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EFFECT OF WATER STRESS ON PROLINE CATABOLISM IN TOBACCO LEAVES

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Abstract—Proline-[^{14}C] infiltrated into leaf disks of tobacco (*Nicotiana tabacum* cv BY-4) in the dark was converted to glutamic acid and then metabolized through the TCA cycle. A smaller amount of proline-[^{14}C] was metabolized when the leaf disks were wilted than when turgid. During a 6 hr period following rehydration, disks converted a larger amount of proline-[^{14}C] to oxidized products than when wilted, although the proline content of rehydrated disks had not declined. These results indicate that proline oxidation is inhibited by water stress.

INTRODUCTION

Water stress induces a characteristic increase in the level of free amino acids, especially proline and amides [1]. The accumulation of amides is attributed to the incorporation of free ammonia released by the deamination of amino acids, which were released by the degradation of protein during water stress [2]. Although several attempts have been made to explain the accumulation of free proline [1, 3, 4], the mechanism is not fully understood. Barnett and Naylor [5] and Morris *et al.* [6] have observed the increased conversion of glutamic acid-[^{14}C] to proline in wilted leaves as compared to turgid leaves of Bermuda grass and barley. They concluded that proline synthesis was stimulated under water stress. Other results [7] have suggested that proline oxidation was inhibited during water stress. However, experiments on proline oxidation are generally complicated by the presence of large pools of unradioactive proline in wilted tissues. This difficulty can be avoided by comparing the proline metabolism of leaves shortly after rehydration with that of wilted leaves.

Table 1. Proline-[^{14}C] metabolism during water stress

	Wilted	Turgid
CO_2	28.4*	54.3
Asp	1.2	1.0
Glu	2.9	0.7
Gln	trace	trace
γ-ABA†	0.3	0.2
Pro	23.4	3.8
Citric acid	8.6	11.7
Malic acid	23.8	1.2
Fumaric acid	1.9	0.2
Succinic acid	trace	0.2
Unknown	trace	0.2
Residue	10.1	26.5

Incubated for 24 hr in the dark.
* Expressed as percentage of total ^{14}C.
† γ-Aminobutyric acid.

RESULTS

Proline metabolism in wilted leaves

Proline-[^{14}C] was fed to wilted and turgid leaf disks. After 24 hr at room temperature, the leaves were wilted to 45% of the fr. wt (Table 1). Proline was converted to CO_2, soluble compounds and insoluble material. In the soluble fraction, measurable radioactivity was recovered in aspartic acid, glutamic acid, γ-aminobutyric acid, citric acid, malic acid, fumaric acid and succinic acid. All the compounds are closely related to the TCA cycle. In a short period (30 min) experiment, glutamic acid was a heavily labelled metabolite. These results indicated that proline was oxidized to glutamic acid which was subsequently metabolized through the TCA cycle.

In wilted leaves, the radioactivity was detected in similar compounds to those observed in turgid leaves. It seemed that proline was metabolized via the same route. In wilted leaves, only 3.8% of ^{14}C was present in proline. In turgid leaves, a larger amount of ^{14}C (67.5%) was detected in oxidized products: CO_2, glutamic acid, aspartic acid, malic acid and so on. A smaller amount of ^{14}C was detected in these compounds in wilted leaves (25.8%). There was little incorporation of ^{14}C from proline into insoluble material during water stress.

Rehydration and proline content

The effect of water stress on the proline content of leaf disks is shown in Fig. 1. Proline content markedly increased under water stress and increased 12-fold in 16 hr. At this point, the moisture content of the leaf had fallen from 88 to 46%. Leaf disks were then placed on filter paper saturated with water. In the initial period (0–6 hr rehydration), proline continued to increase. After 6 hr rehydration, wilted leaves regained water to 90% of initial moisture content and then proline rapidly disappeared. After 16 hr rehydration, the proline content was almost as low as it was prior to wilting.

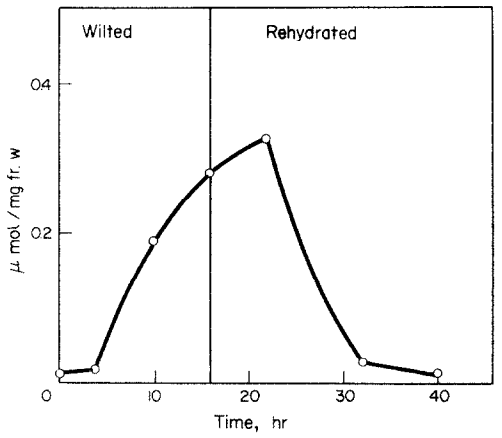

Fig. 1. Effect of water stress on the proline content. The leaf disks were incubated in air for 16 hr and then these disks were placed on moist filter paper. The experiment was performed in the dark. The data are shown in μmol proline per mg fr. wt.

Rate of proline catabolism

A leaf was excised and wilted in air. After 24 hr, the leaf was cut into disks. Proline-[^{14}C] was fed to the leaf disks by vacuum infiltration. Three disks were placed on moist filter paper. Three other disks were placed on filter paper which was in direct contact with silica gel particles. Although the wilted leaves had regained their initial fr. wt during vacuum infiltration, within 40 to 60 min, their moisture content returned to the level prior to infiltration (Table 2). After 6 hr rehydration, proline concentration in rehydrated leaves was almost as high as that in the wilted leaves. Therefore, the diluting effect of radioactivity caused by an enlarged proline pool can be ruled out as an explanation of differences in proline catabolism. In both wilted and rehydrated leaf disks, only trace amounts of proline were incorporated into protein. In both treatments proline was oxidized to glutamic acid, aspartic acid, glutamine, γ-aminobutyric acid, organic acid and CO_2. The wilted leaves converted 24.7% of proline to oxidized products, while in rehydrated leaves, 42.5% of proline was oxidized to other compounds. These results indicated that water stress reduced the rate of proline oxidation.

Table 2. Proline-[^{14}C] degradation under a water-stressed or rehydrated condition

	Wilted	Rehydrated
CO_2	8.0*	9.7
Pro	75.3	57.5
Other amino acids	11.5	13.2
Organic acids	4.9	18.6
Residue	trace	trace
Non-radioactive Pro	0.354†	0.340

After the excised leaf was wilted for 24 hr, proline-[^{14}C] was fed to leaf disks. The leaf disks were incubated for 6 hr under a water-stressed or rehydrated condition. The experiment was performed in the dark.

* Expressed as percentage of total ^{14}C.

† Expressed μmol/mg fr. wt.

DISCUSSION

Table 1 shows that the incorporation of ^{14}C from proline into other compounds was reduced under water stress. The wilted leaves incorporated a small amount of ^{14}C which was detected in oxidized products when wilted rather than when well watered. On the basis of only this result, it could not be concluded that protein synthesis and proline oxidation were inhibited under water stress; because proline accumulated markedly under water stress, the reduced incorporation of ^{14}C from proline into other products might result from the enlarged proline pool. In the experiment shown in Table 2, this possibility could be ruled out, since the proline pool size was almost the same. The radioactivity in proline disappeared more slowly from wilted leaves than from rehydrated leaves. The disappearance of proline did not reflect protein synthesis but proline oxidation. Therefore, it was concluded that proline oxidation was inhibited under water stress and restored by rehydration. While these experiments confirmed the elaborate work of Stewart *et al.* [8], our experiments are sound and the results clear.

The mechanism which regulates proline oxidation remains to be elucidated. At least three possibilities can be considered. First, it has been reported that water stress lowers the levels of various enzymes. For example, Bardzik *et al.* [9] found that the levels of nitrate reductase and phenylalanine ammonia-lyase decreased with water stress and recovered with rehydration. A second possibility is that the high concentration of amino acid [10] or the level of cofactor may regulate the proline oxidizing system. A third possibility is that the water deficit may prevent proline from reaching the site of proline oxidation. Unfortunately, we have little information regarding the effect of water stress on proline oxidizing system. It is necessary to determine which mechanism is involved in the inhibition of proline oxidation and we are continuing to explore this problem.

EXPERIMENTAL

Tobacco (*Nicotiana tabacum* cv BY-4) was grown in a greenhouse. Young fully expanded leaves were used 2–3 months after germination. 1 μCi L-proline-[^{14}C] (225 mCi/mmol) was fed to leaf disks (dia 1 cm) by vacuum infiltration. After infiltration, the leaf disks were placed on a dried filter paper or moist filter paper in a flask and were incubated in the dark at room temp. The respired $^{14}CO_2$ was collected in alkali in a center well in the flask. At the end of incubation period, the leaf disks were ground in a mortar and pestle with 10 ml MeOH and extracted × 3 with 70% MeOH. The extracts were combined and evapd *in vacuo*. The extracts were re-dissolved in H_2O and fractionated into neutral, organic acid and amino acid fractions as described before [11]. The organic acid fraction was analysed according to the method of ref. [12]. The amino acid fraction was analysed with an amino acid analyser (JEOL JLC-6AH) and the eluant monitored by liquid scintillation counting. The remaining insoluble material (residue) was washed with H_2O and then suitable portions were collected on glass-fiber disks. The amount of radioactivity was determined by liquid scintillation counting.

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