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Isolation and Freezing Tolerances of Mesophyll Cells from Cold-hardened and Nonhardened Winter Rye[1]

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ABSTRACT

A method is described for the release of large quantities of mesophyll cells from leaves of both cold-hardened and nonhardened winter rye seedlings by a combination of enzymic and physical maceration. Such preparations usually contain a certain percentage of nonviable cells and are thus not suitable for quantitative biochemical studies. A method is also described whereby pure preparations of viable cells could be obtained by centrifugation on Percoll, using the observation that upon replacement of the isolation medium by water the viable and dead cells exhibited very different buoyant densities. The buoyant density of cells isolated from cold-hardened seedlings differed significantly from their nonhardened counterparts. Survival of the isolated cold-hardened and nonhardened cells following extracellular freezing in water and following plasmolysis in balanced salt solutions was found to be in very close agreement with that of the plants and tissues from which they were isolated.

A recent innovation in the study of the mechanisms of freezing injury and tolerance in cereals is the use of isolated protoplasts (9, 11, 13). Protoplasts not only provide a system in which freezing injury to the plasmalemma could be studied *in situ* (15), but the absence of the cell wall and the separation of cells from bulk tissues overcome infiltration problems encountered during incorporation of membrane labels into the cell (12). The use of protoplasts suffers one important disadvantage, in that protoplasts require the presence of an external osmoticum to prevent cell lysis. Upon initiation of freezing of protoplasts in suspension, channels of high concentrations of solute are formed within the ice. A majority of the protoplasts accumulate in these channels, thus subjecting the cell to a plasmolytic stress and the plasmalemma to a very high external solute stress. In such cases, whether or not the cell is subjected to a freezing stress equivalent to that of the tissues *in situ* may become questionable. The requirement of an external osmoticum could be avoided if single cells, in the presence of the cell wall, but without the infiltration problems usually encountered in whole tissue slices, were used. Furthermore, such isolated cells are probably the best materials to demonstrate the freezing process in cold-hardened and nonhardened cells.

Although mesophyll cells have been isolated from a number of higher plant tissues (3–5, 8, 14), there have not been any reports of the mass isolation of such cells from cereals (such as wheat or rye) which are capable of attaining a high degree of freezing tolerance. This paper describes a method, for the first time, to isolate large quantities of mesophyll cells from cold-hardened and nonhardened winter rye seedlings and describes a method to remove nonviable cells in such preparations, making them suitable for quantitative biochemical studies. The freezing tolerances of these cells in water are also presented.

MATERIALS AND METHODS

Growth of Seedlings. Seeds of *Secale cereale* L. cv. Puma were germinated and grown in two ways. Dark-grown nonhardened plants were obtained as follow: seeds were soaked in 3% hypochlorite solution for 2 min, washed with tap water, and then germinated on moist filter paper in the dark at 17.5 C for 1 week. Plants that had been germinated for 2 days at 25 C, were cold-hardened by growing them at 2 C for 5 weeks in the dark. Nonhardened, light-grown plants were obtained by germinating seeds for 2 weeks in soil in 10-cm deep containers at 20 C-day and 15 C-night temperatures (16 h daylength). Cold-hardening of light-grown plants was achieved by growing the plants at 2 C, (16 h daylength), for 7 weeks following germination for 5 days at higher temperatures as described above.

Isolation of Cells. The top 2 to 3 cm of the epicotyls of dark-grown rye seedlings were harvested (0.7 g fresh weight) and sliced into 0.5 to 1 mm cross-sectional pieces with a razor blade. The chopped tissues were vacuum infiltrated in 5 ml of isolation medium for 30 s in a 10-ml Erlenmeyer flask. The isolation medium contained 0.1 M mannitol, 1.5% glycerol (w/v), 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 0.3 mM L-arginine, 1% crystalline BSA, 3 mM Tris-Mes, pH 6, and 1% dextran sulfate (Pharmacia, Montreal, Quebec). The supernatant was then discarded and replaced by 5 ml of fresh isolation medium. DTT was added to a final concentration of 3 mM and Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Chiba-ken, Japan), was added to a final concentration of 0.0005% w/v. Pectolyase Y-23 is a new type of maceration enzyme prepared from *Aspergillus japonicus* with high activities of endo-pectin lyase (endo-PL, EC 4.2.2.3) and endo-polygalacturonase (endo-PG, EC 3.2.1.15) and contains a factor which stimulates tissue maceration by endo-PL and endo-PG (6). The resultant suspension was incubated with stirring at moderate speed on a magnetic stirrer for 2 h at room temperature. The suspension was filtered through 44 mesh nylon cloth and the filtrate centrifuged at 1,800 rpm (380*g*) for 5 min in a bench-top clinical centrifuge. The resultant pellet was resuspended in 1 ml of isolation medium, and the supernatant was recombined with the undigested tissues and further incubated as described above. After 1 h, the suspension was ground very gently with a mortar and pestle for 1 min and then filtered through nylon cloth and the filtrate centrifuged as described above. The pellets were retained and the supernatant recombined with the undigested tissues and ground, filtered, and centrifuged again. This procedure was repeated once more and all the pellets were then combined and washed twice with 5 ml each of isolation medium minus dextran sulfate. The cell suspension, in 1 ml of isolation medium minus

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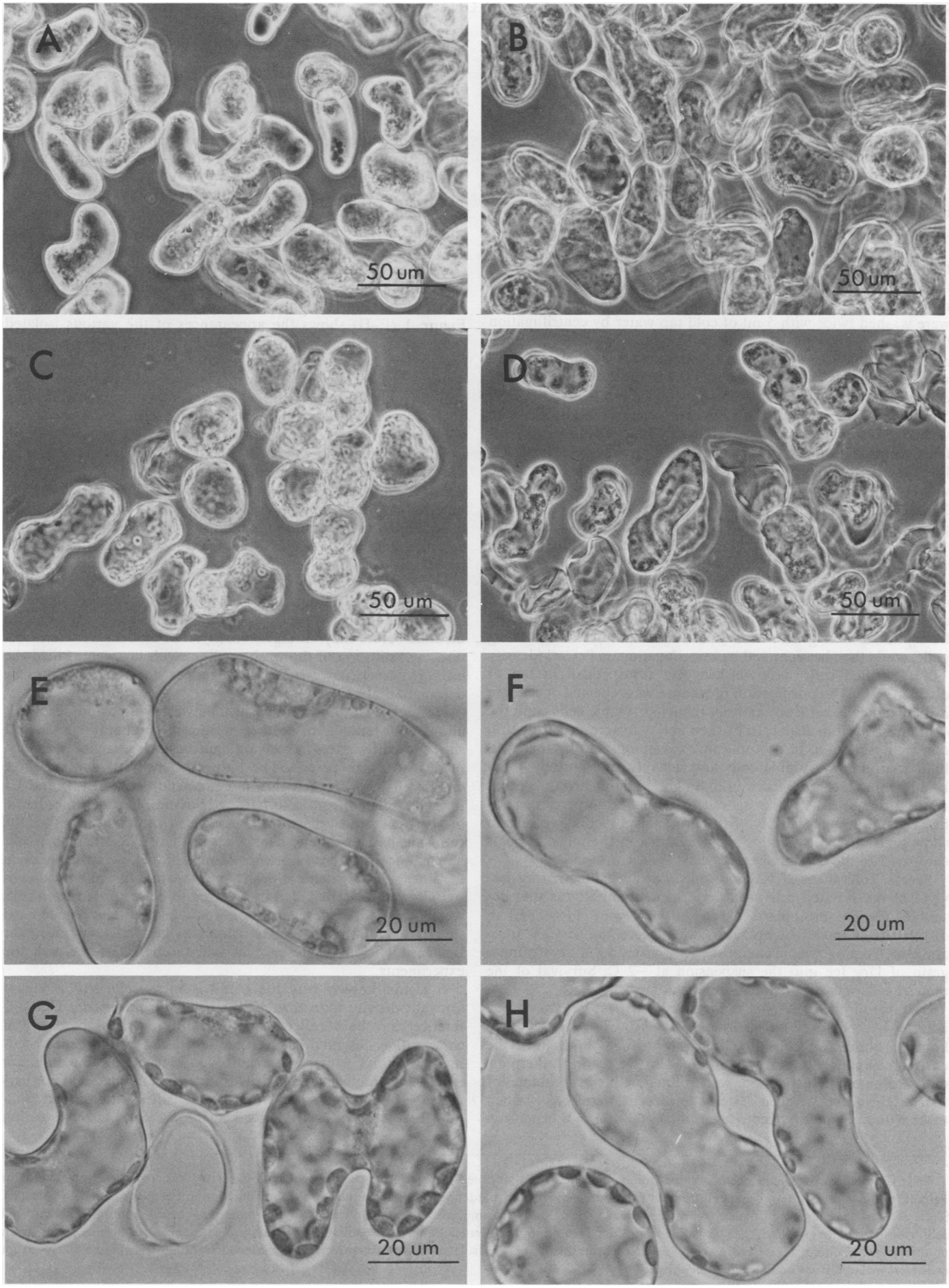

(Legend to Figure 1 on following page.)

dextran sulfate was then dialyzed overnight against 1 liter of tap water at 2 C. At this stage, the cell suspensions consisted of 40 to 50% viable cells mixed with dead and empty cells (cell wall fragments).

The isolation procedure for light-grown tissues was similar to that described above except for the following. One g fresh weight of expanded leaves per 5 ml isolation medium was employed. The leaves were harvested and immersed in 5 ml of isolation medium for 48 h at 2 C prior to cell isolation. This treatment always resulted in a higher yield of viable cells during isolation, especially in the case of nonhardened tissues.

Removal of Nonviable and Empty Cells. All glassware used in this procedure was rinsed with Sigmacote (Sigma) as the cells adhered to untreated glassware. The dialyzed cells isolated from nonhardened light- or dark-grown and hardened light-grown rye were washed twice with 5 ml of cold tap water by centrifugation for 5 min at 380*g* in a clinical centrifuge. The pellet was then resuspended in 10 ml of Percoll adjusted to a final density of 1.06 with distilled H_2O. The resulting suspension, in a 15-ml Corex conical centrifuge tube, was centrifuged at room temperature for 5 min at 2,500 rpm (740*g*) in a benchtop clinical centrifuge fitted with a horizontal rotor. After centrifugation the empty cells pelleted to the bottom, whereas the viable and dead cells formed a layer at the top. The top layer was collected and washed twice with tap water by centrifugation. It was then resuspended in Percoll (1.03 for light-grown nonhardened, and 1.04 for dark-grown nonhardened and light-grown hardened cells) and centrifuged again as above. The viable cells pelleted to the bottom, whereas the dead cells layered at the top. Empty cells from dark-grown, cold-hardened rye were separated simply by centrifugation of the preparation in water at 1,800 rpm. The empty cells formed a white layer at the top of the otherwise yellow pellet and could be aspirated after gentle stirring. Dead cells from the remaining pellet could then be separated by centrifugation on Percoll (density 1.05). Purified cells were washed and resuspended in tap or distilled H_2O. The purification procedure was repeated once more if the dead cells were not entirely removed. Viable cells could be distinguished from dead cells by their ability to accumulate neutral red, by observation of protoplasmic streaming, and by their cellular morphology. Viable cells also actively transported ^{14}C-labeled amino acids (Barran and Singh, unpublished). A rapid method that was used throughout to estimate the percentage of live cells utilizes the ability of the protoplasm of the live cells to show high refractility under phase contrast in the microscope as compared to the dead and empty cells (Fig. 1; A and C).

Freeze Tolerances of Isolated Cells. Extracellular freezing survival of the isolated cells was performed essentially as that described for rye protoplasts (11). The cells were suspended in distilled H_2O and the temperature was lowered at 2.5 C/h. Initiation of freezing was accomplished by dropping a very minute grain of Dry Ice into the suspension at −2 C. Survival of the thawed cells was counted on a hemocytometer.

Survival to plasmolysis of the isolated cells was determined by adding 4 M balanced salt solution ($NaCl:CaCl_2$, 9:1) slowly to known volumes of cells in clinical centrifuge tubes until the desired salt concentration was obtained. After 5 min in the plasmolyzed state, the suspensions were diluted with equal volumes of tap water and centrifuged. The pelleted cells were then washed twice with tap water by centrifugation at 600 rpm in a bench-top clinical centrifuge. Survival was estimated by counting the cells on a hemocytometer.

RESULTS AND CONCLUSION

A method has been developed for the release of mesophyll cells and their purification from tissues of cold-hardened and nonhardened light- and dark-grown winter rye. As far as I am aware, this is the first report of a method of release and purification of mesophyll cells from winter rye. The preparations obtained were chloroplast free and consisted of mostly single cells with some cells adhering to each other in groups of two or three. Figure 1 (A–D) shows the differences in the appearance of cold-hardened and nonhardened viable *versus* dead and empty cell fractions under phase contrast light microscopy after separation on Percoll. Figure 1 (E–H) shows the appearances of the various isolated cells. After dialysis against tap water, the cells showed active protoplasmic streaming and were still viable after 12 days in tap water at 2 C. The yield of purified viable cells from the tissues varied from 0.5 to 1 × 10^6 cells/g fresh weight. The following observations, critical to the isolation procedure, were made. (*a*) With nonhardened, dark-grown tissues, it was not possible to isolate cells in the absence of 1% dextran sulfate as the cells were completely disintegrated even at extremely low concentrations of enzyme. The function of dextran sulfate is obscure, but it has been reported to yield tobacco mesophyll cells of higher quality if included in the maceration medium (4). (*b*) Germination of dark-grown, nonhardened seedlings at 17.5 C instead of 25 C produced higher yields of viable cells. Also, the yield of viable cells was lower if the seedlings were grown for less than 6 to 7 days. (*c*) The yield of live cells from light-grown seedlings was considerably increased if the leaves were soaked for 48 h at 2 C in the isolation medium before enzymic digestion. (*d*) After the cells were released, direct substitution of the wash medium with tap water killed the cells. However, it was possible to replace the wash medium by overnight dialysis against tap water.

Differences in buoyant densities between viable and dead cells allowed for removal of dead cells from the preparations. Table I shows the differences in buoyant densities between viable, empty, or dead cells, in Percoll. The buoyant density of nonhardened, viable cells was between 1.03 and 1.05, and between 1.05 and 1.1 for cells isolated from cold-hardened tissues. On the other hand, in all cases, dead cells with disintegrated protoplasm within the cell walls had a buoyand density lower than 1.03, and empty cells had a buoyant density greater than 1.06. These differences in densities not only made it possible to separate live and dead cells but allowed for cells killed by freezing to be separated from cells which survived. Such a separation would be useful for biochemical studies of membrane lesions caused by nonlethal extracellular freezing, where the presence of killed cells interferes with the experiments.

It is not known why the cold-hardened cells had a higher buoyant density than their nonhardened counterparts. This difference may be a reflection of the differences in biochemical composition observed between hardened and nonhardened seedlings of Puma winter rye (2). In the presence of the isolation media, the viable and dead cells from both hardened and nonhardened rye exhibited similar buoyant densities. Attempts to separate these cells by using Percoll in the presence of the isolation

FIG. 1. Photomicrographs of cells isolated from winter rye. (A), Viable cells isolated from dark-grown, cold-hardened Puma rye after purification in Percoll (phase contrast). (B), Dead cell fraction from dark-grown, cold-hardened Puma rye removed by centrifugation in Percoll (phase contrast). (C), Same as A except cells were isolated from nonhardened seedlings. (D), Same as B except cells were isolated from nonhardened seedlings. (E), Purified cells resuspended in distilled H_2O. Isolated from dark-grown, cold-hardened Puma rye seedlings. (F), Same as E except cells were isolated from nonhardened seedlings. (G), Purified cells resuspended in distilled H_2O. Isolated from light-grown, cold-hardened seedlings. (H), Same as G, except cells were isolated from nonhardened seedlings.

Table I. *Purification of Mesophyll Cells Isolated from Cold-Hardened and Nonhardened Puma Winter Rye Seedlings*

Seedlings of Puma Rye from Which Cells Were Isolated	Buoyant Density			Percent Viable Cells	
	Viable Cells	Dead Cells	Empty Cells	Before Centrifugation	After Centrifugation
	g/cc				
Light-grown					
Nonhardened	1.02–1.04	<1.03	>1.06	40–50	>95
Hardened	1.04–1.05	<1.03	>1.06		
Dark-grown					
Nonhardened	1.04–1.05	<1.03	>1.06	40–50	>95
Hardened	1.06–1.1	<1.03	>1.06		

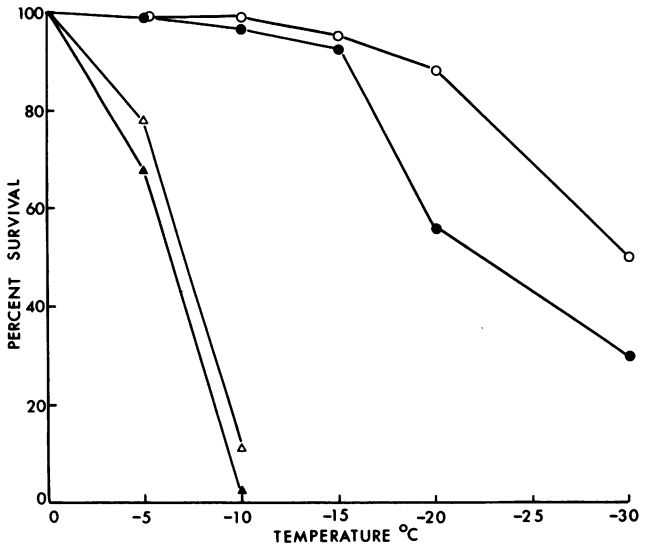

FIG. 2. Survival following extracellular freezing of isolated cells from Puma rye. (○), Cells from light-grown, cold-hardened seedlings. (●), Cells from dark-grown, cold-hardened seedlings. (△), cells from light-grown, nonhardened seedlings.

medium or sucrose before dialysis was unsuccessful.

The freezing tolerance of isolated cells from hardened and nonhardened, light- and dark-grown cells were compared. To simulate freezing conditions of the whole tissues as closely as possible, the cells were frozen in distilled H_2O. Cells isolated from cold-hardened tissues had a higher degree of tolerance for extracellular freezing than those isolated from nonhardened tissues, and also that cells isolated from seedlings which were cold-hardened in the light were more freezing tolerant than their dark grown counterparts (Fig. 2). The approximate LT_{50} (lethal temperature for 50% kill) of −22 C for cold-hardened, dark-grown and −30 C for cold-hardened, light-grown Puma rye cells are in close agreement with −19 and −28 C, respectively, reported for Puma rye seedlings hardened under similar conditions (1, 7). Similarly, as reported for cells of winter rye tissue sections (10), cells isolated from dark-grown, hardened seedlings tolerated a higher degree of plasmolysis than their nonhardened counterparts (Fig. 3). In this case also, light-grown cells showed a higher percentage survival (Fig. 3). These results indicate that cells isolated from cold-hardened winter rye retained their freezing

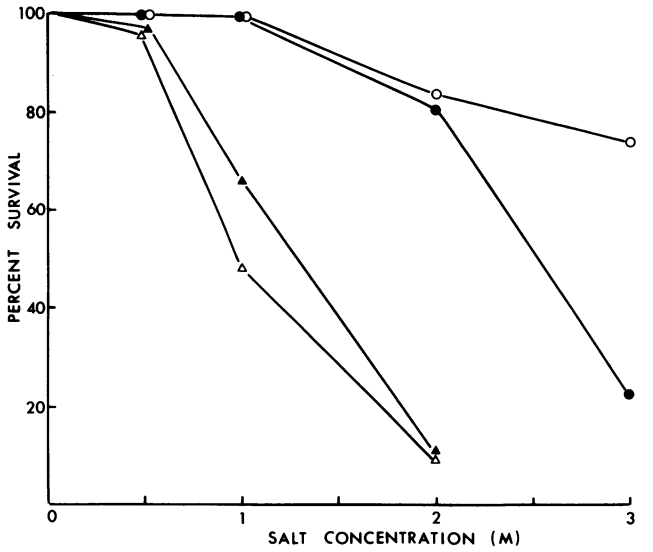

FIG. 3. Survival following plasmolysis in balanced salt solutions and deplasmolysis in tap water of cells isolated from Puma rye. (○), Cells from dark-grown, cold-hardened seedlings. (△), Cells from light-grown, nonhardened seedlings. (▲), Cells from dark-grown, nonhardened seedlings.

tolerances after isolation. Furthermore, the high percentage of viable cells in these preparations, made possible by the purification procedure described above, make them ideally suited for quantitative biochemical and biophysical studies of the mechanisms of freezing tolerance.

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LITERATURE CITED

1. ANDREWS CJ, MK POMEROY 1977 Changes in survival of winter cereals due to ice cover and other simulated winter conditions. Can J Plant Sci 57: 1141–1149
2. DE LA ROCHE AI 1979 Mechanism of thermal tolerance. *In* L Underwood, L Tieszen, A Callahan, G Folk, eds, Comparative Mechanisms of Cold Adaptation. Academic Press, New York, pp 235–257
3. EDWARDS GE, CC BLACK 1971 Isolation of mesophyll cells and bundle sheath cells from *Digitaria sanguinalis* (L.) Scop. leaves and a scanning microscopic study of the internal leaf cell morphology. Plant Physiol 47: 149–156
4. JENSEN RG, RB FRANCKI, M ZAITLIN 1971 Metabolism of separated leaf cells. Plant Physiol 48: 9–13
5. JULLIEN M 1973 Division et croissance *in vitro* des cellules séparées du parenchyme foliaire *d'Asparagus officinalis* L. Z Pflanzenphysiol 69: 129–141
6. NAGATA T, S ISHII 1979 A rapid method for isolation of mesophyll protoplasts. Can J Bot 57: 1820–1823
7. POMEROY MK, CJ ANDREWS 1975 Effect of temperature on respiration of mitochondria and shoot segments from cold-hardened and nonhardened wheat and rye seedlings. Plant Physiol 56: 703–706
8. SERVAITES JC, WL OGREN 1977 Rapid isolation of mesophyll cells from leaves of soybean for photosynthetic studies. Plant Physiol 59: 587–590
9. SIMINOVITCH D, J SINGH, A I DE LA ROCHE AI 1978 Freezing behavior of protoplasts of winter rye. Cryobiology 15: 205–213
10. SINGH J 1979 Ultrastructural alterations in cells of hardened and nonhardened winter rye during hyperosmotic and extracellular freezing stresses. Protoplasma 98: 329–341
11. SINGH J 1979 Freezing of protoplasts isolated from cold hardened and nonhardened winter rye. Plant Sci Lett 16: 195–201
12. SINGH J, RW MILLER 1980 Spin-label studies of membranes in rye protoplasts during extracellular freezing. Plant Physiol 66: 349–352
13. STEPONKUS PL, SC WIEST 1979 Freeze-thaw induced lesions in the plasma membrane. *In* J Lyons, D Graham, J Raison, eds, Low Temperatures Stress in Crop Plants. Academic Press, New York, pp 231–254
14. TAKEBA I, Y OTSUKI, S AOKI 1968 Isolation of tobacco mesophyll cells in intact and active state. Plant Cell Physiol 9: 115–124
15. WIEST SC, PL STEPONKUS 1978 Freeze-thaw injury to isolated spinach protoplasts and its simulation at above freezing temperatures. Plant Physiol 62: 699–705