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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 aie 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 cm^2 pieces and suspended for 10 min in 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 $NaNO_3$. 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 $NaH^{14}CO_3$ into acid stable products. The reaction mixture (2 ml) contained 0·4 M mannitol, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM $NaNO_3$, 1 mM $MnCl_2$, 5 mM K_2HPO_4, 50 mM tricine buffer pH 8·0, 5 mM $NaH^{14}CO_3$ and cell suspension (equivolant 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 ^{14}C 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 intac mesophyll cells on mild maceration (table 1). The washed cells (a few appear as

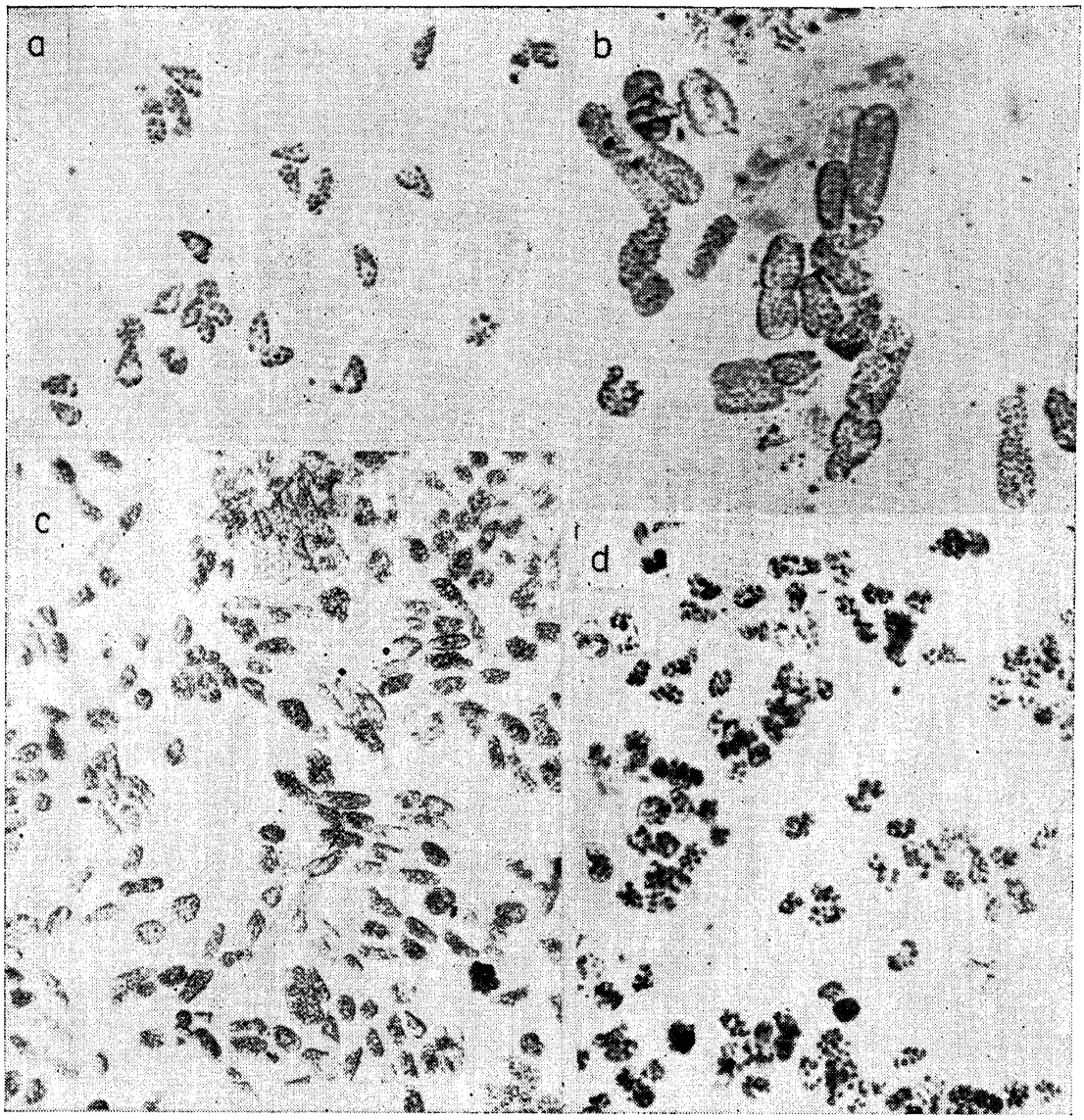

Figure 1. Intact mesophyll cells isolated from leaves of *Commelina benghalensis* (a), *Celosia cristata* (b), *Arachis hypogaea* (c) and *Digitaria adscendens* (d) × 150.

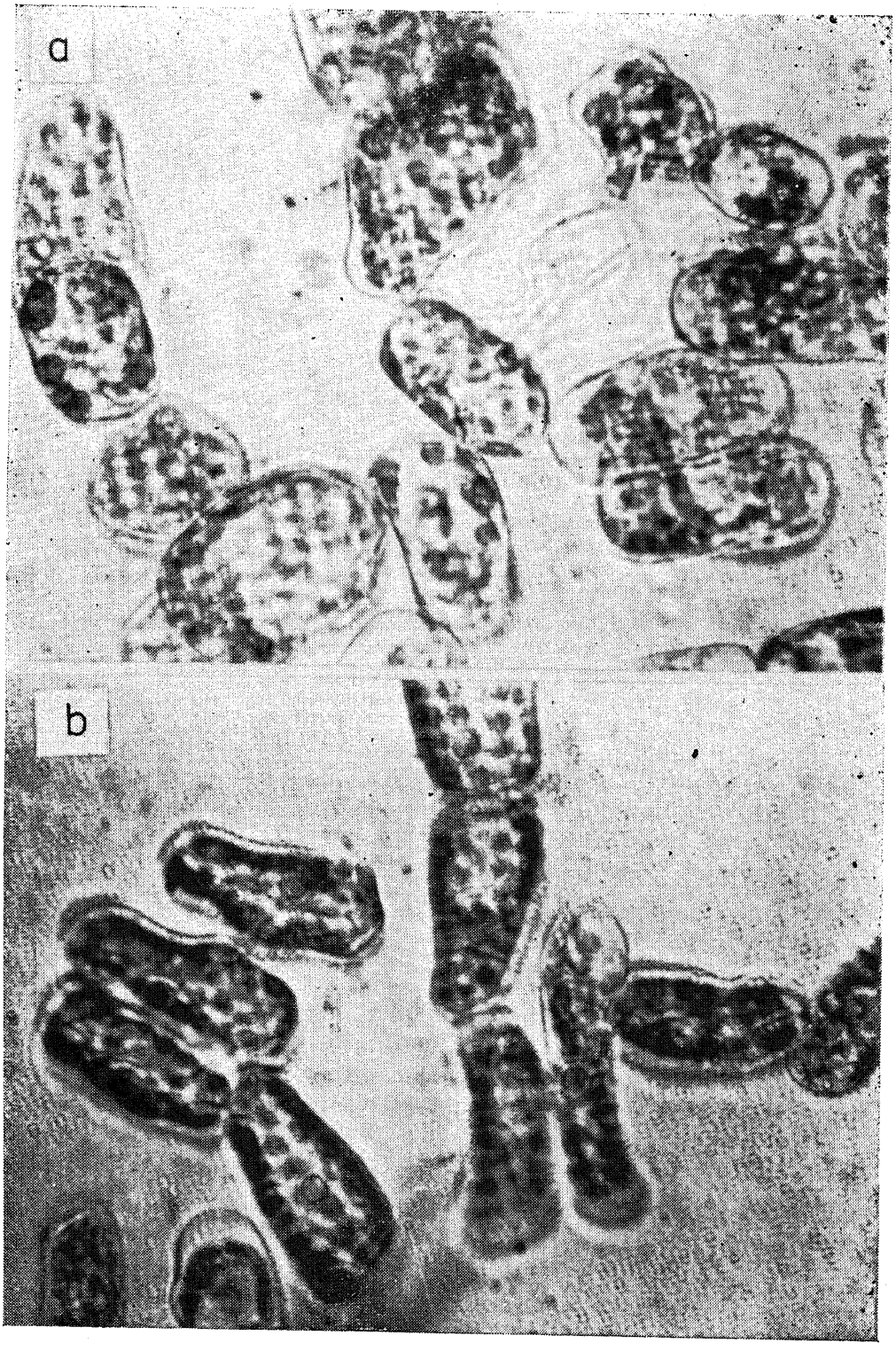

Figure 2. Intact mesophyll cells isolated from leaves of *Digera alternifolia* (a) and *Aster leavis* (b) × 600.

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

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

(Contd.)

Table 1 (*Contd.*)

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

(*Contd.*)

Table 1 (*Contd.*)

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

(*Contd.*)

Table 1 (*Contd.*)

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

(*Contd.*)

Table 1 (*Contd.*)

Plant species	Cell release*
Echinochloa colonum Link	+
Eleusine coracana Gaertn.	0
Heteropogon contortus Beauv.	+
Imperata cylindrica Beauv.	0
Oryza sativa L.	0
Panicum maximum Jacq.	+
P. nodosum Kunth	+
P. psilopodium Trin.	+
P. purpurascens Raoldi	+
Saccharum officinarum L.	+
Sorghum vulgare L.	0
Spinifex squarrosus L.	0
Tragus biflorus Schult.	+
Zea mays 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 μmoles 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 ^{14}C-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

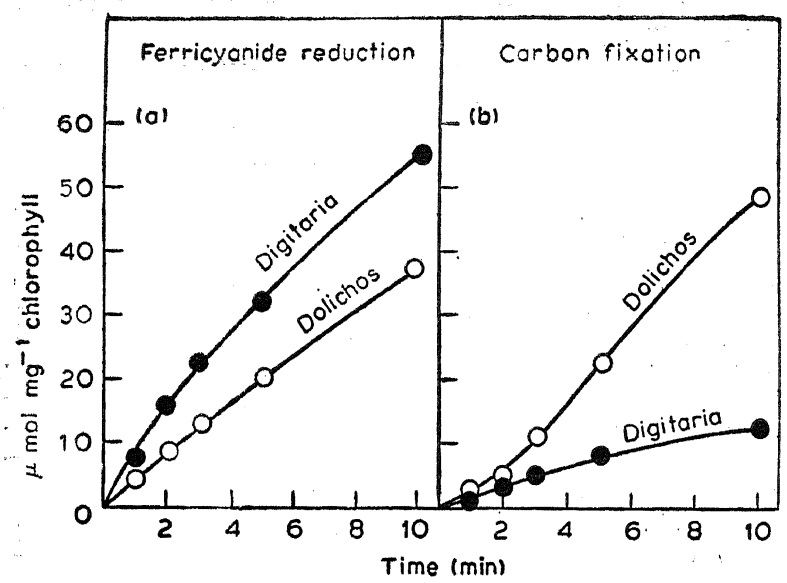

Figure 3. The pattern of ferricyanide reduction (a) and carbon assimilation (b) by isolated mesophyll cells from *Digitaria adscendens* and *Dolichos lablab*.

Table 2. Cell release and chlorophyll recovery of isolated mesophyll cells from different plant species (values are means of five observations ± 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	
Arachis hypogaea	5·60	2·08±0·18	1·59±0·11	76·4±6·2
Dolichos lablab	6·60	2·31±0·17	2·02±0·16	87·4±8·8
Musa paradisiaca	2·30	1·63±0·12	0·73±0·05	44·8±3·9
Justicia tranquebariensis	0·50	3·36±0·26	0·44±0·02	13·2±1·2
Randia malabarica	1·25	1·90±0·12	1·09±0·11	57·4±4·9
Commelina benghalensis	2·15	2·54±0·21	1·21±0·09	47·6±3·6
Chenopodium quinova	1·75	1·51±0·10	0·44±0·03	29·1±2·6
Cleome viscosa	5·16	2·22±0·19	1·37±0·12	61·7±5·7
Digera alternifolia	2·94	1·98±0·11	0·89±0·06	44·9±4·8
Digitaria adscendens	2·04	2·05±0·23	1·36±0·11	66·5±5·9
Achyranthes aspera	4·92	2·78±0·20	1·36±0·14	48·9±4·6
Panicum maximum	0·90	1·42±0·09	0·62±0·05	43·6±3·8
Cyperus rotundus	4·20	1·71±0·12	1·03±0·09	60·2±7·2
Celosia argentea	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}	$^{14}CO_2$ assimilation μ moles mg^{-1} (chlorophyll) h^{-1}
Arachis hypogaea	230·6	110·4
Digera alternifolia	330·8	96·8
Digitaria adscendens	450·2	62·4
Dolichos lablab	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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