Plant Physiol. (1992) 98, 198–205
0032-0889/92/98/0198/08/$01.00/0

Received for publication May 15, 1991
Accepted August 17, 1991

An Improved Method for Using Electrolyte Leakage to Assess Membrane Competence in Plant Tissues[1]

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ABSTRACT

A new expression for ion leakage from plant tissue, the tissue ionic conductance (g_{Ti}), is compared with electrical conductivity (EC) and a commonly used damage index (I_d) to test the ability of each expression to correctly describe leakiness in two model systems representing examples of physiological processes with well-known effects on membrane permeability. In experiments in which drought-acclimated leaves were compared with nonacclimated leaves and senescing leaves were compared with nonsenescing leaves, I_d contradicted our expectation that acclimated tissue would be less leaky than nonacclimated tissue, and g_{Ti} and EC confirmed this expectation. In a comparison of senescing and nonsenescing tissue, I_d again contradicted our expectation that senescing tissue would be more leaky than nonsenescing, and EC and g_{Ti} were confirming. Using a diffusion analysis approach, we show that I_d fails to account for variation in the concentration gradient between the tissue and the bathing solution and variation in the surface area through which efflux occurs. Furthermore, because I_d is a parameter that relates treatment performance to control performance as a percentage value, it distorts the actual differences among treatments. The resulting artifacts lead to a presentation of membrane integrity which is probably incorrect. EC is a more direct measurement of net ion efflux and appears to be less vulnerable to artifact. However, because g_{Ti} is the only expression that explicitly includes chemical driving force and tissue surface area, it is the most reliable of the three expressions.

Measuring solute leakage from plant tissue is a long-standing method for estimating membrane permeability in relation to environmental stresses, growth and development, and genotypic variation. Early published accounts used total electrolyte leakage, expressed as specific conductance (EC[2]) of the aqueous bathing solution in which the tissue was immersed, to indicate degree of damage resulting from chilling injury (3, 4). Recognizing that electrolyte content could vary among samples, Stuart (23) recommended expressing electrolyte leakage as an index percentage of total electrolyte present in the tissue. Flint *et al.* (6) reduced Stuart's calculation to the following form:

$$I_d = 100\ (R_t - Ro)/(1 - R_o) \quad (1)$$

where, according to the terminology of Flint *et al.* (6), I_d is the index of injury, $R_t = EC_{initial}/EC_{total}$ for stressed tissues, $R_o = EC_{initial}/EC_{total}$ for nonstressed tissues, and $EC_{initial}$ is the conductivity of the bathing solution following a given period of leakage, EC_{total} is the conductivity of the bathing solution following heat killing to release all ions from the tissue. This calculation of I_d adjusts all values to a scale of 0 to 100, with higher percentages indicating greater damage.

To generate data for calculating I_d, the standard procedure is to remove appropriate samples of plant tissue, bring the samples to full hydration, rinse off surface adhering electrolytes and contents of cells damaged by excision, and then impose an environmental stress such as dehydration (1, 5, 7, 12, 19), high temperature (8, 13, 18, 21, 25), or low temperature (2–4, 9, 23, 27, 28). The assumption is that it is necessary to stress tissue *in vitro* to elicit differences among samples. Leaf discs cut with a cork borer are common sample units, although stem or root segments have also been used. To obtain data for calculation of R_t, a common procedure is to place samples in standardized volumes of glass-distilled water and allow them to leak solutes into the bathing solution for 12 to 24 h, at which time $EC_{initial}$ for the solutions is measured using a solute bridge. Following measurement, samples are killed by autoclaving or rapid freezing in liquid N_2 and allowed to leak for an additional period, typically 24 h. The conductivity is then remeasured to obtain EC_{total}. R_o is calculated from measurements obtained from nonstressed control samples that have been monitored along with the stressed samples. Many recent applications of the technique have used similar procedures and either the above formula or one that yields the same value (for example, see ref. 13).

This technique has enduring appeal because it is simple, uses readily available and inexpensive equipment, and is suited to analyzing large numbers of samples. Despite these advantages, I_d is flawed in ways that make interpretation difficult and potentially misleading. The lack of adequate theoretical derivation makes it unclear exactly what I_d measures in relation to membrane competence. I_d is a scaled value between 0 and 100 measured at a moment in time following an arbitrary and lengthy incubation period. Although studies using I_d frequently claim to assess "membrane stability" or

[1] Supported by an R.P. White research grant from the Horticultural Research Foundation and Hatch projects NE-27 and NY-430 to T.H.W.

[2] Abbreviations: EC, electrical conductivity; RWC, relative water content.

"membrane permeability," the I_d calculation includes none of the variables typically used to describe ion movement across a membrane. I_d cannot be interpreted as a flux and does not take into account activity or electrical gradients that could be related to membrane permeability in a defined, quantitative fashion. This suggests that I_d cannot discriminate between membrane characteristics and other confounding variables that affect flux. Our preliminary attempts to use I_d to rank genotypes and acclimation treatments in relation to leakiness indicated that both leaf thickness (hence surface area of the cut disc margin) and RWC varied along with the independent treatment variables under study. Failure to account for variation in leaf thickness ignores the fact that flux of any solute varies with the surface area through which flux occurs. Failure to account for variation in RWC neglects the influence of solvent volume on ion concentration inside the leaves, which in turn determines the electrochemical gradient driving ion efflux. As we will show, neglecting these variables causes artifacts that lead to erroneous conclusions about the leakiness of the tissues being compared.

These limitations prompted us to modify the traditional I_d protocol to permit calculation of an empirical coefficient, which we call g_{Ti}, which takes into account variation in tissue thickness and electrolyte concentration and provides a dynamic picture of recovery following stress. Our goal was to retain the convenience of traditional protocols while deliberately factoring out sources of variation not directly related to membrane characteristics. In this paper, we present a method for deriving g_{Ti} and use two prototype systems to compare g_{Ti}, EC, and I_d methods for evaluating tissue leakiness.

METHODS

Derivation of g_{Ti}

In the simple case of an uncharged solute, Fick's first law of diffusion states that flux through a planar surface is a function of driving force and the resistance of the medium through which the flux occurs. In a membrane-bound system, driving force is determined by the concentration gradient across the membrane and resistance is determined by the surface area and permeability of the membrane for the solute. In the case of a charged solute such as an inorganic ion, flux is affected by the gradient in electrical potential across the membrane in addition to the gradient in chemical potential. Ideally, any method purporting to quantify membrane impairment in plant tissue would include direct measurements or estimates of all variables governing flux. With experiments involving many comparisons, as in germplasm screening, it is not practical to measure the electrical gradient and the surface area of the plasma membrane. However, estimates of the average concentration gradient of an ion and the area of the cut surface through which flux occurs are easily obtainable within the framework of familiar protocols. These estimates serve as the basis for the g_{Ti} calculation, which effectively removes them as confounding variables.

Nobel (16) derived the following expression from Fick's first law which shows explicitly how area, concentration gradient, and a conductance coefficient influence flux:

$$J_j = P_j A(c_j^o - c_j^i) \quad (2)$$

where $J_j = ds_j/dt$, the flux of solute species j per unit time, P_j is a permeability coefficient for solute species j, A is the surface area through which diffusion occurs, and $(c_j^o - c_j^i)$ is the difference between outside (or higher) concentration and the inside (or lower) concentration. Drawing on the formalism of Eq. 2 and substituting g_{Ti} for P_j and area of cut surface for A, we solve for g_{Ti}, obtaining:

$$g_{Ti} = J_j/A(c^o - c^i) \quad (3)$$

This empirical expression ignores the contribution of membrane potential to the electrochemical gradient but adjusts for tissue characteristics that might otherwise be confused with behavior of membranes. We make no claim that g_{Ti} is a direct estimate of membrane permeability and discourage others from making similar claims. We justify the liberty of ignoring the electrical component on the grounds that it is preferable to remove known sources of variation that are estimable even though other, essentially nonestimable, sources still remain. We have borrowed the symbol g, for conductance, from leaf gas exchange terminology. We propose the term g_{Ti} instead of P_j not to proliferate new terminology but to emphasize the important departures from the definition of the permeability coefficient. First, g_{Ti} is based on net flux for bulk tissue, not independent measurements of influx and efflux from an isolated cell. As such, g_{Ti} represents average concentration gradient and leakiness of all cells in the sample. Heterogeneity in concentration among cells and poor mixing in the apoplasm will affect the behavior of individual cells that cannot be resolved. Second, g_{Ti} is not based on surface area of the plasma membrane but rather the planar area of the cut disc margin. The true membrane surface area is much larger, and concentration gradients for internal cells that are not directly exposed to the bathing solution are likely to be less steep. Additionally, treating the cut surface as a smooth cylinder is itself an approximation that underestimates total surface area at the disc margin. In reality, the surface is three dimensional and consists of cell wall and cell lumina with adhering fragments of plasmalemma. (Because the cut perimeter of the disc is typically exposed to glass-distilled water, any unbound protoplasts would burst.) Third, P_j is usually interpreted as describing the physical properties of the membrane independently of the membrane potential.

General Experimental Protocol

Our protocol followed standard I_d procedures except that we measured fresh and dry weights and measured EC repeatedly during a 24-h experiment. Leaves were cut before dawn, and petioles were recut under glass-distilled water and allowed to hydrate in a cool (20 ± 2°C) dark laboratory. After 3 h, five 9-mm diameter discs were punched from each leaf with a cork borer and placed in beakers containing 50 mL glass-distilled water to remove the contents of cells lysed by the borer. Punches avoided major veins. Beakers were agitated periodically by hand. A sample consisted of five leaf discs (one disc from each leaf), with eight to 10 replicate samples per treatment. After 1 h, leaf discs were removed from the

water, placed on laboratory tissues (KimWipes) previously saturated with glass-distilled water, and sealed in small plastic bags (WhirlPacs). Half of the samples were randomly selected and removed from the bags, blotted dry, and weighed to obtain fully hydrated weights. These samples were then allowed to dry on the laboratory bench until they reached 80% of their fresh weight. This level of stress had been previously determined to be the operational limit for discs to regain full hydration following stress. Following the secondary dehydration stress, discs were quickly placed in small cylindrical stainless steel screen cages mounted on stainless steel rods and suspended inside test tubes over 7 mL glass-distilled water equilibrated to 25°C in a water bath (model 25, Precision, Chicago, IL) oscillating at 1 cycle s^{-1}. The remaining nonstressed discs were removed from the plastic bags, quickly blotted, weighed, and treated identically with the stressed samples. At time 0, all samples were plunged into the glass-distilled water. At predetermined intervals, the samples were removed from the bathing solutions and the conductivity of the solutions was measured with a conductivity meter (model 32 equipped with a model 3417 electrode, Yellow Springs Instruments, Yellow Springs, OH). The cage facilitated rapid submersion and removal from the distilled water, ensured that all discs were fully submerged, and provided a means for holding the leaf discs in the humid headspace of the tube during those periods when EC was being measured. After the initial 120 min, during which time measurements were frequent, the tubes were covered with Parafilm to retard evaporation. EC of the bathing solution was measured at predetermined intervals for 24 h, at which time all samples in their cages were plunged into liquid N_2 to kill the tissue. Samples were then replaced in their respective tubes for an additional 24 h, at which time final conductivity was measured. Dry weight of each sample was measured after 24 h of drying at 70°C in a convection oven.

Calculations

Several quantities must be measured or derived to solve Eq. 3. These include the following.

Internal Aqueous Volume

Internal aqueous volume was determined gravimetrically. The weight of the leaf discs was measured with an electronic balance with 0.1-mg resolution (model AE 163, Mettler Instrument Corp., Hightstown, NJ). Initial solvent volume of the plant tissue was determined by subtracting oven-dried tissue weight from fresh weight. For nonstressed samples, this value was assumed to be the total internal solvent volume and was constant for the duration of the experiment. Stressed leaf discs that had been dried to 80% of their initial fresh weight experienced a rapid increase in internal water content during rehydration, substantially affecting internal ion concentration and hence the concentration gradient between the discs and the external solution. To account for this, leaf discs were subsampled from the same leaves used in the efflux experiments, dried to 80% fresh weight, and weighed at 1-min intervals until they regained full hydration. Curves from these rehydration experiments were used in conjunction with fresh and dry weights to estimate internal water volume at intermediate times coinciding with *EC* measurement in the main experiment.

External Volume

Glass-distilled water was dispensed with a bottle top dispenser that had been calibrated to deliver precisely 7 mL. We assumed that volume remained constant during the experiment.

Ionic Concentration

At dilute concentrations, EC is a positive, nearly linear function of the concentration of all ions in solution. This relationship is widely applied in routine soil and water analysis. Instead of calculating total ion concentration from EC values using empirical coefficients developed by others, we selected potassium as a representative ion because it is the major inorganic cation present in glycophytes (12, 17). It should be noted that using $[K^+]$ in lieu of EC is not essential but is used in our demonstration because it provided a convenient way to check the accuracy of our estimates of internal concentrations against published values. Palta *et al* (17) provide independent verification of the strong relationship between EC and $[K^+]$ in aqueous solutions containing effusate from plant tissue. In our study, empirical regression equations relating EC to $[K^+]$ were developed for each plant species and leaf type. Bulk subsamples of leaves similar to those used in efflux experiments were washed in glass-distilled water, blotted dry, inserted into 30-mL plastic syringes, and frozen at −20°C. After thawing, leaf sap was squeezed from the leaves, and aqueous serial dilutions were made over a range that bracketed the EC values measured in the external solutions in the efflux experiments. Ion concentration of these solutions was measured using an inductively coupled argon plasma atomic emission spectrometer (Jarrell-Ash model 975).

Because the external volume was a known constant in all experiments, concentrations in the external solution were easily convertible to absolute amounts, and net efflux could be calculated for each time interval. Internal concentrations were calculated by freezing the tissue in liquid N_2 at the end of the experiment and permitting all ions to leak from the tissue. The total concentration in the bathing solution was derived from EC using our regressions equations. Because the ratio of internal to external solvent volumes and the amount of K^+ leaving the leaf discs both could be estimated, internal concentrations could be calculated for any appropriate time interval. The concentration gradient was calculated as the difference between internal and external concentrations.

Area

There are two parallel pathways for ion efflux: one through the intact upper and lower epidermis and another through the cut perimeter of the leaf discs. The resistance of the cut surface is much lower than the resistance of the intact epidermis and cuticle; therefore, the flux through the ab- and adaxial surfaces can be considered negligible (22). Disc thickness was determined by water displacement from a 30-mL pycnometer bottle. Assuming a cylindrical shape,

$$T_{av} = (V/A_d)/n \quad (4)$$

where T_{av} is average disc thickness, V is total displaced volume by the sample, A_d is the circular area of the disc, and n is the number of discs in the sample. Discs were subsampled from each leaf used in the efflux experiment for determination of leaf thickness. Batched samples of 20 to 40 discs were sufficient for accurate determination of T_{av}. Discs were submerged individually to avoid trapping bubbles between them.

Experimental Design and Plant Material

Two model systems were selected to represent different physiological phenomena that have been shown in other studies to be accompanied by changes in electrolyte leakage. These phenomena were stress acclimation and senescence. Stress acclimation has been shown to make membranes less leaky (12), and senescence and aging are associated with increased leakiness to ions (24). These systems allowed us to pose *a priori* hypotheses about experimental outcomes that could be used to test the ability of each computational method (*i.e.* EC, I_d, and g_{Ti}) to correctly describe the behavior of plant tissues. By using two independent examples of processes and species, we intended to establish a general approach with broad application. The specific systems used were drought acclimation in crab apple and senescence in red oak.

Experiment 1: Drought Acclimation in Crab Apple

Hypothesis: Leaves acclimated to slowly imposed, sublethal drought stress will be less leaky to ions than nonacclimated control leaves.

Prairie crab apple (*Malus ioensis* 'Klehm's Improved' scions grafted on *Malus baccata* rootstocks) was selected as a woody species in which drought acclimation was known to occur (20). Thirty bare root 1-m tall whips were trimmed back to three basal buds and planted in a 1:1:1 peat:soil:perlite mix in 130-L plastic trash cans. Plants were kept outdoors, receiving tap water as needed and weekly application of complete nutrient solution (20 N-10 PO_5-20 K_2O applied at a rate equivalent to 200 ppm N). After 14 weeks, all plants were placed in a greenhouse (day 21°C/night 16°C) to permit supplemental illumination with incandescent lights between 5 and 11 PM to delay bud set. Half of the trees were designated as controls and were watered regularly to maintain predawn leaf water potentials of approximately −0.33 MPa. The remaining trees were subjected to drought stress which consisted of withholding all water until predawn leaf water potentials (measured with a SoilMoisture water status console, Santa Barbara, CA) reached approximately −2.0 MPa. This required 27 days. At this time, leaves from five control and stress trees were selected and handled as discussed above in the general protocol.

Experiment 2: Senescence Effects in Red Oak Leaves

Hypothesis: Ion leakage will be greater from tissue at a more advanced stage of autumnal senescence than tissue in which senescence is less advanced.

Individual leaves of many deciduous trees develop autumn color in patches or zones, presumably indicating small-scale variation in onset of senescence in tissue that had previously appeared quite uniform. In red oak, *Quercus rubra*, coloration changes from green to yellow as Chl breaks down and ultimately turns brown just before abscission. On November 3, mottled leaves showing patches of yellow and green coloration were cut from a 69-cm diameter breast height red oak growing on the Cornell campus. The tree was growing in the open under ambient conditions and had shown no previous symptoms of abnormal growing conditions. Five leaf punches were cut from either yellow or green patches on each of eight leaves to yield eight, five-disc batch samples of each color per leaf. This sampling procedure ensured that yellow and green samples were as similar as possible in all respects except degree of senescence. Discs were handled according to the general protocol previously described, with four batches assigned to each treatment.

RESULTS

Experiment 1: Drought Acclimation

After 15 min of leakage, EC in the bathing solution averaged 4.93 μS for dehydrated leaves and 2.72 μS for fully hydrated leaves (Fig. 1A), indicating more leakage of ions from leaves dried to 80% of their fresh weight. Although the difference between dried and nondried leaves decreased over time, dried leaves had consistently higher EC values for 6 h. After 6 h, acclimated tissue had consistently higher EC values than nonacclimated controls regardless of subsequent stress treatment. Within an acclimation treatment, tissue dehydration resulted in higher EC values during the course of the experiment. This is especially apparent after 6 h (Fig. 1B). After 24 h, EC values for drought-acclimated leaves averaged 10.11 and 14.15 μS for control leaves (Fig. 1B).

Tissue ionic conductance showed the greatest differences among treatments during the first 5 min (Fig. 1C). After the first minute, g_{Ti} for dehydrated tissue averaged $5.48 \cdot 10^{-12}$ $m \cdot s^{-1}$, and fully hydrated samples averaged 0.78×10^{-12} $m \cdot s^{-1}$ (Fig. 1C). After 10 min, however, all four treatment combinations converged on an average $g_{Ti} < 0.1 \times 10^{-12}$ $m \cdot s^{-1}$ for the duration of the experiment. Although the differences were small in magnitude, acclimated leaves had lower conductances than control leaves during the entire 24 h time course (Fig. 1D). After 24 h, g_{Ti} values were 0.029 and 0.011 $\times 10^{-12}$ $m \cdot s^{-1}$ for control and acclimated leaves, respectively.

Both EC and g_{Ti} consistently ranked drought-acclimated leaves less leaky than controls. In contrast, I_d ranked acclimated tissues more damaged than controls (Fig. 1, E and F). It is interesting to note that I_d showed both control and acclimation treatments becoming more damaged during the first 10 min of the experiment (Fig. 1E). Because this is not reflected in the primary data (EC), this is almost certainly an artifact of the computation. Comparisons between control samples and those dehydrated to 80% of fresh weight are not possible using the I_d expression because damage is expressed as a percentage of control.

Experiment 2: Senescence in Red Oak Leaves

Red oak leaves followed a pattern similar to crab apple leaves, with EC increasing fastest during the first 15 min and

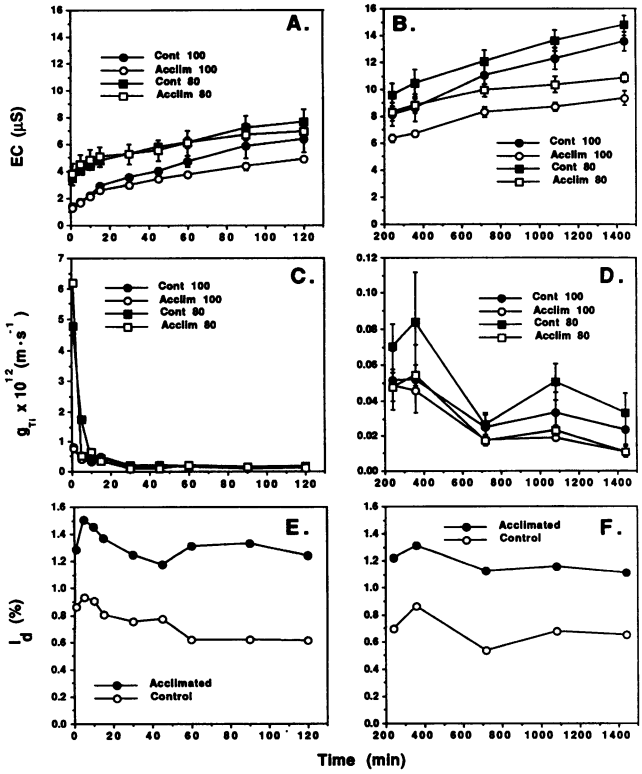

Figure 1. Comparison of apparent ion efflux from drought-acclimated (Acclim) and nonacclimated (Cont) crab apple leaf discs during 24 h. 100 and 80, discs that were either fully hydrated or dried to 80% of fresh weight, respectively. Points, averages of five replicates. Error bars, ± 1 SD; omitted from C for clarity and could not be computed for E and F because I_d uses average values for its calculation (see text). EC and g_{Ti} rank acclimated leaves less leaky than nonacclimated, whereas I_d consistently ranks acclimated leaves as leakier.

then increasing more slowly during the remainder of the experiment (Fig. 2, A and B). Initially, there was a nearly twofold difference in solution EC between control samples and those that had been subjected to a dehydration stress (Fig. 2A). Dried leaves averaged 4.26 μS and nondried averaged 2.42 μS after 15 min. After 4 h, tissue senescence had a stronger influence than the dehydration treatment, with yellow leaves leaking more than green leaves (Fig. 2B).

During the first 15 min, g_{Ti} declined from an initial range of 3.71 to 7.58 × 10^{-12} m·s^{-1} to a range of 2.32 to 7.47 × 10^{-13} m·s^{-1} (Fig. 2C). Tissues that had been dried to 80% of fresh weight were consistently leakier than controls, and within a drying treatment senescing leaves were leakier than green leaves (Fig. 2, C and D). Between 6 and 18 h, yellow leaves became increasingly leaky, and green leaves showed little change (Fig. 2D). After 24 h, g_{Ti} of yellow leaves had decreased to levels similar to green leaves.

Whereas EC and g_{Ti} rank yellow leaf samples leakier than green samples, I_d reverses this ranking consistently during the course of the experiment. The I_d increased slightly between 1 and 5 min and then remained relatively constant between 3 and 5% for 12 h (Fig. 2, E and F). Yellow leaf discs were more damaged than green for the first 2 h but less leaky thereafter. After 12 h, I_d showed that green leaves became slightly more damaged and yellow leaves became less damaged. I_d values for yellow leaves eventually became negative between 18 and 24 h. As in experiment 1, it was not possible to evaluate the effect of the dehydration treatment independently of senescence effects because the I_d calculation expresses dehydrated values as a percentage of control.

DISCUSSION

Results of the experiments reported here indicate that the I_d commonly used to express electrolyte efflux data contradicts our expectations about the relative leakiness of acclimated and senescing leaves and their respective controls. Failure to correctly rank leaf tissues by leakiness indicates that I_d is not merely unreliable, as reported by Murray *et al.* (15), it is fundamentally wrong. To accept the inferences drawn from the I_d calculations in our experiments, we would have to conclude that acclimated tissue was leakier than nonacclimated and that healthy tissue was leakier than senescing tissue. We submit that these conclusions are improbable and that I_d is in error. Although EC achieves rankings

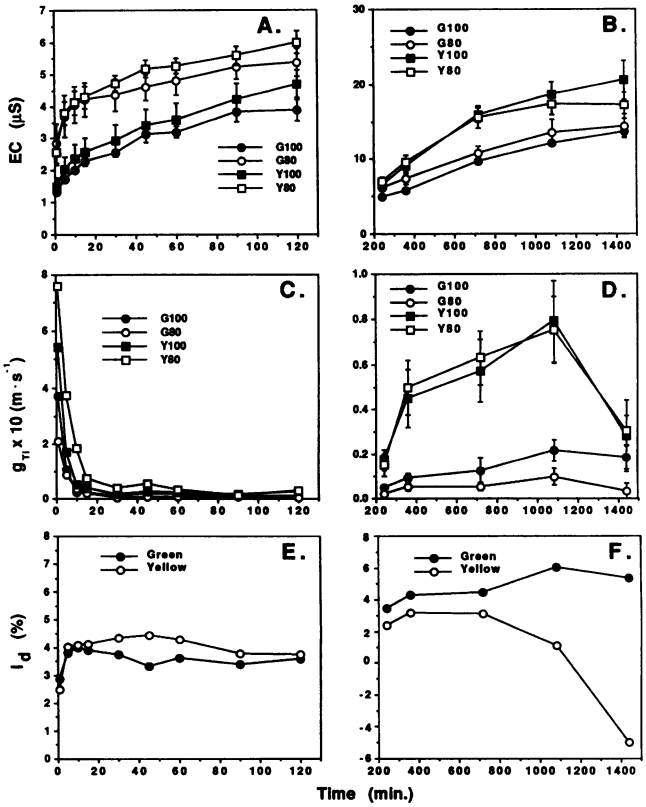

Figure 2. Comparison of apparent ion efflux from red oak leaf discs cut from either green, competent tissue (G) or yellow, senescing tissue (Y) during 24 h. 100 and 80, leaf discs that were either fully hydrated or dried to 80% of fresh weight, respectively. Error bars, ± 1 SD; omitted from C for clarity and could not be computed for E and F because I_d uses average values for its calculation (see text). EC and g_{Ti} show that green tissue is less leaky, whereas I_d shows yellow tissue as being less leaky.

consistent with our expectations, g_{Ti} is the only expression that explicitly corrects for variation in surface area and concentration gradient. Accordingly, it is more likely to yield a realistic picture of membrane competence than either I_d or EC.

The importance of including surface area in presentations of flux data was elegantly demonstrated by Smith and Epstein (22). Using leaf discs of constant thickness but varying diameter, they showed that Rb uptake appeared to decrease when expressed as a function of tissue fresh weight. When the same data were plotted against disc circumference, there was no trend. In this special case, leaf thickness was constant, and therefore, circumference was adequate for resolving fluxes from different samples into equivalent units so that reliable comparisons could be made. If leaf thickness had varied, however, expressing flux in relation to circumference would not have adjusted uptake data so that valid comparisons could be made. Surface area, rather than length, of the cut margin is the more appropriate measurement. Extending this argument to efflux experiments, it is clear that area of the cut surface will affect net efflux. Disc diameter was constant in our experiments, so the major uncontrolled variable affecting cut surface area was leaf thickness. All else being equal, the surface area through which flux occurs will be rate limiting, and unless it is included as a variable, apparent variation in leakage cannot be attributed to alteration of membrane properties. To dramatize the magnitude of variation even among similar samples, consider that, in our study, drought acclimation caused crab apple leaves to increase in average thickness from 0.0206 to 0.0258 cm, corresponding to a 25% increase in cut surface area. Neglecting this difference would tend to make acclimated leaves appear leakier than they really are. This is illustrated by comparing the spread in the data in Figure 1, A and B. Treatment differences suggested by the raw EC of the bathing solution are substantially greater than the differences shown by g_{Ti}, in part because variation in surface area is not included in the EC term. If leaf thickness shows this much phenotypic plasticity within a single vegetative apple clone, it is reasonable to expect similar variation among cultivars and ecotypes of other species and even greater variation among plants from more distantly related taxa.

Concentration gradients are recognized as a major component of the electrochemical driving force for ion movement. Furthermore, it is well-known that both leaf water content and K^+ content, which together determine internal concentration, vary with age, stress history, and other factors (26). I_d confuses ion content with ion concentration and fails to compensate for differences in chemical driving force. The effect of concentration gradient on tissue conductance is shown graphically by comparing the initial behavior of samples that had been dried to 80% of their fresh weight with nondried controls. The apparent rapid decline in g_{Ti} in dehydrated leaves during the first 20 min after submersion reflects the sensitivity of the calculation to rehydration (Figs. 1C and 2C). As water re-enters the discs, the internal solution volume increases, resulting in a decrease in the average internal ion concentration and concomitantly decreasing the the concentration gradient between the leaf disc and the external solution. Of the three expressions, g_{Ti} is most sensitive to this change. It is unlikely that this is a computational artifact because EC, a measure of efflux not subjected to arithmetic manipulation, reflects the same phenomenon by increasing more slowly over time as the discs recover full hydration.

Limitations of Traditional Approaches

In addition to their uncertain relationship to ion transport theory and failure to arrive at a true determination of tissue leakiness, the traditional protocols, EC and I_d have other problems. The fallibility of taking only one measurement is illustrated by the variation in ranking over time in the senescence experiment. EC showed that dehydrated leaves leaked more than fully hydrated leaves for the first 120 min, whereas g_{Ti} consistently showed that yellow leaves were leakier than green regardless of drying treatment. Although it could be argued that leaf color is not a reliable indicator of senescence or that senescing tissue might not always be leakier, it is unlikely that leaf tissue damaged by dehydration would in fact be less leaky than nondamaged control samples. Both EC and g_{Ti} ranked yellow leaves more leaky than green leaves after 24 h, although the absolute differences were small. Again, I_d was contradictory, ranking green leaves leakier than yellow after 24 h and yielding negative values for yellow leaves. This points to a second problem specific to the I_d. It is apparent from Eq. 1 that negative values will result whenever $R_o > R_i$. Although negative I_d values could be interpreted in terms of the relative performance of stressed and nonstressed tissues, this exercise is needlessly convoluted. We suggest that expressing the behavior of stressed tissue as a percentage of nonstressed should be avoided.

Our results show that using EC measurements as a direct estimate of net flux can achieve the correct relative ranking of treatments, although the magnitude of differences may be either exaggerated or diminished. Recently, Murray *et al.* (15) criticized traditional EC methods and advocated calculating rates of change from EC time-course data. As shown earlier, flux is proportional to concentration gradient and surface area. Flux values derived from EC will lead to correct interpretations if, and only if, both variables are constant among samples. The same caveats apply to expressing EC as a percentage of the total in the same sample. When accounting for variation in concentration gradient, one must recognize that concentration is a function of both ion content and the internal aqueous volume.

We emphasize that it is not the use of the specific conductance of the external bathing solution *per se* that causes problems. Although we have converted EC to $[K^+]$ to permit comparison of our estimates with published values for internal concentrations, this is largely unnecessary if a simple ranking of samples is all that is desired. EC could be substituted for ion concentration in the calculation of g_{Ti} with minimal effect, and the units still cancel to meters per second. It should be noted, however, that the relationship between EC and $[K^+]$ is slightly curvilinear even at dilute concentrations, so using EC in the g_{Ti} calculation introduces a small error.

Advantages of a Revised Protocol

The protocol described in this paper has several attributes that make it superior to the alternatives currently available.

Establishing a time course for tissue leakiness is preferable to single point measurement regardless of the way the data are manipulated. Time-course studies are less arbitrary and provide a picture of recovery kinetics that the I_d expression cannot and also permits applying different criteria for damage assessment. For example, failure of stressed tissues to demonstrate recovery of tissue ionic conductances similar to undamaged controls could be used to infer irreversible damage. Additionally, the rapidity of recovery after the stress is relaxed may be of diagnostic value. It is more likely, however, that the initially rapid change is due to processes unrelated to loss of semipermeability and that inferences drawn during this period are misleading. In this way, the initial transience could be used to establish operational criteria for selecting the minimum period required to obtain reliable information.

The expression g_{Ti} has the added advantage of correcting for variation extraneous to membranes and focus on variation in membrane behavior which is of primary interest. Because studies using I_d sample only once during the efflux period and use averages of several replicates in the calculation, noise in the data is usually undetected and is rarely addressed. In a noteworthy exception comparing six tree species during a growing season, Martin *et al.* (12) resorted to seventh order polynomial transformations to eliminate scatter in their data. Our experiments indicate that environmental history, phenology, and small-scale differences over the surface of an individual leaf all contribute variability. Although statistical transformations are powerful tools with many useful applications, higher order polynomial equations are difficult to interpret and contribute little to our understanding of physiological mechanism. We suspect that much of the unexplained variation will be eliminated if the nonmembrane tissue attributes affecting ion efflux are taken into account. In any case, we can think of no reason for not applying corrections for easily measured variables such as concentration gradients and tissue thickness that affect ion efflux independently of membrane characteristics.

A final advantage is that g_{Ti} calculations give access to the entire data set because the control and stress samples are not combined in a single index. This suggests the possibility of directly assessing tissue leakiness without having to impose a stress (dehydration, chilling, heating, etc.) as well as providing an opportunity to evaluate differences that are apparent only when tissues are stressed. This is especially attractive because traditional electrolyte studies have sometimes imposed arbitrarily severe stresses to elicit differences (14). And, because any experimentally applied stress is likely to affect the aqueous volume of the tissue, cell geometry, and the configuration of free space in addition to compromising membranes, the net effect of stress to the tissue will be complex and ill-defined. Against this background, unambiguous inferences about membrane behavior are made even more difficult. It would be advantageous to avoid deliberately introducing these problems.

Potential Limitations of g_{Ti}

Despite the many advantages, we recognize that the g_{Ti} protocol is subject to the same limitations present in any study using bulk tissues. For example, inadequate mixing of the apoplasmic and bathing solutions at the disc margin would reduce the apparent flux to the outside. In our experiments, uptake from the apoplasm surrounding internal cells is also likely to affect net flux at the margin of the leaf disc. By comparison, uptake from the external solution was probably quite low even assuming the operation of a high-affinity uptake system (10) because external [K^+] never exceeded 78 μM even after 24 h of efflux.

We caution that, although the EC efflux curves resemble washout curves used for compartmental analysis, typical electrolyte efflux studies are inherently nonsteady state and hence violate a fundamental assumption of compartmental analysis. It would be feasible, and we suggest worthwhile, to interpret a *bona fide* steady-state compartmental analysis in terms of variation in membrane competence in response to various treatments. Differences in pool sizes, rate constants, and half-times for different compartments could be diagnostically useful.

The typical objective of electrolyte leakage studies is to assess injury, presumably at the membrane level, resulting from an environmental stress. Leakiness will vary in relation to the membranes' abilities to take up and retain solutes and, therefore, will reflect stress-induced changes in both membrane potentials and membrane permeability (14). We suggest that g_{Ti} is sensitive to the net changes in both, although independent membrane potential measurements would be required to determine the relative contributions of these components to flux. This is not a problem as long as the experimental objectives are appropriately coarse, as in screening studies. Inappropriate applications of the technique include prediction of the direction of flux or estimation of unidirectional fluxes. In the experiments reported here (and probably all similar ion efflux experiments), the plant tissue is the sole source of solutes in the bathing solution and net outward flux occurs throughout the experiment; hence, the issue of the direction of flux typically will not be of concern. Furthermore, observations indicate that even after 24 h, a substantial concentration gradient still existed while efflux had slowed and was not changing rapidly with time, indicating that membranes were still capable of regulating efflux. These conditions may not hold for all systems, however, which underscores the need for time-course experiments.

There is nothing to suggest the plant tissues used in our experiments were unusual in ways that might compromise the general application of our findings. EC values have not been manipulated arithmetically and provide qualitative evidence that our systems are not idiosyncratic. Although the exact shape of the curves varies, in both of our experiments, plots of EC against time show a decline in efflux over time which is consistent with similar reports of efflux kinetics (7, 11). Finally, although the I_d values are small in relation to many reports using leaves from herbaceous species, our values are within the range reported for red, white, and black oak and black walnut that had been dehydrated to water potentials of -1.5 to -2.0 MPa (15).

CONCLUSION

The expression tissue g_{Ti} is an improvement over traditional expressions for drawing inferences about membrane behavior

from observations of electrolyte leakage. In contrast, I_d is highly susceptible to artifacts and should be abandoned. The reliability of conclusions drawn from the numerous studies in which I_d has been used to evaluate membrane injury is thus in question. It would be prudent to reevaluate any studies that may have served as the basis for breeding programs and other long-term research. Raw EC of the bathing solution and EC expressed as a percentage of the total for a given sample may be useful for determining relative tissue leakiness if internal ion concentration (not content) and cut surface area are constant among samples.

ACKNOWLEDGMENTS

We have appreciated the stimulating discussions with Drs. Arnold Bloom, Jaleh Daie, Jay Jacobson, Carl Leopold, Richard Lovelace, Jack Paul, Roger Spanswick, Robert Turgeon, and Anne Wrona during the course of this study. We offer special thanks to Drs. Leon Kochian, Peter Marks, Kenneth Mudge, and David Wolfe for their conscientious reviews of the manuscript and the comments of three anonymous reviewers.

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