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Comparison of Ethylenediaminetetraacetate-Enhanced Exudation from Detached and Translocation from Attached Bean Leaves[1]

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ABSTRACT

A technique for collection of phloem exudate from detached leaves using 20 millimolar EDTA (pH 7.0) has previously been developed (King, Zeevaart 1974 Plant Physiol 53: 96–103). It was the aim of the present study to determine the efficiency of this technique in relation to undisturbed export from attached leaves. Paired primary leaves of bean seedlings (*Phaseolus vulgaris* L. cv Montcalm) were used to minimize variations in plant material. Attached leaves, exposed to $^{14}CO_2$ for 10 minutes with subsequent excision of one of the leaves and collection of the exudate over a 12-hour period, showed a 25% export of total assimilated ^{14}C from the attached *versus* 15% of total assimilated ^{14}C in the form of exudation from the detached ones. Leaf excision changed the labeling pattern within the leaf, increasing % total leaf ^{14}C-activity in the ethanolic fraction, while decreasing activity in the starch fraction, as compared to attached leaves. This was presumably caused by a lack of translocation from the detached leaves. Excision did not affect dark respiration. However, measurements of total nonstructural carbohydrates in leaf starch and neutral fractions indicated no significant differences between attached and leaves detached in EDTA. Thus, in terms of actual carbon export, and accompanying distribution of nonexported carbohydrate within the leaf, EDTA-enhanced exudation compares favorably with translocation from attached leaves.

Few plant species are capable of continuous phloem exudation from cut stems or petioles (11) which makes analysis of fluid from the sieve tubes difficult, if not impossible. Therefore, for qualitative and quantitative analyses of translocated substances, various workers have used radioisotopes and subsequently extracted relatively large amounts of tissue remote from the site of label application. King and Zeevaart (8) have demonstrated that treating the cut ends of petioles of several species with 20 mM EDTA at pH 7.0 will yield a considerable amount of exudate as compared to control leaves kept in water. This technique has subsequently been used by other workers with a variety of plants (1, 4, 10, 11, 13, 14–16, 18).

Dickson (1) has previously performed a comparison between the rate of exudation of ^{14}C-labeled compounds into an EDTA solution and translocation from attached cottonwood and bean leaves. He reported that bean was capable of EDTA-enhanced exudation at much higher rates than cottonwood and that the amount and composition of the ^{14}C-bean exudate was similar to the material normally translocated from an attached leaf. However, although he did demonstrate these similarities, no comparison was made as to the distribution of label within the non-water-alcohol soluble compounds (*i.e.* starch) within the leaf, in either the attached or detached condition. In fact, no estimates of total nonstructural carbohydrate levels as affected by excision and EDTA-enhanced exudation are available. It was therefore the aim of this present study to compare these parameters in attached and detached leaves. The results obtained indicate that EDTA-enhanced exudation from primary leaves of *Phaseolus vulgaris* compares favorably with translocation from attached leaves.

MATERIALS AND METHODS

Plant Material. Bean seeds (*Phaseolus vulgaris* L. cv Montcalm) were planted in 950-ml containers in a mixture of vermiculite: perlite:peat (3:3:4, v/v/v). Plants were grown in a growth chamber under the same conditions as described before (19). The plants were watered with half-strength Hoagland solution in the morning and with deionized H_2O in the afternoon.

Labeling Experiments. Procedures were similar to those described previously (19). Both primary leaves of 15-d-old plants were placed in a Plexiglas chamber attached to a closed loop system. $^{14}CO_2$ (60 μCi) was generated from $Ba^{14}CO_3$ (59.7 mCi/mmol) by injection of 20% lactic acid. After a 10-min labeling period, the chamber was flushed with air for another 10 min. One of the leaves was then excised and the petiole placed either in distilled H_2O, 20 mM citrate (pH 7.0), or 20 mM Na_2-EDTA (pH 7.0). The detached leaves were placed in darkness alongside the paired attached leaves for 12 h. Control plants were similarly labeled and placed in darkness, but without excision of the leaves.

At the time of harvest, leaves were frozen in liquid N_2 and lyophilized. The material was then weighed, ground to small particles in a Micro-Mill (Technilab. Instruments, Pequannock, New York), and aliquots were taken for carbohydrate analyses as described below, as well as for determination of ^{14}C-content of the neutral, basic, and acidic fractions. Sugars in the neutral fraction were separated by TLC (5) and identified by co-chromatography of the appropriate standards. Bands were scraped into scintillation vials with cocktail, and the amount of radioactivity was determined by scintillation spectrometry. For determining total radioactivity, additional aliquots were oxidized in a Packard model 306 Tri-Carb sample oxidizer as described previously (19).

Total Nonstructural Carbohydrate Analysis. Thirteen-d-old plants were transferred from the growth chamber to a greenhouse (average light intensity during summer at noon, 24–28 mw cm^{-2}) for 48 h prior to sampling leaves at 8:00 PM. Plants were selected for uniformity of the paired primary leaves. One leaf was excised and the petiole recut and maintained either in distilled H_2O, 20 mM citrate (pH 7.0), or 20 mM Na_2-EDTA (pH 7.0). Both detached and attached leaves were placed in darkness along with intact control plants. After 12 h, the attached leaves were excised and all leaves were frozen in liquid N_2 and lyophilized. The dried leaves were weighed, ground, and 50-mg aliquots extracted according to

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the method of Dickson (2) which consisted of sequential extraction with a mixture of methanol:chloroform:H_2O (12:5:3, v/v/v), and 80% ethanol. This yielded a water-soluble fraction which was passed through coupled anion (Dowex 1, formate) and cation (Dowex 50, H^+) columns to obtain the neutral sugar fraction.

Starch was extracted from the pellet using an enzymic digestion step with 1% (w/v) Clarase 900 (Miles Laboratories, Elkhart, IN) in acetate buffer at pH 4.5 (2). Neutral sugars and starch extracts were both assayed with the phenol-sulfuric acid method (total sugars) and the reducing sugar assay of Somogyi as modified by Nelson (7). Sucrose was quantitated by the Somogyi-Nelson method following incubation with invertase (200 units/ml) at 45°C and pH 4.5 for 1 h. Total organic carbon of nonextracted material was determined according to the method of Ho (6).

Gas Exchange Measurements. Measurements of photosynthesis, dark respiration, and stomatal conductance were made on attached and detached leaves with the system described in (3). Paired chambers permitted simultaneous measurements on attached and detached leaves of the same plant. The light intensity for the photosynthesis measurements was 10.8 mw cm^{-2}. Assimilation and respiration rates were determined using internal CO_2 concentrations (3).

RESULTS AND DISCUSSION

The EDTA exudation technique has provided a simple means for analyzing phloem fluid from detached leaves (1, 8, 14–16), cut fruits (4, 11), or senescing flowers (18). In a number of studies, the efficiency of the EDTA technique was determined by comparing the amount of ^{14}C-activity exuded into EDTA with the radioactivity detected in water (8, 13, 15, 16). In the present study, the loss of radioactivity from detached leaves placed in EDTA was compared with the radioactivity lost from leaves left *in situ*. After 12 h, detached primary leaves of bean had exuded 15.0 ± 2.0% (six replicates) of the total ^{14}C-photosynthate recovered from the detached source leaves, whereas the attached source leaves labeled at the same time had exported 25.0 ± 3.0% of the ^{14}C-activity recovered from the entire plant. Leaves labeled with $^{14}CO_2$ and detached after 20 min did not exude ^{14}C into water without EDTA. This difference (15 *versus* 25%) in total ^{14}C-export between the detached and attached leaves could have been due to (a) damage to the transport system by the excision and EDTA uptake, (b) the subsequent formation of callose in spite of the presence of EDTA, or (c) an effect of excision on the partitioning of ^{14}C within the leaf. Care was taken to minimize possibility (a), but some damage would seem inevitable. EDTA has been shown to reduce callose formation in *Perilla* (8) significantly, although some may have been formed in bean petioles after 12 h. The possibility of EDTA effects on the distribution of ^{14}C among various pools was discounted in work by Dickson (1) with leaves of bean and cottonwood. However, Köcher and Leonard (9) did show significant differences in ^{14}C-label distribution in bean leaves which were excised and kept in moist Petri dishes when compared with attached leaves. Watson and Wardlaw (17) also reported that prevention of translocation of photosynthate out of leaves of wheat and sorghum by steam killing the base of the blade changed the pattern of labeling of metabolites following a pulse of $^{14}CO_2$. It was important, therefore, in the present experiment to determine the effect of leaf excision (*i.e.* complete elimination of translocation) on the ^{14}C-labeling pattern. The results in Table I indicate that after 12 h a greater proportion of the radioactivity within the leaf was found in the ethanolic fraction (mainly sucrose and fructose) in detached leaves than in attached leaves, whereas the reverse was true for the starch fraction. These differences presumably reflect the lack of translocation from the detached leaf. The attached leaf would have exported a significant amount of the label initially present in the ethanolic fraction as sugar (see above) after 12 h, thus changing the ratios between the sugar and starch pools. No differences, however, were evident in the lipid or structural fractions indicating that these may not be as responsive to alterations in export capabilities as the nonstructural carbohydrates.

Table I. *Effect of Excision on ^{14}C-Assimilate Distribution in Bean Leaves*

Primary leaves on bean plants were exposed to $^{14}CO_2$ after which one of the leaves was excised and placed with its petiole in water. The excised leaf was placed alongside the remaining attached leaf in darkness for 12 h. Data are averages of six plants.

Fraction	Attached	Detached
	% of total ^{14}C in leaves	
Ethanolic	25.5 ± 3.0	35.3 ± 2.1
Lipid	11.5 ± 3.0	11.0 ± 2.1
Starch	41.5 ± 3.5	30.0 ± 4.2
Structural	21.5 ± 3.5	23.7 ± 2.8

Inasmuch as the amount of ^{14}C assimilated during a pulse is only a small fraction of the total carbon assimilated and maintained in the leaf during its development, we deemed it essential to analyze the total nonstructural carbohydrates of bean leaves and see how these are affected in excised leaves by various test solutions. As part of this analysis, net assimilation, respiration, and stomatal conductance were also determined. Detached leaves with the petioles maintained in water exhibited no significant differences in assimilation rate as compared to attached ones: detached 12 ± 3 mg $CO_2 \cdot dm^{-2}\ h^{-1}$ *versus* attached 11 ± 2 mg $CO_2 \cdot dm^{-2}\ h^{-1}$ after 8 h. Conductance increased significantly for the detached leaves after 1 h and remained so for 8 h: detached 0.75 ± 0.08 cm s^{-1} *versus* attached 0.5 ± 0.1 cm s^{-1}. This is in contrast to the girdling effects on soybean leaves observed by Setter *et al.* (12), and indicates that the damage from excision may not have been as severe as girdling for potential ABA accumulation and subsequent stomatal closure (12).

Excised leaves placed in EDTA and kept in the light exhibited a 20% decrease in assimilation rate after 1 h as compared to the attached control leaves, and a 70% decrease after 6 h. This decrease was determined using internal CO_2 concentrations and was mesophyll in origin, although stomatal conductance showed a similar decline. The assimilation rate of leaves immersed in citrate had also declined by 10% after 1 h, and by 40% after 6 h. Conductance declined to the same extent. It was therefore apparent that the chelating agents were affecting photosynthetic mechanisms directly. To avoid this, experiments were thereafter conducted in the dark to reduce transpiration and subsequent uptake of the chelating agents into the leaf. Dark respiration was not affected by any of the solutions bathing the petioles: the average respiration rate was 0.25 ± 0.06 mg $CO_2\ dm^{-2}\ h^{-1}$ after 8 h. Conductance rates were also similar for all leaves maintained in the dark.

On the basis of these results, it was expected that detached leaves placed in water (with no exudation) would be heavier than their comparable control leaves left on the plant. However, due to variations among the leaves of even carefully selected and paired primary leaves, no significant differences in dry weight were found between the detached and attached leaves, although a clear cut trend was evident (Table II). It should be noted that the mean of the EDTA material was closest to the control and also had the smallest SD.

Total organic carbon determinations (6) demonstrated no significant differences between the attached and detached leaves: 89.5 ± 0.8% *versus* 89.4 ± 1.0%, respectively. This indicates that the differences between the treatments as observed in the ^{14}C experiments may be in the allocation of carbon within the leaf. When the amounts (mg g^{-1} dry weight) of nonstructural carbohydrate from attached primary leaves were compared with their paired excised leaves placed in either water, citrate, or EDTA, significant differences were observed (Table III). The largest of

Table II. *Ratio of Dry Weight of Detached over Attached Paired Primary Leaves of Bean as Affected by the Composition of the Solution Bathing the Petioles*

Leaves were excised and maintained with their petioles in distilled H_2O, 20 mM citrate (pH 7.0), or 20 mM Na_2-EDTA (pH 7.0) for 12 h in darkness alongside the attached leaves. Data are averages of 18 plants per treatment with SD. No significant differences were evident between treatments as determined by a single tailed *t* test.

Treatment	Dry Wt Detached/Attached ± SD
Water	1.18 ± 0.10
Citrate	1.04 ± 0.13
Na_2-EDTA	1.01 ± 0.08
Control[a]	0.98 ± 0.17

[a] Both leaves attached.

Table III. *Ratios of Nonstructural Carbohydrates of Detached over Attached Paired Primary Leaves of Bean as Affected by the Composition of the Solution Bathing the Petioles*

The detached leaves were cut and maintained with their petioles in distilled H_2O, 20 mM citrate (pH 7.0), or 20 mM Na_2-EDTA (pH 7.0) for 12 h in the dark alongside the remaining attached leaves. Control plants with neither leaf excised were also placed in darkness. Data are averages of six plants per treatment with SD. Significant differences as determined by a paired single tailed *t* test are given as superscripts.

Fraction	Water	Citrate	Na_2-EDTA	Control
		ratio[a]		
Starch	0.8 ± 0.30[b]	0.9 ± 0.10[b]	1.0 ± 0.07	1.1 ± 0.09
Neutral	3.4 ± 0.05[b]	1.7 ± 0.67[b]	0.9 ± 0.25	1.0 ± 0.05
Reducing sugars	2.1 ± 0.02[c]	1.2 ± 0.30[b]	0.9 ± 0.15	1.1 ± 0.27
Sucrose	3.8 ± 0.28[c]	2.1 ± 0.40[c]	2.1 ± 0.03[b]	1.1 ± 0.24
Total	1.7 ± 0.03[b]	1.3 ± 0.16[b]	1.1 ± 0.16	1.1 ± 0.14

[a] Ratio of detached/attached leaves.
[b] Level of significance >0.05.
[c] Level of significance >0.01.

these differences occurred in the neutral fraction and its components in those excised leaves whose petioles had been placed in water as is evident by the mg g^{-1} dry weight ratios of detached/attached leaves.

The higher amount of carbohydrate in the sugar fractions of the excised leaves most likely reflects blockage of the translocation pathway from the leaf, since no carbohydrate was found in the exudation solution of the water treatments. Further, the samples included tissue from both the lamina and petiole, and it has been previously shown by Köcher and Leonard (9) and Dickson (1) that the petioles of excised bean leaves rapidly accumulate sugars exported by the lamina in the absence of transport to the rest of the plant.

The ratios of the starch fractions from the excised leaves in water are also significantly different from the controls (Table III) indicating a loss of starch in the excised leaves. This observation confirms that of the ^{14}C experiments (Table I). However, use of a pulse-chase technique should always be accompanied by total carbohydrate determinations for reliability.

Ratios of the citrate-treated leaves were significantly lower than the controls in the starch fraction, but higher in the neutral sugar fraction (Table III). The rate of exudation was also very low for citrate treatments averaging about 1.5 ± 0.3 mg 12 h^{-1} with the exudate containing mostly sucrose (>95% of total carbohydrates). This was only about 20% of that exuded into the EDTA (7.8 ± 1.2 mg 12 h^{-1}) with the same carbohydrate composition. This clearly indicates that, at least in bean, EDTA is superior to citrate in enhancing exudation from detached leaves.

The EDTA-treated leaves also appeared to reflect more closely the distribution of control leaf nonstructural carbohydrates in every fraction, except sucrose (Table III). It must be noted, however, that the actual amount of sucrose in a leaf on a dry weight basis (4–7%) was only one-third that of the reducing sugars. Small changes in amounts of sucrose, due perhaps to even slight reductions in export, result in relatively large changes in the ratios. The EDTA sucrose ratios were still, however, almost half that of the water-treated leaves.

In conclusion, the data presented above indicate that EDTA-enhanced phloem exudate from detached bean leaves compares favorably with the amount of material exported from attached leaves over a 12-h period. This is evidenced by a lack of significant differences in nonstructural carbohydrate partitioning (mg g^{-1} dry weight) within the leaf and petiole as compared to attached leaves. Measurements of this type are also important in experiments where total percent ^{14}C-assimilate partitioning may be affected through excision and lack of export.

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