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Communication

Protoplast Volume:Water Potential Relationship and Bound Water Fraction in Spinach Leaves[1]

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ABSTRACT

Methods used to estimate the (nonosmotic) bound water fraction (BWF) (*i.e.* apoplast water) of spinach (*Spinacia oleracea* L.) leaves were evaluated. Studies using three different methods of pressure/volume (P/V) curve construction all resulted in a similar calculation of BWF; approximately 40%. The theoretically derived BWF, and the water potential (Ψ_w)/relative water content relationship established from P/V curves were used to establish the relationship between protoplast (*i.e.* symplast) volume and Ψ_w. Another method of establishing the protoplast volume/Ψ_w relationship in spinach leaves was compared with the results from P/V curve experiments. This second technique involved the vacuum infiltration of solutions at a range of osmotic potentials into discs cut from spinach leaves. These solutions contained radioactively labeled H_2O and sorbitol. This dual label infiltration technique allowed for simultaneous measurement of the total and apoplast volumes in leaf tissue; the difference yielded the protoplast volume. The dual label infiltration experiments and the P/V curve constructions both showed that below −1 megapascals, protoplast volume decreases sharply with decreasing water potential; with 50% reduction in protoplast volume occurring at −1.8 megapascals leaf water potential.

Recent studies (3, 19) have indicated that in leaves of plants exposed to water deficits, loss of photosynthetic capacity by chloroplasts may be more closely related to the extent of protoplast and/or chloroplast volume reduction than the low Ψ_w[2] induced in dehydrated cells. The measurement of protoplast (*i.e.* symplast) volume changes in leaves of plants subjected to water deficits may be, therefore, an important indicator of physiological competence of cells in water stressed leaves.

Direct measurement of the volume/Ψ_w relationship in conjunction with photosynthesis studies has delineated clear differences between cultivars of an agronomic crop with regards to stress resistance (*i.e.* maintenance of photosynthetic capacity at low Ψ_w) as shown by Sen Gupta and Berkowitz (19). However, there is at present only one widely accepted experimental technique which can be used to monitor the relationship between protoplast volume and Ψ_w: P/V curve analysis. As discussed in reviews by Turner (22) and Boyer (5), construction of a P/V curve for a leaf can allow for estimation of relative protoplast volume with varying leaf Ψ_w. This relationship is established by estimation of the (nonosmotic) extraprotoplast bound water fraction of the leaf, and removing this value (which remains constant as a leaf dehydrates; 5, 22) from RWC measurements taken at a range of water potentials. The technical problems and disadvantages of this measurement have been reviewed elsewhere (6, 22, 23). Briefly, the establishment of a protoplast volume/Ψ_w relationship using P/V curves depends on the accurate estimation of a theoretically derived value (BWF), it can take up to several hours to obtain the data for a single P/V curve and the volume/Ψ_w relationship established by a P/V curve is based on data obtained from a single leaf.

Berkowitz and co-workers (3, 19) have used an entirely different experimental approach first developed by Kaiser (11) to directly measure relative changes in protoplast volume at varying leaf Ψ_w. This technique involves the vacuum infiltration of solutions (at a range of Ψ_s) containing radiolabeled H_2O and sorbitol into leaf tissue. The difference between the ^3H_2O-space (total volume) and [^{14}C]sorbitol space (apoplast volume) yields a direct measurement of protoplast volume. The potential sources of error with this technique are significant (*e.g.* possible equilibration of [^{14}C]sorbitol into the symplast due to vacuum infiltration-induced loss of membrane integrity). However, the advantages of this technique are that many replications of a treatment can be made, and the resulting volume/Ψ_w relationship can be established using a large number of leaves.

One objective of the work outlined in this report was to compare the protoplast volume/Ψ_w relationship derived from P/V curve analysis, and the dual label infiltration technique. Attempts were made to validate the usefulness of these techniques for examining the volume/Ψ_w relationship in leaf cells.

A second objective was to evaluate the significance of the P/V curve-derived BWF measurement, in terms of its influence on the protoplast volume/Ψ_w relationship. If the BWF is high, then the degree of protoplast volume change occurring as leaf Ψ_w declines can be quite substantial, and cannot be estimated from RWC measurements alone. For example, at

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[2] Abbreviations: Ψ_w, water potential; BWF, bound water fraction; Ψ_s, osmotic potential; P/V, pressure volume; RWC, relative water content.

a BWF of 50%, a 15% reduction in RWC would be associated with a reduction, by nearly a third, in the osmotic volume of cells. The concentrating effect of this volume change on some key cell solutes may be responsible for water deficit-induced perturbations in cell metabolism (2, 12, 17, 20). However, in many studies (*e.g.* 9, 10, 21), RWC changes alone are used to quantitate the solute-concentrating effects of dehydration as leaf Ψ_w declines. In these cases, the deleterious effects of volume changes at low Ψ_w may be underestimated.

MATERIALS AND METHODS

Plant Material

Spinach seeds (*Spinacia oleracea*, var 'Melody') were germinated in flats of vermiculite for 1 week. Seedlings (3/pot) were transplanted to pots containing 4.3 dm^3 of 1:1 peat/vermiculite in a growth chamber. Plants were irrigated with standard commercial (Peter's 'Geranium Special') fertilizer twice a week, and once with just water. The conditions in the growth chamber were: 22°C and 50% RH constant, with an 11 h light period (250 $\mu E \cdot m^{-2} \cdot s^{-1}$). Plants were used 6 to 8 weeks after transplanting. Fully expanded nonsenescing leaves were used for all measurements.

Pressure/Volume Curves

P/V curves were constructed for individual spinach leaves using several different techniques. One method used was that described by Turner (22) involving leaf overpressure and xylem sap collection. Fully turgid leaves (petioles of excised leaves were recut twice under water prior to hydration at 4°C for a minimum of 4 h) were placed in a pressure chamber (Soil Moisture Equip. Corp., Santa Barbara, CA). The chamber walls were lined with moistened paper, and the leaf was covered with plastic wrap. The overpressure series was typically 11 steps. The overpressure steps were approximately 0.3 MPa over balance pressure for 1 to 2 min between leaf Ψ_w of 0 and −1.3 MPa, and 0.2 MPa over balance pressure for 4 to 5 min for leaf Ψ_w below −1.3 MPa (usually down to −2.5 MPa). This overpressure regime was selected so that at each step the leaf Ψ_w was near to equilibrating to the overpressure, but was not left at the overpressure without sap exudating for significant periods of time. The leaf was rarely more than 0.1 MPa away from equilibrating to the balance pressure at each step. Another important consideration in designing the overpressure series was to allow for the maximum number of data points to be collected on the linear portion of the P/V curve. The number of data points collected on the linear portion of the P/V curves ranged between 7 and 9. During the overpressure series, xylem sap was collected by placing a 7 mL plastic vial filled with absorbent cotton over the end of the petiole protruding from the pressure chamber. After each overpressure step and subsequent lowering of the chamber pressure, the vials were immediately capped and weighed. At the end of each overpressure series, the fresh and dry weights of the leaf was obtained. These measurements allowed for calculation of the percentage recovery of total water lost during the overpressure series as xylem exudate, and for conversion of xylem sap exudate measurements to RWC values. Typical recovery of xylem sap exudate (*i.e.* leaf fresh weight loss recovered as total increase in weight of vials) was 95%. For conversion of vial weight change to RWC, this 5% difference between weight loss and recovery was accounted for.

A second technique used for P/V curve construction followed the method of Wilson *et al.* (24). Fully turgid (*i.e.* hydrated to 100% RWC), excised leaves were allowed to air-dry on the laboratory bench. At appropriate intervals, leaf fresh weight, and then immediately Ψ_w (ascertained using the pressure chamber) were recorded. Subsequent measurements of the dry weight of the sample leaf allowed for conversion of fresh weight loss to RWC at each Ψ_w. On occasion, this technique was used to develop P/V curves for leaves which had the major veins removed. In this case, the midrib leading to the petiole was left intact, but the major veins diverging from the midrib were excised. It was found that these veins comprised 28.3% ± 1.6 (n = 15) of the fresh weight of the leaves used in these experiments.

A third procedure used to develop P/V curves for spinach leaves involved removing samples from a dehydrating leaf for simultaneous measurements of RWC and Ψ_s. In these studies, an excised spinach leaf was allowed to air dry. At appropriate intervals, five 4.6 mm diameter discs were cut from the interveinal region of the leaf blade for RWC determination. Two discs of similar size were also cut for Ψ_s measurements. These discs were wrapped in plastic, frozen in liquid nitrogen, and stored at −20°C. After defrosting, the Ψ_s of the discs was ascertained by thermocouple psychrometry with a Wescor (Logan, UT) HR33T microvoltmeter and C-52 sample chambers.

Results of this third method of P/V curve construction were analyzed differently from the other methods to avoid the problem pointed out by Turner (22) regarding Ψ_w measurements of previously frozen leaf tissue with thermocouple psychrometers. After a freeze/thaw cycle, the protoplast osmoticum can be diluted upon mixing with the apoplast BWF. As discussed by Tyree (23), this can induce significant errors in a P/V curve. Therefore, as suggested by Scholander *et al.* (18) and Markhardt *et al.* (14), thermocouple psychrometer measurements of Ψ_s were assumed to be increased by free cell solute dilution across the total BWF. Corrections were made to account for the diluting effects of cell sap mixture with the BWF by using BWF and Ψ_s at 100% RWC values from other P/V curves, and assuming that the change in ln Ψ_s is a linear function of changes in the ln of the solvent volume (*i.e.* protoplast water content and total water content) as discussed by Sen Gupta and Berkowitz (19). The adjusted Ψ_s values were replotted with the RWC values to generate a P/V curve.

Dual Label Infiltration Measurement of Protoplast Volume

The relative protoplast volume of leaf tissue at a range of water potentials was ascertained as described previously (19). Six 4.6 mm diameter discs were placed in 1 mL of infiltration medium (25 mM Hepes [pH 7.6], 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 3.125 μCi/mL $[^3H]H_2O$, 0.5 μCi/mL [^{14}C]sorbitol, and varying concentrations of sorbitol). The sorbitol concentration was varied so as to obtain a range of osmotic potentials (solution Ψ_s was ascertained using thermocouple psychrometry). Two discs were cut from each of

three leaves for each infiltration tube. Three replicate infiltration tubes were used at each Ψ_s. Discs cut from the same three leaves were used for all infiltration tubes (representing a range of osmotic potentials) in each experiment. Discs were vacuum-infiltrated twice, incubated for 1 h, rinsed twice with 2 mL of unlabeled infiltration medium, and then frozen in liquid N_2. After the liquid N_2 evaporated, 5 mL of 96% ethanol with 10 N formic acid was added to the tubes, and the label was leached out of the discs for 24 h by shaking in this extraction solution. The label which had equilibrated in the discs was measured by assaying the ethanol extract with a liquid scintillation spectrophotometer (Beckman 3801). Measurement of ^3H_2O leached out of the discs represented the summed apoplastic and symplastic volume of the leaf tissue, and the apoplastic volume was ascertained from the [^{14}C]sorbitol value. The difference represented the symplastic (protoplast) volume of the leaf tissue at a given leaf Ψ_w (the leaf Ψ_w equilibrated with the solution Ψ_s). The data from these experiments are expressed as percentage change in protoplast volume at a given Ψ_w (the maximum leaf Ψ_w typically occurs at a solution Ψ_s close to 0 MPa; 3, 19).

RESULTS AND DISCUSSION

Bound Water Fraction in Spinach Leaves

In a review of P/V curve construction, and use for analysis of cell water relations parameters, Turner (22) indicated that P/V analyses yield leaf BWF values of up to 50%. Scholander *et al.* (18) reported a BWF of 33% for juniper leaves using the xylem sap collection method of P/V curve construction. Boyer (4) determined a BWF of 26% for rhododendron leaves using entirely different methodology (electron micrograph analysis of cell wall volume in leaves). Despite these indications that a substantial portion of the total water in leaf cells of some species may be nonosmotic, BWF is typically not considered in analyses of the effects of Ψ_w depression on symplast volume (*e.g.* 9, 10, 21).

Using the 'standard' P/V curve technique (*i.e.* xylem sap collection), we found the BWF in spinach leaves to be nearly 40% (Table I). Using the air-drying method (*i.e.* intermittent determinations of leaf Ψ_w using a pressure chamber as a leaf is air-dried) of P/V curve construction, the BWF in spinach leaves was again determined to be nearly 40% (Table I). Theoretical considerations suggest that these two methods of BWF determination could be significantly influenced by artifacts of the assay procedures. With the leaf overpressure/sap collection technique, conversion of xylem sap loss to RWC change could be in error, and bulk leaf dehydration at each balance pressure could possibly be not in equilibrium with the dehydration in the vascular system due to xylem sap loss. With the air-drying technique, repeated pressurizations with intermittent air-drying could cause xylem cavitation and induction of embolisms in the vascular system. Both techniques suffer from the possible effect of altered water relations parameters of the structural (parenchyma and collenchyma) cells of the vascular system influencing the calculated relationship for the leaf mesophyll. Also, repeated pressurization injures the petiole such that the minimum RWC which the leaf can be brought to is limited (Table I). This last problem results in a large extrapolation between the range of RWC at which data are collected, and the theoretical RWC at which the BWF is determined.

Further studies were undertaken to determine if these potential artifacts of the assay systems influenced the BWF calculation. To examine whether the thickened walls of the vascular system structural cells unduly influenced the calculated BWF for the bulk mesophyll, P/V curves were constructed using the air-drying technique for leaves which had the secondary veins removed (Table I). This vascular/structural tissue represents a substantial portion of the spinach leaf fresh weight (28.3 ± 1.6%), and is characterized by thickened cell walls (7) which can influence BWF of a leaf (4, 16). If a high BWF in the vascular tissue was influencing the calculated BWF for the whole leaf, then removal of the secondary veins of the leaf should have reduced the BWF. As shown in Table I, this was not the case; BWF of leaves with veins removed still was near 40%.

The possibility of artifacts in the xylem sap collection and air-drying methods of P/V curve construction influencing the BWF estimation was also addressed by constructing P/V curves using a third technique. In this case, the Ψ_s of interveinal mesophyll tissue of leaves was determined with thermocouple psychrometry. RWC was also determined for the interveinal sections of the spinach leaf laminae. Using this technique, many of the potential problems of the sap collection and air-drying techniques (which both involve repeated pressurizations of a leaf) are obviated. As shown in Table I, the psychrometric method of P/V curve construction also resulted in a BWF near 40%.

The minimum RWC which the leaf can be dehydrated to can be lower with the psychrometric method than the other two methods of P/V curve construction (Table I). For our experiments, the minimum RWC obtained with the psychrometric method was 45%, which considerably reduces the degree to which the linear portion of the P/V curve needs to be extrapolated in order to calculate BWF (22).

As noted in "Materials and Methods," the construction of a P/V curve with psychrometry-derived Ψ_s values necessitates that the effect of cell sap dilution by BWF be accounted for. This correction was made using Ψ_s at 100% RWC and BWF values generated from P/V curves using the other two methods of data collection. The BWF values generated using the psychrometric method, therefore, are not truly an independent verification of the first two methods. However, the fact that the results from this third method agree well with the other methods supports our contention that the data generated from any one of the techniques was likely not substantially influenced by artifacts inherent in the particular methodology used to generate the P/V curve.

As shown in Table I, the three different techniques of P/V curve construction all yielded similar BWF estimates. Also, removal of the secondary veins did not influence the BWF estimation. These results suggest that the BWF estimation of approximately 40% was likely not significantly influenced by artifacts inherent in each of the three assay systems.

The different techniques of P/V curve construction were also used to determine the Ψ_s of leaf tissue at 100% RWC (Table I). Results indicate that in addition to similar estimates

Table I. *Pressure/Volume Curve Analysis of the Bound Water Fraction in Spinach Leaves*

Three different techniques of P/V curve construction were used. The 'sap collection' method involved dehydrating leaves by overpressurization in a pressure chamber with RWC change monitored by xylem sap collection and Ψ_w change monitored by determining the balance pressure in the pressure chamber. The 'air-drying' method refers to the dehydration of leaves by air-drying, with RWC change measured by weighing the leaf, and Ψ_w change monitored by determining the balance pressure in a pressure chamber. The 'psychrometry' technique involved leaf dehydration by air-drying, RWC measurement by leaf weights, and Ψ_s measurement using thermocouple psychrometry. For the sap collection method, the percentage fresh weight loss from the sample leaf recovered as xylem sap exudate was 95.1 ± 1.2%. A minimum of three P/V curves were constructed using each of the techniques. All data are presented as means ± SE. The 'r^2' value refers to the correlation coefficient for the linear regression of the inverse Ψ_w/RWC relationship at RWC below the turgor loss point.

Method of P/V Curve Construction	Bound Water Fraction	Osmotic Potential at 100% RWC	r^2	Measurement Range	
				Maximum	Minimum
	% of total	*−MPa*		*% RWC*	
Sap collection	38.91 ± 1.78	0.945 ± 0.028	0.981 ± 0.013	95.4 ± 2.3	61.0 ± 3.8
Air-drying	39.28 ± 4.96	0.972 ± 0.040	0.971 ± 0.014	92.2 ± 5.1	65.7 ± 5.0
Psychrometry	39.41 ± 2.25	0.940 ± 0.060	0.995 ± 0.004	72.3 ± 4.5	45.3 ± 1.3
Air-drying (veins removed)	39.38 ± 1.67	0.907 ± 0.0347	0.970 ± 0.007	80.0 ± 1.9	55.4 ± 0.3

of BWF, these techniques also yield similar values of Ψ_s at full turgor. This value of about −0.95 MPa Ψ_s at 100% RWC is similar to that reported previously for spinach leaves (1). The similarity of the values of Ψ_s at 100% RWC determined using the different P/V techniques further substantiates the likelihood that the BWF estimates of approximately 40% are not the result of artifacts of the assay methods.

If the estimation of nearly 40% BWF in spinach leaves, as is suggested from the data in Table I, is accepted as valid, then a possible contradiction should be noted. The BWF of a leaf is generally acknowledged to be primarily associated with the cell wall (4, 5). Estimation of the wall volume in spinach leaf cells from photomicrographs (8, 15) suggests that the cell wall volume in spinach leaves is no more than 25%. Therefore, either the BWF estimates as shown in Table I are not accurate, or cell wall volume estimates from two-dimensional micrographs are not reliable, or part of the BWF is associated with micromolecules other than the cellulose of cell walls. Estimates of cell wall volume (*i.e.* maximum BWF) from electron micrographs should be made with caution, as the apparent cell wall volume could change substantially when tissue is dehydrated and fixed in glutaraldehyde.

Comparison of P/V Curve and Dual Label Infiltration Analysis of the Volume/Potential Relationship

The BWF values determined using the various P/V curve construction techniques were used to convert the RWC/Ψ_w relationship measured in spinach leaves into protoplast volume/Ψ_w values using the analysis described by Turner (22). These data, along with dual label infiltration determinations of the same relationship, are shown in Figure 1. The dual label infiltration data show that leaf Ψ_w depression down to −1.0 MPa caused only a slight (10%) reduction in protoplast volume (Fig. 1). With further declines in leaf Ψ_w, protoplast volume reduction is more pronounced; at −1.8 MPa, protoplast volume is reduced by 50%. The results of previous work in this laboratory (3, 19, 20) and others (11) have led to the speculation that dual label infiltration measurements of the protoplast volume/Ψ_w relationship in leaves may be a convenient screening procedure to access the extent of water deficit inhibition of chloroplast metabolism in leaves, and also differences in cellular-level acclimation to low Ψ_w. However, the dual label infiltration technique exposes leaf tissue to the potentially injurious step of vacuum infiltration. Liquid injection (especially at nonisotonic Ψ_s) into the apoplast of the leaf interior may result in cell damage (although direct examination of this problem suggests otherwise; 13). Control experiments were undertaken to ascertain the extent to which tissue damage at low infiltration solution Ψ_s could have caused [^{14}C]sorbitol uptake into the symplast. Increased cell permeability to [^{14}C]sorbitol at low Ψ_s would result in a reduction in calculated protoplast volume which would not necessarily be caused by protoplast volume reduction. Dual label vacuum infiltration experiments with ^3H_2O replaced by [^{3}H]inulin indicated that at low Ψ_s, the 'apparent' increase in apoplast volume (reducing the calculated protoplast volume; 11) as evidenced by an increase in disc-associated [^{14}C]-sorbitol was matched by a proportionate increase in disc-associated [^{3}H] inulin (data not shown). Inulin is a much larger molecule than sorbitol (mol wt of 5,200 versus 200 for sorbitol), and therefore should not penetrate damaged cell membranes as readily as sorbitol.

In order to evaluate the physiological relevance of dual label infiltration measurements of the protoplast volume/Ψ_w relationship, the results of these studies were compared with the P/V analyses (Fig. 1). Although there are some variations among individual leaves when P/V curves were constructed using the xylem sap collection method, these data generally follow the volume/Ψ_w relationship obtained from the dual label infiltration analysis (Fig. 1A). When the air-drying method of P/V curve construction was compared with the infiltration method (Fig. 1B), there were some differences noted. The air-drying P/V curve data followed the relationship developed using the dual label infiltration method at

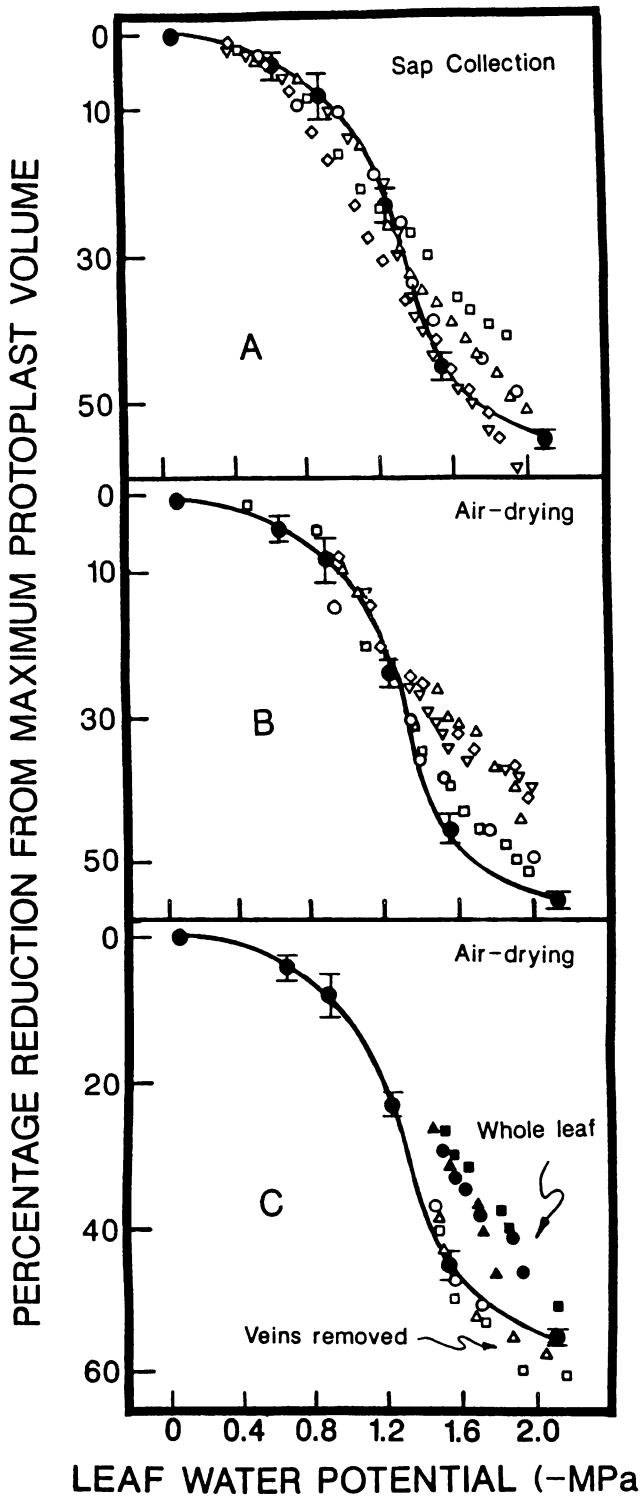

Figure 1. Effect of leaf Ψ_w depression on protoplast volume in spinach leaves. The closed circles with standard error bars connected by the solid line in each of the panels represents the relationship as determined using the dual label infiltration technique. These data are compared to results of P/V analysis of the same relationship. P/V curves were constructed using the sap collection (A) or air-drying (B, C) techniques. In Figure 1C, data from P/V curves constructed with whole leaves (small solid symbols) and leaves with secondary veins removed (open symbols) are shown. Different symbols (○, △, ▽, □, ◇, ■, ▲) represent data from different P/V curves. The data points from the dual label infiltration experiment are the means of nine values; experiments were performed with three replications at each leaf Ψ_w within each experiment. The mean maximum protoplast volume (obtained in each case at the leaf Ψ_w approaching 0 MPa) in each of the three experiments was 1.437 ± 0.026, 1.536 ± 0.241, and 2.265 ± 0.207 mL/dm^2 leaf area.

high Ψ_w (between 0 and −1.2 MPa). However, at low Ψ_w, these P/V curve data showed less protoplast volume reduction than was found with the dual label infiltration technique (Fig. 1B). Therefore, the volume/Ψ_w relationship delineated from P/V curves using the air-drying technique was investigated further. P/V curves were constructed using the air-drying technique on leaves with, and without the secondary veins. The volume/Ψ_w relationship at leaf Ψ_w below −1.2 MPa was evaluated from these P/V curves (Fig. 1C). It was thought that since the dual label infiltration technique uses only interveinal areas of the leaf laminae, then removal of the secondary veins may cause the P/V curve data to more closely follow the relationship developed from the dual label infiltration technique. As shown in Figure 1C, this was the case. The data points from P/V curves constructed on leaves with secondary veins removed fell on the line developed from the dual label infiltration experiments, with approximately 50% protoplast volume reduction occurring at −1.8 MPa.

We interpret the data presented in Figure 1 as indicating that the dual label infiltration technique delineates essentially the same relationship of declining protoplast volume at low Ψ_w as determined from P/V curve experiments. These experiments, then, validate the usefulness of the dual label infiltration technique for examining the extent of protoplast volume reduction at declining leaf Ψ_w.

Evaluation of the RWC/Protoplast Volume Relationship

P/V curve analyses and dual label infiltration studies indicate that as leaf Ψ_w drops below −1 MPa in spinach leaves, substantial protoplast volume reduction can occur (Fig. 1). These results suggest that RWC may not be an adequate indicator of low Ψ_w effects on cell metabolism, if the adverse effects of low Ψ_w are caused by dehydration-induced concentration of cell solutes.

This point is supported by the results shown in Figure 2. Data from P/V curve constructions using the xylem sap collection technique (Fig. 2A), and the air-drying technique (Fig. 2B) were used to investigate the relationship between RWC and protoplast volume reduction. The line drawn through the origin in Figure 2, A and B, delineates this relationship at BWF of 0%. Deviations of the data from this line are due to the effect of the calculated BWF (approximately 40% in both cases, as shown in Table I). These data indicate that in spinach leaves, especially at low RWC, estimation of the extent of protoplast volume reduction by RWC values may be in error. For example, at a RWC of 75%, protoplast volume is reduced by approximately 40% (Fig. 2, A and B).

SUMMARY

The results of experiments described in this report are interpreted as validating the BWF estimation in spinach leaves

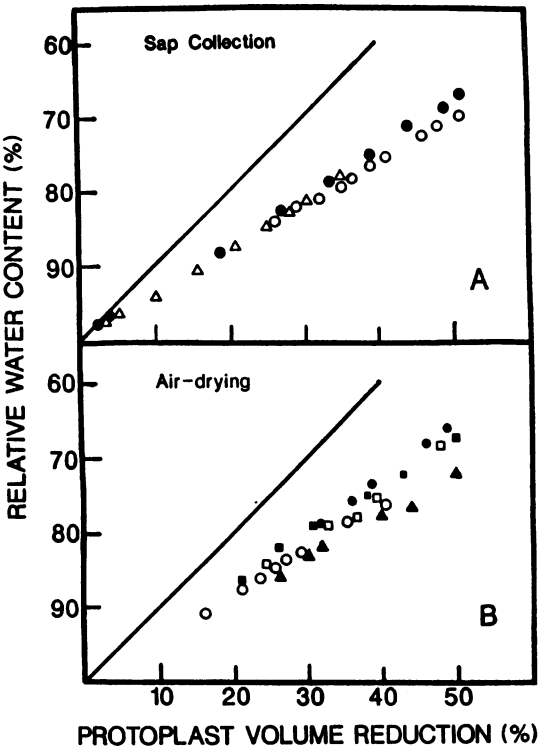

Figure 2. Relationship between relative protoplast volume and RWC as determined with P/V curves constructed using the sap collection (A) or air-drying (B) techniques. The solid line passing through the origin in each of the panels represents the theoretical volume/RWC relationship of BWF = 0.

from P/V curve analysis. This high (40%) BWF value results in the calculation of substantial protoplast volume reduction at leaf Ψ_w below −1.0 MPa. Dual label infiltration measurement of this relationship yielded similar results. It was also concluded that especially at low RWC, water deficit-induced protoplast volume reduction in the mesophyll of spinach leaves may be substantially underestimated by the RWC measurements.

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