Isolation of intact mesophyll cells from the leaves of higher plants

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Abstract. A total of 146 species of angiosperms belonging to 35 taxonomically diverse families were screened for the isolation of living mesophyll cells from the leaves. Seventy-three species belonging to 22 families, on mild maceration in mortar with the isolation medium (pH 5.8) containing 0.7 M mannitol, 2 mM EDTA, 5 mM $MgCl_2$, 5 mM K_2HPO_4 and 1 mM $NaNO_3$, followed by fractional centrifugation, yielded intact mesophyll cells as seen under a research microscope. The high frequency of cell release, associated with the high percentage recovery of chlorophyll in cells was a common feature of most of the plant species examined by us. Nearly 87% of the chlorophyll present in the leaf could be recovered from the isolated cells in *Dolichos lablab*. The isolated cells retained active photosynthetic carbon metabolism as evidenced by high rates of ferricyanide reduction as well as carbon assimilation.

Keywords. Isolated cells; photosynthesis; maceration.

1. Introduction

Isolated intact plant cells form an ideal system for understanding metabolism. Suspensions of isolated leaf cells are particularly promising for photosynthetic studies. According to Jensen *et al* (1971) they are useful for both short term experiments and for metabolic studies even up to about 36 hr after preparation.

Photosynthetically active cells have been isolated from leaves of higher plants by mild maceration in a few instances (Racusen and Arnoff 1953; Zaitlin 1959; Gnanam and Kulandaivelu 1969; Jullien and Rossini 1977). The technique of isolating leaf cells with the help of fungal enzymes in a hypertonic medium has been recently gaining popularity (Takebe *et al* 1968; Power and Cocking 1970; Jensen *et al* 1971; Evans *et al* 1972; Cataldo and Berlyn 1974; Schieder 1975; Bajaj 1977; Gamborg 1977). Yet the technique of cell isolation by mere maceration easily yields good cellular suspension, without implying the use of pectinases.

We have screened a large number of plants of the Tirupati region (as many as 32 mono- and 114 dicotyledons including 33 crop plants) for the isolation of intact mesophyll cells by using the simple technique of mild maceration followed by fractional centrifugation.

2. Materials and methods

The plants used were grown under natural field conditions in the campus of Sri Venkateswara University (approximate 12 hr photoperiod with temperatures of 35° C day and 25° C night). Some of the plants were ornamentals raised in the Botanical Garden. Fully expanded leaves were picked from the plants. The leaves were thoroughly washed with tap water followed by distilled water. The laminar tissue was cut into Ca 0.25–0.50 cm² pieces and suspended for 10 min in the isolation medium (pH 5.8) containing 0.7 M mannitol, 2 mM EDTA, 5 mM MgCl₂, 5 mM K₂HPO₄ and 1 mM NaNO₃. The leaf pieces were ground very gently in 10 ml of the isolation medium using a mortar and pestle. The degree of cell breakage and cell separation during mild maceration were observed periodically with a research microscope. The homogenate was poured into a centrifuge tube and allowed to settle for about 10 min. The supernatant (which usually contained broken cells) was decanted and replaced by fresh isolation medium. This procedure was repeated thrice. The cell suspension was then filtered through two layers of cheese cloth and the cell-clumps retained on the cheese cloth were thoroughly washed and collected in the same medium. The cells were subsequently collected by centrifugation at 250 g for 2 min. The pellet was again suspended in the fresh isolation medium. Centrifugation and resuspension were continued (usually 2 or 3 times) until the supernatant fluid was free from green colour. The cells free from debris were examined under a research microscope.

The cell number in the suspensions (50 µl) was determined using Neubauer improved double Haemocytometer. The total chlorophyll content of the cell suspensions as well as whole leaves was determined according to the method of Arnon (1949).

The carbon assimilation capacity of isolated mesophyll cells was determined in terms of incorporation of radioactivity from NaH¹⁴CO₃ into acid stable products. The reaction mixture (2 ml) contained 0.4 M mannitol, 2 mM EDTA, 5 mM MgCl₂, 1 mM NaNO₃, 1 mM MnCl₂, 5 mM K₂HPO₄, 50 mM tricine buffer pH 8.0, 5 mM NaH¹⁴CO₃ and cell suspension (equivalent to 15–20 µg chlorophyll/ml). The illumination was provided by incandescent bulbs and the light intensity after passing through a 10 cm water filter was 200 w m⁻². The reaction was terminated by the addition of 0.5 ml of 5 N HCl. The ¹⁴C incorporation was determined using a GM counter.

Ferricyanide reduction was followed as the decrease in absorbance at 420 nm (Raghavendra and Das 1978b).

3. Results

Out of the 146 species screened, 73 species belonging to 22 families yielded intact mesophyll cells on mild maceration (table 1). The washed cells (a few appear as

Figure 1. Intact mesophyll cells isolated from leaves of *Commelina benghalensis* (a), *Celosia cristata* (b), *Arachis hypogaea* (c) and *Digitaria adscendens* (d) $\times 150$.

Figure 2. Intact mesophyll cells isolated from leaves of *Digera alternifolia* (a) and *Aster leavis* (b) $\times 600$.

Table 1. List of plant species screened for yield of intact mesophyll cell suspensions.

Plant species	Cell release*
<i>Dicots</i>	
1. <i>Acanthaceae</i>	
<i>Andrographis echinoides</i> (L.) Nees	+
<i>Barleria buxifolia</i> L.	+
<i>Blepharis molluginifolia</i> Pers.	++
<i>Crossandra infundibuliformis</i> (L.) Nees	0
<i>Justicia prostrata</i> Gamb.	+
<i>J. tranquebariensis</i> L.f.	+
2. <i>Aizoaceae</i>	
<i>Mollugo microphylla</i> R.	0
<i>M. nudicaulis</i> Lamk.	0
<i>M. pentaphylla</i> L.	0
3. <i>Amaranthaceae</i>	
<i>Achyranthes aspera</i> L.	+++
<i>Allmania nodiflora</i> R.Br.	0
<i>Alternanthera pungens</i> H.B.K.	0
<i>Amaranthus tricolor</i> L.	0
<i>A. spinosus</i> L.	+
<i>A. viridis</i> L.	0
<i>Celosia argentea</i> L.	+
<i>C. cristata</i> L.	+
<i>Digera alternifolia</i> (L.) Aschers.	+++
<i>Gomphrena celosioides</i> Mart.	0
<i>G. globosa</i> L.	0
<i>Alternanthera ficoidea</i> (L.) R.Br. ex R.	0
4. <i>Apocynaceae</i>	
<i>Carissa spinarum</i> L.	0
<i>Rauvolfia serpentina</i> Benth.	0
<i>Catharanthus roseus</i> (L.) G.Don.	0
5. <i>Asclepiadaceae</i>	
<i>Calotropis gigantea</i> R.Br.	+
<i>Leptadenia reticulata</i> W & A.	+
6. <i>Asteraceae</i> (Compositae)	
<i>Acanthospermum hispidum</i> DC.	0
<i>Aster leavis</i> L.	+++
<i>Carthamus tinctorius</i> L.	0
<i>Eclipta prostrata</i> (L.) L.	0
<i>Guizotia abyssinica</i> Cass.	0
<i>Glossocardia bosvallea</i> DC.	0
<i>Gaillardia</i> sp.	+++
<i>Helianthus annuus</i> L.	+
<i>Tagetes patula</i> L.	0
<i>Tridax procumbens</i> L.	0
<i>Vernonia cinerea</i> (L.) Less.	0
<i>Zinnia elegans</i> Jacq.	+++

(Contd.)

Table 1. (Contd.)

Plant species	Cell release*
7. Capparidaceae	
<i>Cleome viscosa</i> L.	+++
<i>Cleome gynandra</i> (L.) Briv.	0
8. Casuarinaceae	
<i>Casuarina equisetifolia</i> Forst.	0
9. Caryophyllaceae	
<i>Polycarpaea corymbosa</i> (L.) Lamk.	0
10. Chenopodiaceae	
<i>Chenopodium amaranticolor</i> Coste and Reyn	+
<i>Chenopodium quinova</i>	+
11. Convolvulaceae	
<i>Evolvulus alsinoides</i> L.	+
<i>Ipomoea tridentata</i> Roth.	+
<i>I. tuberosa</i> L.	++
12. Cruciferae (Brassicaceae)	
<i>Brassica juncea</i> (L.) Czern. and Coss.	0
<i>Raphanus sativus</i> L.	0
13. Euphorbiaceae	
<i>Acalypha indica</i> L.	0
<i>Euphorbia hirta</i> L.	0
<i>E. pulcherrima</i> Willd.	+
<i>E. milii</i> Ch.-des-Moullins	0
<i>Phyllanthus maderaspatensis</i> L.	+
<i>P. fraternus</i> Webster	++
<i>Ricinus communis</i> L.	0
<i>Sebastiania chaemaelea</i> M.-Arg.	+
14. Balsaminaceae	
<i>Impatiens balsamina</i> L.	+
15. Labiatae (Lamiaceae)	
<i>Anisomeles malabarica</i> R.Br.	+
<i>Coleus aromaticus</i> Benth.	0
<i>Leucas aspera</i> Spr.	0
<i>L. linifolia</i> Spr.	0
<i>Ocimum basilicum</i> L.	0
<i>O. sanctum</i> L.	0
16. Leguminosae (Fabaceae)	
<i>Acacia leucophloea</i> (Roxb.) Willd.	++
<i>A. sundra</i> DC.	++
<i>A. latronum</i> Willd.	++
<i>Alysicarpus monilifer</i> DC.	0
<i>Arachis hypogaea</i> Willd.	+++
<i>Cajanus cajan</i> (L.) Millsp.	+

(Contd.)

Table 1 (Contd.)

Plant species	Cell release*
<i>Cassia auriculata</i> L.	0
<i>C. raxburghii</i> DC.	0
<i>Crotalaria retusa</i> L.	0
<i>Cyamopsis tetragonoloba</i> (L.) Taub.	+
<i>Dolichos lablab</i> L.	+++
<i>Heylandia latebrösa</i> DC.	+
<i>Indigofera linnaei</i> Ali.	++
<i>Prosopis juliflora</i> (SW.) DC.	++
<i>Rhynchosia minima</i> (L.) DC.	++
<i>Teramnus labialis</i> (L.f.) Spr.	+
<i>Vigna sinensis</i> (L.) Saviex Hassak.	0
<i>Zornia diphylla</i> (L.) Pers.	++
17. Malvaceae	
<i>Gossypium hirsutum</i> L.	0
<i>Hibiscus micranthus</i> L.f.	0
<i>Pavonia zeylanica</i> Cav.	+
<i>Sida acuta</i> Burm.f.	++
<i>S. glutinosa</i> Cav.	+
18. Myrtaceae	
<i>Eucalyptus globulus</i> Labill.	0
<i>Psidium guajava</i> L.	0
19. Nyctaginaceae	
<i>Boerhavia diffusa</i> L.	0
<i>B. repanda</i> Willd.	0
<i>Bougainvillea spectabilis</i> Willd.	0
20. Pedaliaceae	
<i>Sesamum orientale</i> L.	0
21. Piperaceae	
<i>Peperomia hybrida</i> L.	0
22. Portulacaceae	
<i>Portulaca oleracea</i> L.	0
23. Rhamnaceae	
<i>Zizyphus mauritiana</i> Lamk.	+++
24. Rubiaceae	
<i>Borreria articularis</i> (L.f.) F.N. Williams	+
<i>Chomelia asiatica</i> O. Kze.	0
<i>Oldenlandia umbellata</i> L.	+
<i>Randia dumetorum</i> Lamk.	+
<i>R. malabarica</i> Lamk.	++
25. Sapindaceae	
<i>Dodonaea viscosa</i> Jacq.	+

(Contd.)

Table 1 (Contd.)

Plant species		Cell release*
26. Solanaceae		
	<i>Capsicum frutescens</i> L.	0
	<i>Datura fastuosa</i> L.	0
	<i>Lycopersicum esculentum</i> Mill.	0
	<i>Nicotiana tabacum</i> L.	0
	<i>Physalis minima</i> L.	0
	<i>Solanum melongena</i> L.	0
	<i>S. nigrum</i> L.	0
27. Umbelliferae (Apiaceae)		
	<i>Coriandrum sativum</i> L.	0
28. Urticaceae		
	<i>Pilea microphylla</i> (L.) Liebm.	0
29. Verbenaceae		
	<i>Clerodendrum inerme</i> Gaertn.	0
	<i>Lantana camara</i> L.	++
30. Zygophyllaceae		
	<i>Tribulus terrestris</i> L.	0
	Monocots	
31. Commelinaceae		
	<i>Commelina benghalensis</i> L.	+++
	<i>C. clavata</i> Cl.	++
32. Cyperaceae		
	<i>Bulbostylis barbata</i> Kunth	+
	<i>Cyperus compressus</i> L.	++
	<i>C. rotundus</i> L.	+++
33. Musaceae		
	<i>Musa paradisiaca</i> L.	++
34. Typhaceae		
	<i>Typha latifolia</i> L.	+++
35. Gramineae (Poaceae)		
	<i>Alloteropsis cimicina</i> Stapf	0
	<i>Aristida hystrix</i> L.	0
	<i>Bambusa arundinacea</i> (Retz.) Willd.	0
	<i>Brachiaria mutica</i> Stapf	+
	<i>Cenchrus ciliaris</i> L.	+
	<i>Chloris barbata</i> Sw.	+
	<i>C. gayana</i> Kunth	+
	<i>Coix aquatica</i>	0
	<i>Cynodon dactylon</i> Pers.	+
	<i>Dactyloctenium aegyptium</i> Beauv.	+
	<i>Digitaria adscendens</i> Henr.	+++

(Contd.)

Table 1 (Contd.)

Plant species	Cell release*
<i>Echinochloa colonum</i> Link	+
<i>Eleusine coracana</i> Gaertn.	0
<i>Heteropogon contortus</i> Beauv.	+
<i>Imperata cylindrica</i> Beauv.	0
<i>Oryza sativa</i> L.	0
<i>Panicum maximum</i> Jacq.	+
<i>P. nodosum</i> Kunth	+
<i>P. psilopodium</i> Trin.	+
<i>P. purpurascens</i> Raoldi	+
<i>Saccharum officinarum</i> L.	+
<i>Sorghum vulgare</i> L.	0
<i>Spinifex squarrosus</i> L.	0
<i>Tragus biflorus</i> Schult.	+
<i>Zea mays</i> L.	0

* Degree of cell release: + poor; ++ average; +++ good; 0 without cells.

clumps) were morphologically intact as demonstrated by photomicrographs (figures 1 and 2).

The high frequency of cell release, associated with the high percentage recovery of chlorophyll in cells was a common feature of most plants excepting *Digitaria adscendens*, *Digera alternifolia* and *Randia malabarica*. These three species show low cell number per gram fresh weight with high percentage recovery of chlorophyll in cells (table 2). *Dolichos lablab* was most ideally suited for cell isolation as indicated by the maximum recovery of chlorophyll in the cells.

The isolated mesophyll cells were photosynthetically active as indicated by active carbon fixation as well as ferricyanide reduction. The carbon fixation rates of four species ranged from 62–124 μ moles mg chl⁻¹ hr⁻¹ (table 3). The rate of ferricyanide reduction by the cells of *Dolichos* exhibited linearity even up to 10 min unlike those of *Digitaria* in which linearity was evident only for 3 min (figure 3). The cells of *Dolichos* exhibited a lag phase before attaining maximal rates of ¹⁴C-assimilation. The cells of *Digitaria* did not show any lag phase during carbon fixation.

4. Discussion

Racusen and Arnoff (1953) found for the first time that soybean leaf cells could be separated by mild grinding in a mortar. Gnanam and Kulandaivelu (1969) were able to separate intact cells from several plants belonging to 17 families including monocotyledons by mild grinding in a mortar. In the present study, a large number of plant species have yielded intact mesophyll cells and thus have added to the list of plant species studied earlier (Gnanam and Kulandaivelu 1969; Bajaj 1977; Jullien and Rossini 1977). Although in majority of the plant species, the high frequency of cell release was always associated with a high percentage recovery

Figure 3. The pattern of ferricyanide reduction (a) and carbon assimilation (b) by isolated mesophyll cells from *Digitaria adscendens* and *Dolichos lablab*.

Table 2. Cell release and chlorophyll recovery of isolated mesophyll cells from different plant species (values are means of five observations $\pm$ SE).

Plant species	Number of cells released per g fresh weight ($\times 10^7$)	Total chlorophyll (mg g^{-1} fresh weight)		Per cent recovery of chlorophyll in cells
		Leaves	Cells	
<i>Arachis hypogaea</i>	5.60	2.08 ± 0.18	1.59 ± 0.11	76.4 ± 6.2
<i>Dolichos lablab</i>	6.60	2.31 ± 0.17	2.02 ± 0.16	87.4 ± 8.8
<i>Musa paradisiaca</i>	2.30	1.63 ± 0.12	0.73 ± 0.05	44.8 ± 3.9
<i>Justicia tranquebariensis</i>	0.50	3.36 ± 0.26	0.44 ± 0.02	13.2 ± 1.2
<i>Randia malabarica</i>	1.25	1.90 ± 0.12	1.09 ± 0.11	57.4 ± 4.9
<i>Commelina benghalensis</i>	2.15	2.54 ± 0.21	1.21 ± 0.09	47.6 ± 3.6
<i>Chenopodium quinova</i>	1.75	1.51 ± 0.10	0.44 ± 0.03	29.1 ± 2.6
<i>Cleome viscosa</i>	5.16	2.22 ± 0.19	1.37 ± 0.12	61.7 ± 5.7
<i>Digera alternifolia</i>	2.94	1.98 ± 0.11	0.89 ± 0.06	44.9 ± 4.8
<i>Digitaria adscendens</i>	2.04	2.05 ± 0.23	1.36 ± 0.11	66.5 ± 5.9
<i>Achyranthes aspera</i>	4.92	2.78 ± 0.20	1.36 ± 0.14	48.9 ± 4.6
<i>Panicum maximum</i>	0.90	1.42 ± 0.09	0.62 ± 0.05	43.6 ± 3.8
<i>Cyperus rotundus</i>	4.20	1.71 ± 0.12	1.03 ± 0.09	60.2 ± 7.2
<i>Celosia argentea</i>	0.42	1.62 ± 0.15	0.54 ± 0.03	33.3 ± 2.8

of chlorophyll in cells. There were exceptions like *Digitaria adscendens*, *Digera alternifolia* and *R. malabarica* (table 2).

The fact that isolated mesophyll cells are a useful system to study the photosynthetic reactions was demonstrated by the high rates of ferricyanide reduction

Table 3. Photosynthetic carbon fixation and ferricyanide reduction rates by isolated cells.

Plant species	Ferricyanide reduction μ moles mg^{-1} (chlorophyll) h^{-1}	$^{14}\text{CO}_2$ assimilation μ moles mg^{-1} (chlorophyll) h^{-1}
<i>Arachis hypogaea</i>	230.6	110.4
<i>Digera alternifolia</i>	330.8	96.8
<i>Digitaria adscendens</i>	450.2	62.4
<i>Dolichos lablab</i>	280.0	124.2

and carbon fixation capacity of intact cells (table 3). The carbon fixation rates recorded in the present investigation appear to be high as compared to those obtained in the earlier attempts, i.e., without the addition of any substrate (Edwards *et al* 1970; Edwards and Black 1971; Jensen *et al* 1971; Rehlfeld and Jensen 1973).

The data on time course of ferricyanide reduction and carbon fixation by intact mesophyll cells are particularly interesting and resemble the pattern in isolated chloroplasts. The cells of *Dolichos* (a C_3 plant) exhibited an initial lag phase during carbon fixation and the rates of ferricyanide reduction were linear for longer period. On the other hand the absence of lag phase during carbon fixation by cells of *Digitaria* (a C_4 plant) was associated with maintenance of linearity in ferricyanide reduction for only shorter durations. The chloroplasts from the leaves of C_4 plants, *Amaranthus paniculatus*, *Pennisetum typhoides* and *Setaria italica* do not have a lag phase during carbon fixation (Raghavendra and Das 1978b) and also maintain linear rates of not only carbon fixation but also photochemical activities for shorter duration as compared to those from C_3 plants, *Oryza sativa* and *Rumex vesicarius* (Raghavendra and Das 1978a).

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