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Plant Viability Assay[1]

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In its broadest sense, viability is the ability to live, grow, and develop. Ideally, therefore, a viability assay should measure all three of these properties. In practice, however, viability is often used in a narrower sense, e.g., the vitality of a mature cell which is no longer able to grow or develop. Most assays, therefore, measure, only one of these three properties, or even a single component of one of them. Even for such more limited purposes, viability assays have not been adequately standardized. There are several reasons for this deficiency. (i) In a multicellular organism (as in any population of cells) all cells do not die simultaneously. (ii) Even within a cell, different organelles cease to function at different times. (iii) In order to be truly useful, the test should determine not only whether or not the treated cell, tissue, organ, or organism is alive at the time of assay; but also how well it will continue to fulfill its normal life functions in relation to a control, untreated cell, tissue, organ, or organism. The last of these problems is the most difficult one to deal with.

In this review, an attempt has been made to analyze some of the viability tests used most commonly by plant scientists, without attempting to be all inclusive. They will be classified according to the functions of the living plant material which they are intended to measure.

GROWTH

Ability of the plant material to continue normal growth seems to be the ultimate criterion of viability. The survival of cells after freezing, for instance, has been assayed by their ability to grow and form a distinct colony on agar (15, 40, 41). There are, however, some problems with this test. It is time consuming and difficult to quantify. Furthermore, a great many cells of plants are in the living state and yet not growing, for instance, bulb tissue of onion and cells in various tissues of trees. Growth has been more commonly used as a control for other tests. Yield of dry matter per unit area has also been used for estimating the extent of injury to a given crop (34).

PLASMALEMMA AND TONOPLAST MEMBRANE FUNCTIONS

Biological membranes are differentially permeable (30). Changes in the permeabil-

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ity properties of the protoplasmic layer were proposed by Osterhout (24) as a sensitive and precise indicator of the vitality of the tissue. The most popular way to evaluate the intactness of the membrane has been to test the ability of cells to plasmolyse in hypertonic, relatively impermeable, salt or sugar solution (10, 24). Plasmolysis has been used also as a standard for comparison with other tests and as a test for evaluating frost hardiness (20, 26, 27, 32, 41). It is sometimes difficult to distinguish between injured but still living and uninjured cells by this method (26, 27). Similarly, the absence of plasmolysis usually indicates cell death; but certain plant cells do not plasmolyse under natural conditions due to high cell wall attachment of the protoplast or impermeability of the cell wall to certain plasmolytica (cf. Ref. 35).

Considerable amounts of solutes are lost after cell damage and cell death. Often this leakage is considered an indication of irreversible damage, disregarding the possibility of cell recovery (25). On the basis of this leakage, Dexter *et al.* (9) showed that the measurement of electrical conductivity of the effusate provides a quantitative method for evaluating damage to plants by freezing stress. This method has become very popular for testing relative hardiness of plants (5, 17, 31, 38, 39, 42, 43). In the recent literature, the assumption has been made that the efflux of ions is mainly, if not solely, from dead cells. Therefore, the conductivity method is assumed to measure the freezing injury by determining the percentage of cells killed (31, 38, 39). We found this assumption to be invalid since injured yet viable cells (able to plasmolyse and stain vitally) can suffer extensive efflux of ions and sugars (26) which may be reversible or irreversible depending upon the extent of damage (27). Siminovitch *et al.* (33) found a very good relationship between amino acids released by the cells and their ability to stain vitally. Although this method seems to measure the dead cells, an advantage over the conductivity method, it has not yet been used very widely. Further work needs to be done to realize the full use of this method.

The ability of cell membranes to allow the molecules of a vital stain to permeate and accumulate inside the vacuole has been used as a very good criterion of membrane intactness. Many vital stains which can be used for this purpose are neutral red, acridine orange, toluidine blue, thionine and rhodamine B. Among these, neutral red has been used most commonly by many workers (20, 33). A disadvantage of this method is that one cannot distinguish between injured and uninjured cells since injured cells may also accumulate stains in the vacuole depending upon the extent of the injury whereas dead cells do not. The ability of the cell membranes to exclude certain other dyes, e.g., Evan's blue, has also been used as an indication of membrane intactness (11). Dead cells are stained by these dyes, whereas nonstained cells may be either injured but still living or uninjured.

Luyet and Gehenio (22) advanced the idea that living tissues have a double freezing point, whereas dead tissue has only a single freezing point. Based on this concept, McLeester *et al.* (23) developed a technique for rapidly evaluating the viability of freeze-injured plant tissues. Based on this method, cell damage has also been found to be related to spikes in the freezing curves (3) and to the low-temperature exotherms (12). Although this method may have potential for determining relative hardiness of different plants, the absence of a second freezing point in many tissues, the occurrence of two freezing points occasionally in dead tissue, as well as its qualitative nature are some of the problems associated with this method (37). Damage to the cell membranes has been found to be correlated with the drop in

TABLE 1

A List of Plant Viability Assays

Basis for the test	Viability test used	V[a]	I[b]	Reference
I. Growth	Ability to continue normal growth	+	1	8, 34
	Callus formation (tissue culture)	+	1	16, 40, 41
II. Plasmalemma and tonoplast membrane functions				
A. Passive functions	Plasmolysis frequency test	+	1 or 2	10, 24, 26, 27, 32
Differential permeability (leakage of ions, sugars, and amino acids, etc.)	Conductivity method	+	2 or 3	1, 5, 8, 9, 17, 25–27, 31, 38, 39, 42
Permeability to dyes	Ninhydrin method	+	2 or 3	33
	Vital staining of vacuole	+	1 or 2	20, 21 33
Impermeability to dyes	Absence of vacuole staining	−	3	11
Membrane intactness	Multiple freezing point method	−	2 or 3	22, 23, 37
	Impedance method	−	2 or 3	13, 42
Infiltration of tissue	Visual observations	+	2 or 3	6, 28
Ability to maintain turgor	Visual observations	−	2 or 3	4, 28
B. Active functions	No standard, easily applied test developed as yet			
Maintenance of electric potential		+	1 or 2	
Maintenance of active transport		+	1 or 2	
Activity of membrane associated enzymes		−	2 or 3	
III. Mesoplasmic functions				
A. Enzymatic functions	Activity of specific enzymes	+	1 or 2	18
	Browning test	+	3	5, 17
	TTC Reduction	+	2 or 3	19, 36, 37
Ability to respire	Oxygen uptake	+	1 or 2	2, 7, 29
	Carbon dioxide evolution	+	1 or 2	2, 7
Ability to metabolize	Labeled substance	−	2 or 3	44
B. Nonenzymatic functions	Cytoplasmic streaming	+	1 or 2	26–28
Ability to accumulate vital stains inside the protoplasm	Vital staining of protoplasm (fluorescent stains like uranin and acridine orange)	−	1 or 2	15, 41, 35[a]

[a] V = Validity of tests; valid only when result is as indicated (either positive or negative).
[b] I = Interpretation of results: (1) uninjured; (2) reversible or irreversible injury; (3) dead.

electrical resistance at a given temperature (13, 42). Although these methods have the potential for testing relative hardiness of different plants, they are only of limited value as viability assays. Moreover they are qualitative in nature.

Infiltration of the tissue with water and loss of turgor by the tissue (soft and soaked) are very commonly associated with freezing injury. They provide a qualitative estimate of viability of the frozen and thawed tissue (4). Usually infiltration of a tissue is taken as a sign of death (6). This is, however, not always true, as infiltration can be reversed depending upon the injury (28).

Active transport properties of the plant cell membranes have been found to be associated with various ATPases which are intrinsic membrane proteins. These ion

pumps have been reported to be the target of initial steps in freezing injury (27). They are responsible for maintenance of electric potentials across the cell membrane. Activity of these ATPases or direct measurement of electric potential could serve as a good criterion of viability of plant cells. They are not yet popular with frost hardiness researchers presumably because they are very laborious and time-consuming methods and require high skill. There is a promising future for the use of active transport as a criterion of viability.

MESOPLASMIC FUNCTIONS

The mesoplasm is the bulk of the protoplasm between the plasma membranes (plasmalemma and tonoplast) which contain the cell organelles and nonliving inclusions. The basic metabolic processes of the cell take place in the mesoplasm. Various mesoplasmic functions have been used as criteria of viability. These can be divided into two major groups, enzymatic and nonenzymatic. Among the various viability assays based on enzymatic functions, oxidative browning and reduction of 2-3-5-triphenyltetrazolium chloride (TTC) have been used most frequently (5, 17, 19, 36, 40). The browning test requires about 1 to 2 weeks for evaluation and is very qualitative in nature. It has been more commonly used as a control for other tests. The TTC method was developed by Kuhn and Jerchel (19) and has been used to test the viability of seeds as well as for the evaluation of frost injury. Although various improvements of this method have been made (36), the large variability among the replicates remains one of the major problems (37).

Heber and Santarius (14) have reported the effect of freezing on ATP synthesis. In addition, enzymes of the respiratory chain have been implicated as a target of freezing injury. Based on these results, respiratory oxygen uptake and carbon dioxide evolution have been used as criteria of viability (2, 7, 29). In plant tissues, however, the degree of injury is not always consistent with their rate of oxygen uptake. They can convert, for instance, to a completely anaerobic type of respiration. Moreover, injury to tissues frequently increases respiration rates, which further complicates the use of these methods. The ability of the plant tissue to metabolize some labeled substances has also been used as a criterion of viability (44). A serious drawback of all these enzymatic methods is that they do not demonstrate much more than the presence or absence of a particular cell function at a particular time under particular conditions.

Among the nonenzymatic functions associated with mesoplasm, the occurrence of cytoplasmic streaming and the ability of cytoplasm to accumulate vital fluorescent stains have been used as viability assays (15, 26–28, 41). Cytoplasmic streaming can be easily observed in large plant cells like onion epidermal cells, but it is quite difficult to observe in small cells in woody tissue. Fluorochromes on irradiation with uv give secondary fluorescence which can be easily observed using dark-room conditions. Dead cells do not accumulate certain fluorescent stains. The most commonly used fluorescent dyes are acridine orange, fluorescein diacetate, and uranin. One of the complications in the use of these dyes is due to the fact that a number of naturally occurring substances in plant cells (cell wall substances chlorophyll *a* and *b*, riboflavin) possess primary fluorescence.

INTERPRETATION OF VIABILITY TESTS

One of the most serious problems associated with plant viability assays is the interpretation of the results obtained from these tests. These assays test only an individual function or a few specific functions of the cell; furthermore, positive results may mean something, whereas nega-

tive results may be inconclusive and vice versa. Finally, as pointed out earlier, most of these viability assays give some idea about the present status of the cell or tissue but fail to predict the final performance of the plant. It has been shown that freezing injury in onion bulbs can be completely reversed depending upon the extent of the injury at the time of thawing (27, 28). The problems of interpretation are evident from the list given in Table 1 containing some of these viability assays. For example, in the tests using ability to continue normal growth, callus formation, ability to plasmolyse and cytoplasmic streaming as criteria only a positive result can rule out death but a negative result is inconclusive. Even a positive result in the tests using plasmolysis, cytoplasmic streaming, oxygen uptake, carbon dioxide evolution, activity of specific enzymes, and measurement of active transport properties does not allow distinction between uninjured and reversibly and irreversibly injured cells. Similarly a positive result in the conductivity test, ninhydrin test, TTC reduction test, and infiltration of the tissue does not allow one to decide between the injured and dead tissue although a negative result most of the time would indicate absence of injury.

One way to increase the validity of the results from one viability test is to correlate it with the results of other viability tests. Some workers use two or more tests simultaneously. There again interpretation can vary depending upon the results from accompanying tests. An example of this is shown in Table 2 and concerns our recent experiments on freezing injury to cells of onion bulbs (26, 27). Clearly, depending upon the conductivity of the effusate combined with vital staining of protoplasm and ability of the cells to plasmolyse, we were able to decide if the cells were uninjured, injured but living, or dead. By performing these tests in the post-thaw period, reversible injury could be distinguished from irreversible injury (Table 2).

TABLE 2

Interpretation of Viability Tests

Results of the viability tests		Interpretation
Conductivity of effusate	Vital staining of protoplasm and ability of the cell to plasmolyse	
Immediately after thawing		
No change	+	Uninjured
Increase	+	Injured
Increase	−	Dead
During the post-thaw period		
Decrease	+	Repair
Increase	+	Increased injury

SUMMARY

There is no single method in the long list given in Table 1 which can be used as an unequivocal criterion of viability. Many of the present methods of assay do correlate to various degrees with the final performance of the cell, tissue, organ, or the plant as a whole. Use of parallel viability tests indicating different cell functions is highly recommended. Great care should be taken to interpret the results of these assays. With several parallel tests, the validity of the interpretation can be enhanced, and in many cases, the interpretation may change considerably, depending upon the results from other tests. Since active transport systems have been implicated as one of the primary sites of freezing injury, more effort needs to be devoted to standardize viability assays based on this cell property. In general, the most popular viability assays for plants are based on biophysical rather than biochemical or metabolic functions. A specific test may be suited to a certain material more than another, yet our goal should be to devise a unique assay which will reflect the threshold of vitality versus death.

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