, covering with plastic wrap, and placing in the growth chamber under the standard day/night regime. Excised organs comprised leaf blades, leaf blades with culm attached, or culm alone. Excised roots and intact plants were kept in the pots. Water status of the-excised parts remained essentially unchanged during the 24-h treatment period, as judged by absence of change in fresh weight.

**14CO2 Fixation and Translocation.** Gaseous 14CO₂ was generated by mixing 2 µl of NaH14CO₃ solution (4 µCi, 60 mCi/mmol, Amersham) with a drop of 50% lactic acid in a 5-ml syringe barrel with the plunger at the 4-ml mark. One ml (1 µCi) of the 14CO₂ was injected into each of four leaf-feeding chambers. The chambers were glass tubes (1.5 × 10.5 cm) with a serum cap at one end and a split stopper at the other. Into each chamber was inserted the end 10 cm of intact second leaf blades attached to plants under varying degrees of stress. The 14CO₂ was administered for 15 min with illumination. The leaf ends were then removed from the chambers, and the plants were returned to the illuminated growth chamber. Whole plants were harvested 5 h after label application and divided into the 10-cm ped tips, and all other organs combined. The parts were frozen in liquid N₂, dried to 60 C, and combusted in a Packard model 306 Tri-Carb sample oxidizer. The amount of 14CO₂ fixation was taken as the total 14C recovered from the plant, and translocation activity was assessed from the proportion of ¹⁴C that was recovered from the unfed parts of the plant.

**RESULTS**

**N Redistribution in Proctor during Stress.** The changes in Kjeldahl N of the various organs of intact Proctor barley plants during 4 days of stress are presented in Figure 2A. The pooled leaf blades (leaves 1–3) lost about 28% (1 mg) of their total N. Total N in the roots remained essentially constant, while the N content of the culms increased slightly. As a net result, each plant as a whole lost 0.86 mg of total N. The loss of N was not through the gas phase, because when a stream of air was passed continuously over a similar group of stressed plants and bubbled through an HCl trap to collect volatile N compounds, the trap contained none. We (limit of detection 2 ng, or 0.027% of total N). The loss of N in the culms is essentially unchanged during stress (Table 1), since the trend (in a similar experiment) was for the NO₃⁻ in the second leaf blade to increase slightly during stress (Table 1), with the increase being small compared to the change in Kjeldahl N.

Changes in total Kjeldahl N and various soluble N pools in second leaf blades of Proctor during stress are shown in Figure 2B. Although there was a net loss in total N from the second leaf blade of 24% (0.41 mg), the pool of proline N increased significantly. After 4 days of stress, proline N constituted 5.3% of the total N. The α-amino N content (corrected to exclude proline) increased slightly over the stress period, reaching 3.8% of the total N at day 4. Amide N in second leaf blades rose slightly from 7 µg at day 0 to 12 µg at day 4, whereas NH₄⁺ N remained at less than 4 µg throughout the stress period.

The Kjeldahl N lost from the leaves was not recovered from the rest of the plant, except for perhaps a slight increase in the culm (Fig. 2A). The loss of N from the plant presumably occurred as secretion from the roots into the potting medium. Liberation of amino acids from the roots of drought-stressed barley and several other plants has already been demonstrated (10).

**Effect of Stress on 14CO2 Fixation and Translocation in Proctor.**
Fig. 2. Kjeldahl N in various organs of Proctor plants (A) and distribution of N among various fractions of Proctor second leaf blades (B) during 4 days of PEG stress. (O—O): Unstressed controls. In B, α-amino N does not include proline. Also, note the break and change in scale in B; both proline N and α-amino N contents are very small compared to total N. Free NH₃ in the second leaf blade remained essentially constant at <4 μg N over 4 days, and amide N increased from 7 μg at day 0 to 12 μg at day 4 (not shown). Kjeldahl N content remained essentially constant after day 4 of stress, declining to 1.19 ± 0.05 mg by day 9. Data points for Kjeldahl digests in A and B are means of eight replicates; other points in B are means of four replicates.

Table 1. Nitrate Content of Proctor Second Leaf Blades During PEG Stress

<table>
<thead>
<tr>
<th>Days of Stress</th>
<th>mg NO₃⁻ N/Blade</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.21</td>
</tr>
<tr>
<td>1</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>LSD, P &lt; 5%</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The results of the ¹⁴CO₂ feeding and translocation experiment with Proctor are shown in Figure 3, along with Pₛₑ.XPath and proline levels measured in comparable second leaf blades. Relative to unstressed plants (day 0), ¹⁴CO₂ fixation was inhibited 46% by day 1 and 88% by day 2 of stress. The proportion of total ¹⁴C fixed that was translocated from the fed leaf tip, however, remained essentially unchanged at day 1, but had fallen sharply by day 2. The proline accumulated in the blade during the 1st day of stress was only 0.07 μmol, but then rose to 4.00 μmol during the next 3 days, subsequent to the inhibition of translocation.

Effect of Excisions, Cold Jacket, and Heat Girdle. The leaf blades, culms, and roots of stressed Excelsior plants kept intact during the treatment period contained roughly equal amounts of proline (Table II). When these organs were excised from plants 5 h after stress initiation and kept isolated for an additional 24 h, the leaves accumulated over five times their usual amount of proline, while the culms and roots accumulated much less than those of stressed intact plants. The culm contained a high level of proline only when attached to the leaf blades, and the roots only when attached to the culm with the leaf blades. Intact unstressed plants and their isolated parts accumulated only very little proline.

The effect of a cold jacket was similar to that of leaf excision (Table III). The cold jacket depressed proline accumulation in the culm and roots, and enhanced accumulation in the leaf blades.

Fig. 3. Stress inhibition of ¹⁴CO₂ assimilation and translocation in Proctor barley. Individual second leaf blades were offered 1.0 μCi of ¹⁴CO₂ as described in the text. Fixation and translocation of label were assessed 5 h later. A: total ¹⁴C in all plant parts after 5 h. B: per cent of total ¹⁴C outside fed area after 5 h. C and D: proline content and Pₛₑ.XPath of second leaf blades from a parallel experiment. All data points are means of four replicates.

Results of excisions and cold-jacketing performed on Proctor barley were qualitatively the same as with Excelsior.

Figure 4 shows the effect of a heat girdle on proline levels and Pₛₑ.XPath of Proctor second leaf blades. Although the Pₛₑ.XPath values of both stressed-girdled and stressed-ungirdled plants were very
**Table II.** Effect of Various Excisions on Proline Distribution in Excelsior Barley

Plants were treated with PEG and allowed to wilt for 5 h after which time excisions were performed. Excised organs were held at constant water status for 24 h and were then assayed for proline. The three excision treatments were: (a) keeping plants intact; (b) separating into three parts; and (c) keeping leaves attached to culms but excising roots. Results are means of four replicates.

<table>
<thead>
<tr>
<th>umoles Proline/organ</th>
<th>(a) Intact</th>
<th>(b) 3 Parts</th>
<th>(c) Leaf-Culms</th>
<th>LSD, P &lt; 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>culm</td>
<td>0.54</td>
<td>3.0</td>
<td>2.7</td>
<td>1.59</td>
</tr>
<tr>
<td>roots</td>
<td>0.41</td>
<td>0.14</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>unstressed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>culm</td>
<td>0.15</td>
<td>0.27</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>roots</td>
<td>0.060</td>
<td>0.065</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

**Table III.** Effect of Cold Jacket on Proline Distribution in Excelsior Barley

Plants were treated with PEG and allowed to wilt for 5 h after which time cooling jackets were attached to culms. Control plants were manipulated similarly, but no coolant was circulated. Plants were harvested 24 h later, and organs extracted and assayed for proline. Temperature of cooled culms was 6°C (thermocouple). Results are means of four replicates.

<table>
<thead>
<tr>
<th>umoles Proline/organ</th>
<th>Stressed Uncooled</th>
<th>Stressed Cooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>leaves</td>
<td>0.65</td>
<td>2.17*</td>
</tr>
<tr>
<td>Culm</td>
<td>1.18</td>
<td>0.57</td>
</tr>
<tr>
<td>Roots</td>
<td>0.78</td>
<td>0.47*</td>
</tr>
<tr>
<td>Total</td>
<td>2.41</td>
<td>3.21 n.s.</td>
</tr>
</tbody>
</table>

*p < 5%

n.s. not significant

**Table IV.** Effect of Heat Girdle on N Loss from Excelsior Second Leaf Blades

Excelsior leaf sheaths were heat girdled at a point just below the blade immediately before the plants were PEG-stressed. Second leaf blades were harvested and Kjeldahl N determined after 3 days of stress. Control blades were taken immediately before stress initiation (day 0). Results are means of eight replicates.

<table>
<thead>
<tr>
<th>Kjeldahl N (mg/blade)</th>
<th>Dry Weight (mg/blade)</th>
<th>△leaf (bars)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Control</td>
<td>1.28</td>
</tr>
<tr>
<td>Day 3 Girdled</td>
<td>1.24</td>
<td>33.4</td>
</tr>
<tr>
<td>Day 3 Uncooled</td>
<td>0.97</td>
<td>23.4</td>
</tr>
<tr>
<td>LSD, P &lt; 5%</td>
<td>0.19</td>
<td>3.7</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Most of the experiments described in this paper were conducted using both Proctor and Excelsior barley, with generally similar results. There were no differences in response that could not be ascribed to the faster decline of P₅₀ in Proctor, which has been reported previously (7).

**Nitrogen Mobilization from Stressed Leaves.** There was a significant remobilization of N from the leaf blades during stress (Fig. 2A), although far less of the N was salvaged from stressed leaf blades than from those senescing normally (e.g. 5). This loss of N was presumably through the phloem, because: (a) N losses through volatilization were ruled out; and (b) heat-girdled second leaf blades did not exhibit a stress-induced N loss (Table IV).

Little proline accumulated in the second leaf until after translocation of N out of the leaf had greatly slowed, i.e. after day 2 (Fig. 2B). Further, proline began to accumulate immediately after heat-girdling (Fig. 4). This correlation between the cessation of translocation and the start of rapid proline accumulation is further demonstrated in Figure 3, where translocation was assessed as movement of recent ¹⁴C-photosynthate. Rapid proline accumulation did not begin until translocation had markedly declined.

Using the data from the second leaf blade of Proctor (Fig. 2, Table IV, and ref. 7), a simplified comparison can be made of the N budgets of stressed and unstressed blades. This is shown schematically in Figure 5.

In unstressed plants 17 to 19 days old, the second leaf blades (Fig. 5A) were essentially in N balance insofar as no changes took place in their contents of NO₃⁻, NH₄⁺, Kjeldahl N, α-amino N, amide N, and proline N. The fluxes of NO₃⁻ into the second leaf
Proline Accumulation and Export from Stressed Leaves. The data of Table II extend the observations of Singh et al. (19) on proline accumulation in excised parts of barley seedlings; it appears that proline, or a proline precursor, originates in the leaf blades during stress and is transported to the sheaths and roots. This is supported by the results of the cold-jacketing and heat-girdling treatments (Table III and Fig. 4). Cold-jacketing slowed or inhibited translocation through the phloem in the sheaths, so that leaf blades accumulated more proline than usual and the culms and roots less, with little change per whole plant. Likewise, heat-girdling the sheath of the second leaf caused proline to accumulate sooner and more rapidly in the blade of this leaf, even though NO₃ was the same in both girdled and ungirdled leaf blades. Although these surgical and girdling experiments do not prove that proline itself is exported from stressed leaf blades, this is the simplest interpretation.

In Figure 5 proline accumulation is shown in relation to the other general changes in N metabolism that occur in attached second leaf blades during stress. At day 1 of stress, very little N (2.5 µg) was present as proline. Although by day 4 proline was a major form of soluble N, the total amount of proline N (67 µg) was small compared both to the Kjeldahl N remaining in the blade (1270 µg) and to the amount of N withdrawn from the blade (410 µg). Moreover, much of the free proline was at this stage present in the killed part of the blade (7). Quantitatively, proline would thus seem not to be an important N reserve within the stressed leaf, especially because the localization of a major part of it within killed tissue precludes its reuse upon relief of stress.

If proline is exported from the blades as part of the N remobilization process, an estimate of the rate of proline export can be made by assuming that its rate of removal from leaves during the first 2 days of stress is equal to the increased rate of proline accumulation caused by heat-girdling (Fig. 4). In this case, the rate of proline export = (6.0 - 1.2)/2 = 2.4 µmol/day, or 34 µg N/day. Since this is only 13% of the rate of N loss from the second blade at this time (= 260 µg N/day, Fig. 5B), proline appears to be only a relatively minor component of the N translocated from stressed leaves, despite the sharp rise in its contribution to the free amino acid pool as severe stress develops. Our results leave open the question of the forms in which N is moved from stressed leaves, and whether these are different from those exported from unstressed leaves in N balance.

Survival Value of Proline Accumulation during Water Stress. Proline is neither of major importance as a reserve of N after stress, nor is it a major translocated N species during stress. These conclusions alone cast doubt on (a) the possibility that proline accumulation confers significant survival value during water stress; and (b) attempts to use proline accumulation as a positive index of drought resistance (e.g. 18).

The results on proline translocation lead to another conclusion. A high rate of proline accumulation could arise either from a high rate of synthesis or a low rate of export. If, within a range of crop genotypes, a high rate of proline accumulation is associated with an early and fast loss of the capacity to translocate N (and presumably carbon) from drought-stressed leaves, rapid proline accumulation would seem more likely to be symptomatic of a deleterious response to water stress than to be an adaptive feature with survival value. We have reached a similar conclusion in a comparative study of proline accumulation in the contrasting barley varieties Proctor and Excelsior, and in the progeny of a Proctor × Excelsior cross (7, 8).

We therefore caution against the assumption that the capacity for proline accumulation is positively correlated with drought resistance.

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Fig. 5. Flow diagram for N in the second leaf blade of Proctor barley plants at the three-leaf stage. A: unstressed plants, 18 days after sowing; B: plants stressed for 1 day (NO₃ lowered by about 10 bars); C: plants stressed for 4 days (NO₃ lowered by >30 bars). Units are µg N. Heavy solid arrows indicate probable major paths of N flow, and light broken arrows indicate flows that are probably small. Light solid arrows designate flows of uncertain size. Double bars on arrows show processes known to be inhibited during water stress. Areas of the square pools of NO₃-N, free α-amino N, and free proline N are proportional to their N content. Values for α-amino N exclude proline N. Large boxes containing the various pools represent second leaf blades, which import NO₃⁻ from the xylem at the left, and export reduced N via the phloem at the right.
LITERATURE CITED