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Short Communication

Isolation of Mesophyll Protoplasts from Mature Leaves of Soybeans[1]

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ABSTRACT

A procedure based on a combined cellulase-Pectolyase Y-23 enzyme digestion and metrizamide-sorbitol gradient purification protocol was developed for isolating mesophyll protoplasts from mature leaves of soybean (*Glycine max* L. Merr.). Based on chlorophyll content, this procedure results in a 10 to 15% protoplast yield from fully expanded mature leaves and a 20 to 30% yield from young (expanding) leaves within 3 hours. Isolated protoplasts displayed high rates of HCO_3^--dependent photosynthesis; greater than 75 micromoles O_2 evolved per milligram chlorophyll per hour at 25°C. This photosynthetic rate is comparable to that of mesophyll cells isolated mechanically from the same leaves.

Isolated leaf mesophyll protoplasts have found wide application in the study of photosynthesis, cellular metabolism, solute transport, and metabolite compartmentation (2, 3, 5, 6). Unfortunately, mature leaves of certain important crop species, such as soybean and sugar beet, have presented problems to many investigators because of the difficulty in obtaining viable protoplasts by enzyme digestion procedures. This report describes a procedure for the rapid isolation, in high yield, of photosynthetically active mesophyll protoplasts from mature leaves of soybean. This procedure should facilitate cell physiological studies in species where isolation of protoplasts may be difficult to achieve.

MATERIALS AND METHODS

Plant Growth Conditions. Soybeans (*Glycine max* L. Merr. cv Wye) were grown in 15-cm pots (two plants per pot) with Metro Mix 350 (Grace Horticultural and Agricultural Product Co., Cambridge, MA) and subirrigated with Hoagland II solution (4). Plants were grown under the following conditions: 450 $\mu E\ m^{-2}\ s^{-1}$ (middle canopy level), 12-h photoperiod (23.5°C, light/18.3°C, dark), and 60 to 70% RH. After 5 weeks, leaves were taken from the plants less than 1 h into the light period for protoplast and cell isolations. Fully expanded leaves, with an area greater than 50 cm^2 per leaflet and containing more than 3.5 mg Chl/g fresh weight, were used as mature leaves, while leaves less than 12 cm^2 per leaflet area and containing less than 2 mg Chl/g fresh weight leaves were used as immature developing leaves.

Protoplast and Cell Isolations. Detached leaves were rinsed with distilled H_2O, blotted dry, and midribs removed. Leaves were sliced with a razor blade into 1- to 2-mm strips and placed in a 0.65 M sorbitol, 10 mM $CaCl_2$, 25 mM Hepes (pH 6.0), 0.5 mM DTT holding solution. About 1 g of mature leaf or 0.5 g of young leaf tissue was used in each preparation. After slicing, leaf strips were rinsed once with the same holding solution and incubated after 30 s vacuum infiltration with 25 ml of cell wall digestion enzyme mixture consisting of 2% purified cellulase CELF (Worthington Diagnostics, NJ), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Japan), 0.75 M mannitol, 0.2 mM $CaCl_2$, 0.1% BSA, and 0.5 mM DTT at pH 5.5 for 90 to 120 min with constant shaking (50 cycles/min) at 30°C. Digested tissue samples were filtered through a 150-μm nylon mesh and rinsed once with 30 ml of the holding solution. Crude protoplasts were spun from the filtrate at 150*g* for 3 min. After two washes with the holding solution, 5 ml of crude protoplast suspension were layered on the top of a metrizamide-sorbitol gradient for purification. The gradient consisted of 6 ml each of 0.2 M metrizamide—0.39 M sorbitol, 0.1 M metrizamide—0.52 M sorbitol, and 0.05 M metrizamide—0.585 M sorbitol in 10 mM $CaCl_2$ and 25 mM Mes (pH 6.0). Purified protoplasts were collected at the bottom interface for the mature leaf and next to the bottom interface for the young leaf after the gradient was centrifuged at 150*g* for 3 min. Purified protoplasts were pelleted by adding 20 ml of holding solution and centrifuging at 100*g* for 3 min. With the exception of incubation, all procedures were performed at room temperature. All solutions used were sterilized by passing them through a 0.45-μm Millipore filter.

Mesophyll cells were mechanically isolated according to the method of Schwenk (7). Counts of protoplasts and cells were made using a Hausser Hy-Lite Ultra Place counting chamber (Arthur Thomas Co., PA).

Photosynthesis Assay and Chl Determination. HCO_3^--dependent O_2 evolution by isolated protoplasts and cells was measured at 25°C according to the procedure of Edwards *et al.* (2). Each assay contained 15 to 20 μg Chl of protoplasts or cells. For comparison, some cells were assayed in the absence of sorbitol with 25 mM Hepes-NaOH, pH 7.55. Chl content of protoplasts and cells was determined by the method of Arnon (1). Chl was also extracted from about 0.1 g fresh weight leaf tissue with 5 ml of 80% acetone and determined by the same method (1).

RESULTS AND DISCUSSION

Figure 1 (A and B) shows that the protoplasts isolated from mature leaves by this method were more than twice the size of

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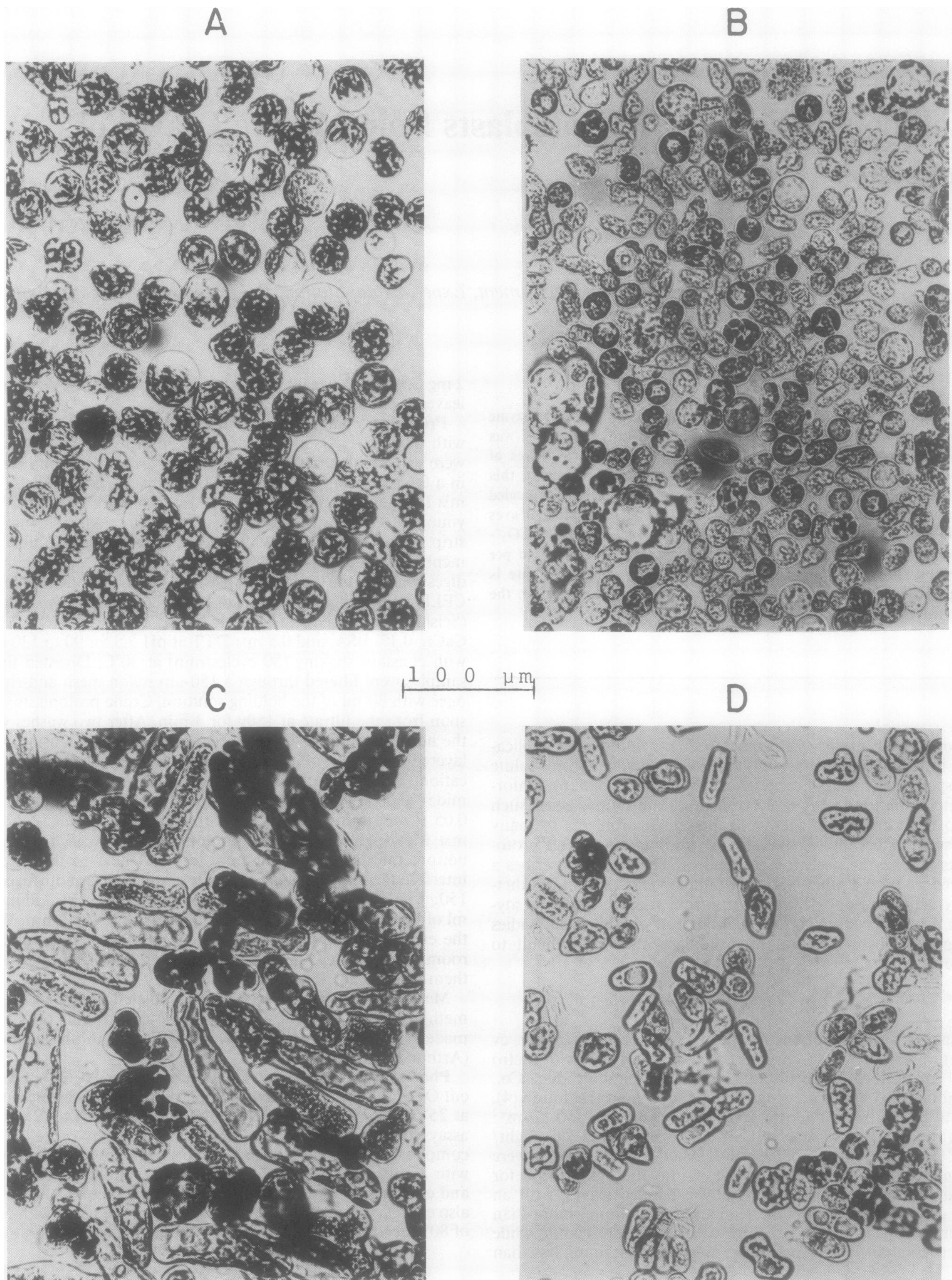

FIG. 1. Light micrographs of mesophyll protoplasts and cells isolated from soybean leaves. A, Protoplasts from mature leaves; B, protoplasts from young leaves; C, cells from mature leaves; D, cells from young leaves.

Table I. *Comparison of Photosynthesis, Chl Content, and Yield of Protoplasts and Cells Isolated from Soybean Leaves*

	O_2 Evolution	Chl/Protoplasts or Cells	Yield
	μmol/mg Chl·h	*μg/10⁶*	*%*
Mature leaves			
Protoplasts	85[a]	90	11
Cells	90[a]	87	6
	70[b]		
Tissue		3.7[c]	
Young leaves			
Protoplasts	82[a]	14	30
Cells	85[a]	15	14
	75[b]		
Tissue		2.0[c]	

[a] Assayed in 0.6 M sorbitol, 25 mM Hepes-NaOH (pH 7.55).
[b] Assayed in 25 mM Hepes-NaOH (pH 7.55) minus sorbitol.
[c] mg Chl/g fresh weight.

those isolated from young leaves and contained a 6-fold higher Chl content per protoplast (Table I). Similar results were found with mechanically isolated cells (Fig. 1, C and D; Table I).

Protoplasts isolated by this method were capable of relatively high rates of HCO_3^--dependent O_2 evolution (Table I). Photosynthetic activities per unit Chl were the same in all protoplast and cell populations isolated from both mature and young leaves. However, as expected, since mature leaf cells contained higher amounts of Chl (Table I), the photosynthetic activity per cell unit was higher in mature leaves than in the young leaves. Table I also shows that photosynthetic activity in isolated cells was not affected by high osmoticum (it was even slightly stimulated). Based on the Chl content in the starting leaf material and final protoplast and cell preparations (Table I), the yield of protoplasts per g leaf tissue was estimated to be about 10% and 30% for mature and young leaves, respectively.

The described method was also used to isolate successfully mesophyll protoplasts from mature leaves of sugar beet, cotton, peanut, and tobacco (data not shown).

In conclusion, the described method is satisfactory for the isolation of protoplasts with high photosynthetic activities from several mature leaf tissues. This method should be a valuable tool to facilitate the study of several physiological processes in plant tissues.

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LITERATURE CITED

1. Arnon DI 1949 Copper enzymes in chloroplasts. Polyphenoloxidases in *Beta vulgaris*. Plant Physiol 24: 1–15
2. Edwards GE, SP Robinson, NJC Tyler, DA Walker 1978 Photosynthesis by isolated protoplasts, protoplast extracts, and chloroplasts of wheat. Plant Physiol 62: 313–319
3. Gay M, L Reinhold, GG Laties 1978 Membrane transport of sugars and amino acids in isolated protoplasts. Plant Physiol 61: 593–596
4. Hoagland DR, DI Arnon 1938 The water-culture method for growing plants without soil. U Calif Agric Exp Stu Circ 347
5. Huber SC, GE Edwards 1975 An evaluation of some parameters for the enzymatic isolation of cells and protoplasts with CO_2 fixation capacity from C_3 and C_4 grasses. Physiol Plant 35: 203–209
6. McC Lilley R, M Stitt, G Mader, HW Heldt 1982 Rapid fractionation of wheat leaf protoplasts using membrane filtration. The determination of metabolite levels in the chloroplasts, cytosol, and mitochondria. Plant Physiol 70: 965–970
7. Schwenk FW 1981 Callus formation from mechanically isolated mesophyll cells of soybean and sweet potato. Plant Sci Lett 23: 147–151