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Isolation of protoplasts from cardamom (*Elettaria cardamomum* Maton.) and ginger (*Zingiber officinale* Rosc.)

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Abstract

Protoplasts were isolated from leaf mesophyll tissue, collected from *in vitro* grown plantlets and cell suspension cultures of cardamom (*Elettaria cardamomum*) and ginger (*Zingiber officinale*). In cardamom, a protoplast yield of 3.5 x 10^5/g of leaf tissue was obtained when incubated in an enzyme solution containing 0.5% macerozyme R10, 2% cellulase Onozuka R10 and 9% mannitol for 18–20 h at 25°C in dark. The yield of protoplasts from cell suspension culture was 1.5 x 10^5 /g tissue, when incubated in 1% macerozyme R10 and 2% cellulase Onozuka R10 for 24 h at 25°C with gentle shaking at 53 rpm in dark. The viability of leaf mesophyll protoplast was 75% and that of cell suspension was 40% on Evan's blue staining. In ginger, a protoplast yield of 2.5 x 10^5 /g of leaf tissue was obtained on digestion in an enzyme solution containing 0.5% macerozyme R10, 3% hemicellulase and 5% cellulase Onozuka R10, when incubated for 10 h at 15°C followed by 6 h at 30°C. The protoplast viability was 55%. Protoplast yield from cell suspension culture was 1 x 10^5 /g of callus when digested with an enzyme solution of 1% macerozyme R10, 3% hemicellulase and 6% cellulase Onozuka R10 and incubated for 10h at 15°C and later at 30°C for 8 h. Seventy two per cent of the protoplasts were viable. The protoplasts from both the species could be cultured and made to develop up to microcalli stage.

Key words: cardamom, *Elettaria cardamomum*, ginger, protoplast, *Zingiber officinale*.

Abbreviations: BAP : N^6-Benzyl aminopurine; CPW : Cell Protoplast Washing Medium; 2, 4-D : 2, 4-Dichlorophenoxyacetic acid; MS : Murashige and Skoog medium; NAA : α - Napthaleneacetic acid.

Introduction

Protoplasts are suitable for a variety of manipulations, which are not normally possible with intact cells and form an important tool for parasexual modification of genetic content of cells (Vasil & Vasil 1980). Protoplasts can be isolated from a variety of tissues like leaves (Power & Cocking 1970; Kartha *et al.* 1974), cladodes (Ha & Mackenzie 1973), shoot apices, fruits (Gregory & Cocking 1965), roots (Davey *et al.* 1973), coleoptiles (Ruesink & Thimann 1965; Hall & Cocking 1974), aleurone layer of cereals (Taiz & Jones 1971), microspore mother cells (Ito & Maede 1973), microspore tetrads (Bhojwani & Cocking 1972; Bajaj 1974) and pollen (Bajaj 1983) by enzymatic digestion of cell wall (Cocking 1960; Gregory & Cocking 1965; Ruesink & Thimann 1965). Development of an efficient regeneration protocol from cultured protoplasts of crop plants is thus important for taking advantage of cell manipulation technologies for crop improvement. This paper reports the successful isolation, culture and microcallus development from protoplast of *in vitro* derived mesophyll tissue and cell suspension cultures of cardamom (*Elettaria cardamomum* Maton) and ginger (Zingiber *officinale* Rosc).

Materials and methods

Source tissue

Mesophyll tissues of *in vitro* derived leaves and actively growing callus tissue from 5 to 7 day old suspension culture were used as source material. The lower surface of the leaves were punctured and then cut into small pieces, as it was difficult to peel off the epidermis, to ensure proper enzymatic digestion. The suspension culture was centrifuged and 1 g of the pelletted callus tissue was used for enzymatic digestion.

Enzymatic digestion

One step method of enzyme digestion was followed ie, the tissue was digested with a mixture of macerozyme and cellulase and cellulase Onozuka R10, at different concentrations. The enzyme solution was prepared in CPW medium with different concentrations of the osmoticum (Mannitol) (Tables 1 and 2).

Protoplast isolation

The source tissues were incubated in CPW medium with different concentrations of mannitol to induce pre-plasmolysis of the cells. Tissues were used directly without the pre-plasmolysis step also. One gram each of mechanically macerated leaves from *in vitro* cultured plants and fragile calli from cell suspension culture were immersed in 10 ml each of the enzyme solution (Table 1) and incubated in dark. In cardamom, the leaf tissue was incubated for 18–20 h at 25°C without agitation and callus tissue for 24 h at 25°C with gentle agitation at 53 rpm. In ginger, the leaf tissue was incubated for 16 h (initially for 10 h at 15°C followed by 6 h at 30°C) and callus tissue for 18 h (initially for 10 h at 15°C followed by 8 h at 30°C) with a gentle shaking at 53 rpm. The changes occurring during incubation were observed at hourly intervals.

Protoplast purification

A combination of filtration, centrifugation, washing and floatation centrifugation was used to purify the protoplasts. After digestion, the enzyme solution containing protoplasts was filtered through a stainless steel mesh (60 mesh size, from Sigma) to remove undigested tissue and cell clumps. A sample was observed under the inverted microscope to confirm enzymatic digestion and release of protoplasts. The filtrate was distributed into sterilized screw capped centrifuge tubes and centrifuged in Beckman table top centrifuge for 10 min at 700 rpm. The protoplasts formed a pellet at the bottom of the tube.

The supernatant enzyme solution was removed using a Pasteur pipette without distrubing the pellet. The pellet was suspended in CPW medium. The centrifugation and re-suspension in fresh medium was repeated three times so as to wash the protoplasts and remove traces of enzyme solution. After washing, the pellet of protoplasts was re-suspended in 1 ml of the CPW medium and layered on top of 9 ml of floatation medium (Table 2) and centrifuged at 700 rpm for 10 min. The living protoplasts formed a band at the interphase, which was collected and used for assessing its viability and culturing.

Protoplast viability

For estimation of viability, a sample of the protoplast was stained with Evan's blue stain. The density of the protoplast was estimated with a haemocytometer.

Portoplast culture

Isolated protoplasts were cultured initially in liquid culture medium (Table 2) in petridishes. Five drops of the culture medium containing protoplasts were placed in the bottom half of the petridish covered with lid, sealed with parafilm and incubated in dark at 25°C. Fresh culture medium was added every 7 days to replenish the nutrients. The samples were periodically observed for cell wall regeneration and cell division. After 40–60 days of culture, they were plated on 0.25% agarose medium (Culture medium II) and monitored for further development and microcallus formation.

Results and discussion

Isolation of protoplasts from in vitro *leaf tissue*

Periodical obervation of changes occurring during enzyme digestion of *in vitro*-derived leaf tissue of cardamom revealed that maceration of tissue was initiated 30 min after incubation. The leaf tissue lost its integrity and clumps of cells start separating. After 4 h of incubation, liberation of cell clumps and cells with partially digested wall were observed. Further incubation for 6–10 h resulted in the liberation of large number of cells/cell clumps. After 18–24 h, cells devoid of cell wall were observed; thus 18–20 h was ideal for obtaining viable protoplasts and further incubation leads to plasmolysis or protoplast lysis based on the concentration of osmoticum used. After incubation, the protoplasts entangled in the undigested debris could be liberated by gentle shaking. Out of 42 enzyme solutions tried, the one with 0.5% macerozyme, 2% cellulase Onozuka R10 and 9% mannitol was sufficient to give good yield of protoplasts (Fig. 1A). Extending the incubation period beyond 20 h resulted in

Table 1. Composition of enzyme solutions utilized for isolation of protoplasts in cardamom and ginger

ES	Mannitol (%)	Pectinase (%)	Macerozyme R10 (%)	Hemicellulase (%)	Onozuka cellulase R10 (%)	Cellulase (%)
ES-1	10.0	-	0.5	-	1.0	-
ES-2	9.0	-	0.5	-	1.0	-
ES-3	8.0	-	0.5	-	1.0	-
ES-4	7.0	-	0.5	-	1.0	-
ES-5	6.0	-	0.5	-	1.0	-
ES-6	5.0	-	0.5	-	1.0	-
ES-7	10.0	-	0.5	-	2.0	-
ES-8	9.0	-	0.5	-	2.0	-
ES-9	8.0	-	0.5	-	2.0	-
ES-10	7.0	-	0.5	-	2.0	-
ES-11	6.0	-	0.5	-	2.0	-
ES-12	5.0	-	0.5	-	2.0	-
ES-13	10.0	0.5	0.5	0.5	2.0	1.0
ES-14	9.0	0.5	0.5	0.5	2.0	1.0
ES-15	8.0	0.5	0.5	0.5	2.0	1.0
ES-16	7.0	0.5	0.5	0.5	2.0	1.0
ES-17	6.0	0.5	0.5	0.5	2.0	1.0
ES-18	5.0	0.5	0.5	0.5	2.0	1.0
ES-19	10.0	-	1.0	-	2.0	-
ES-20	9.0	-	1.0	-	2.0	-
ES-21	8.0	-	1.0	-	2.0	-
ES-22	7.0	-	1.0	-	2.0	-
ES-23	6.0	-	1.0	-	2.0	-
ES-24	5.0	-	1.0	-	2.0	-
ES-25	10.0	-	1.0	-	4.0	-
ES-26	9.0	-	1.0	-	4.0	-
ES-27	8.0	-	1.0	-	4.0	-
ES-28	7.0	-	1.0	-	4.0	-
ES-29	6.0	-	1.0	-	4.0	-
ES-30	5.0	-	1.0	-	4.0	-
ES-31	10.0	-	0.5	3.0	5.0	-
ES-32	9.0	-	0.5	3.0	5.0	-
ES-33	8.0	-	0.5	3.0	5.0	-
ES-34	7.0	-	0.5	3.0	5.0	-
ES-35	6.0	-	0.5	3.0	5.0	-
ES-36	5.0	-	0.5	3.0	5.0	-
ES-37	10.0	-	1.0	3.0	6.0	-
ES-38	9.0	-	1.0	3.0	6.0	-
ES-39	8.0	-	1.0	3.0	6.0	-
ES-40	7.0	-	1.0	3.0	6.0	-
ES-41	6.0	-	1.0	3.0	6.0	-
ES-42	5.0	-	1.0	3.0	6.0	-

ES = Enzyme solution

Table 2. Composition of media utilized for protoplast isolation and culture in cardamom and ginger

Component	Cell protoplast washing medium (mgl^{-1})	Floatation medium (mgl^{-1})	Protoplast culture media (mgl^{-1}) I	Protoplast culture media (mgl^{-1}) II
NH_4NO_3	-	-	1650.000	1650.000
KNO_3	101.000	101.000	1900.000	1900.000
$CaCl_22H_2O$	1480.000	1480.000	440.000	440.000
$MgSO_4 7H_2O$	246.000	246.000	370.000	370.000
KH_2PO_4	27.200	27.200	170.000	170.000
KI	0.160	0.160	0.830	0.830
H_3BO_3	-	-	6.200	6.200
$MnSO_44H_2O$	-	-	22.300	22.300
$ZnSO_47H_2O$	-	-	8.700	8.700
$Na_2MoO_42H_2O$	-	-	0.250	0.250
$CuSO_45H_2O$	0.025	0.025	0.025	0.025
$CoCl_26H_2O$	-	-	0.025	0.025
$FeSO_47H_2O$	-	-	27.800	27.800
$Na_2EDTA\ 2H_2O$	-	-	37.300	37.300
Myo-inositol	-	-	100.000	100.000
Nicotinic acid	-	-	0.500	0.500
Thiamine HCl	-	-	0.500	0.500
Pyridoxine HCl	-	-	0.500	0.500
Glycine	-	-	2.000	2.000
Sucrose	-	21%	2%	3%
Mannitol	7–10%	-	7%	4%
BAP	-	-	0.500	1.000
NAA	-	-	0.500	1.000
2,4-D	-	-	0.500	-

protoplast lysis. The same enzyme concentration with higher level of mannitol was not favourable as the protoplast showed a high degree of plasmolysis. Mannitol at lower levels yielded more than 70% of broken protoplasts. At low levels of enzyme concentration, the liberation of cells or protoplasts was not satisfactory. In cardamom a protoplast yield of 3.5 x 10^5 protoplasts per g leaf tissue was obtained. The viability of the protoplast was 72–75% on Evan's blue staining.

In ginger, the liberation of cell clusters and individual cells was observed immediately after mechanical maceration, even before enzymatic digestion (Fig. 1D). Since mechanical maceration itself resulted in liberation of individual cells and cell clusters, macerozyme at 0.5% was sufficient to digest the middle lamella and separate the remaining cells. Among the enzyme solutions tried, only the one containing 0.5% macerozyme R10, 3% hemicellulase and 5% cellulase Onozuka R10 was efficient in liberation of protoplasts, after incubation for 16 h (the tissue was incubated initially for 10 h at 15°C followed by 6 h at 30°C with gentle shaking at 53 rpm in dark) (Table 3). The isolation solution containing 7% mannitol was necessary for releasing and maintaining viable protoplasts. The protoplast yield was 2.5 x 10^5 per g of leaf tissue in ginger. The protoplast viability as assessed by Evan's blue staining was

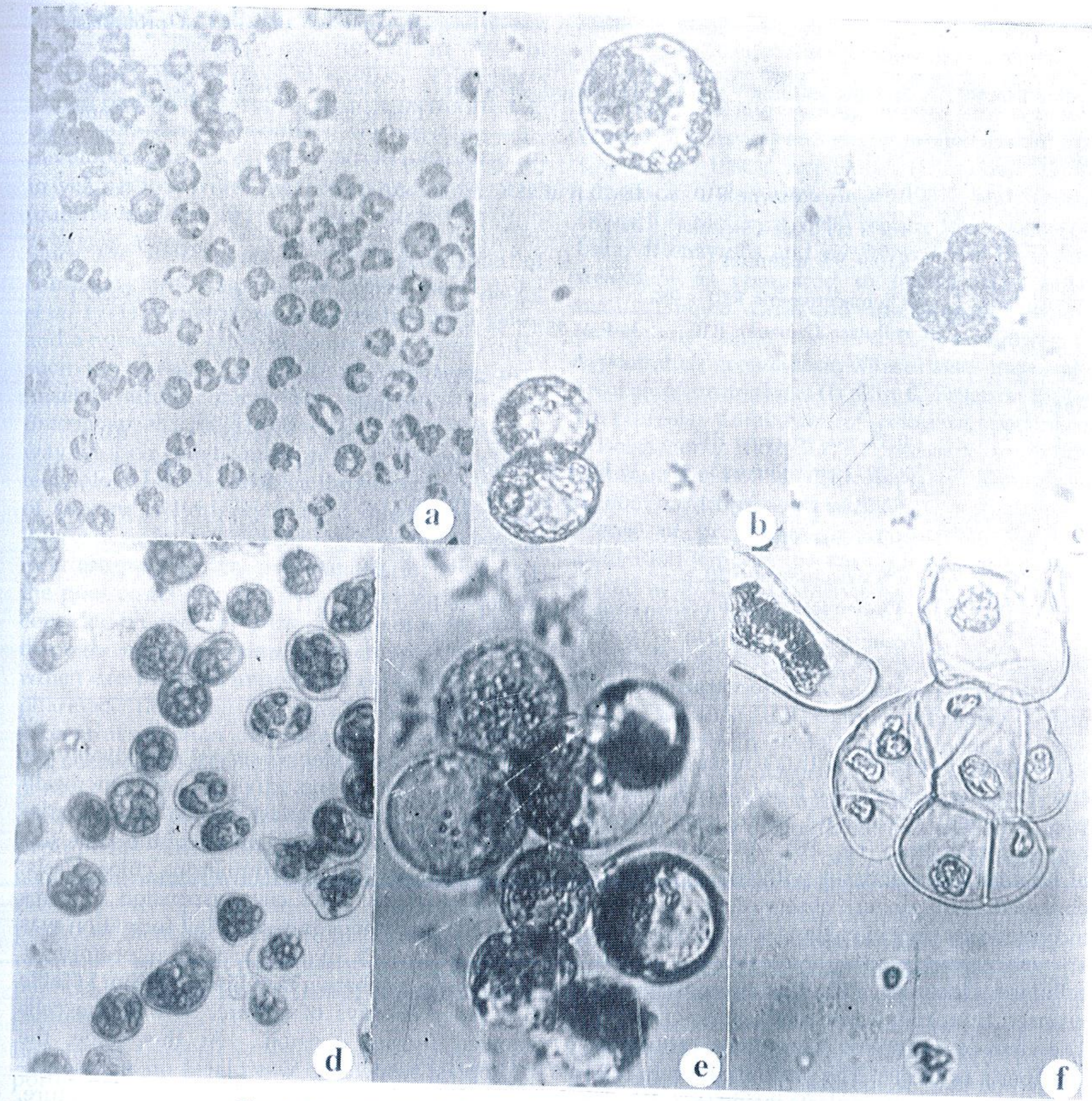

Fig. 1. Protoplast isolation and culture in cardamom and ginger

a. Protoplasts from leaf tissue of cardamom b. Protoplasts from cell suspension culture of cardamom c. First division of cardamom protoplast d. Cells liberated on mechanical maceration of leaf tissue in ginger e. Protoplast from cell suspension culture of ginger f. Microcallus formation from ginger protoplast.

55% (Table 3).

Isolation from callus and cell suspension clutures

Cell suspension cultures were initiated from creamy white fragile callus in MS basal medium with 2 mgl^{-1} 2,4-D and kept on a rotary shaker at 80 rpm. In 5 to 7 days, a cell suspension containing individual as well as clusters of cells was established. The suspension contained newly formed round cells with dense cytoplasm and older elongated and vacuolated cells. Approximately 1 g of callus containing both the cells types, were treated with enzyme solution.

The cell suspension culture consisted of two types of cells, namely highly vacuolated cells with less cytoplasm and slightly vacuolated cells with dense cytoplasm. In cardamom no enzyme

Table 3. Effect of source tissue, enzyme concentration and incubation condition on yield of protoplasts in cardamom and ginger

Source tissue	Enzyme solution	Incubation condition	Yield (per g tissue)	Viability (%)	Size (mm)
Cardamom					
Leaf	0.5% macerozyme R10 + 2% cellulase Onozuka R10 + 9% mannitol	18–20 h at 25°C in dark	3.5 x 10^5	75	0.015
Callus	1% macerozyme R10 + 2% cellulase Onozuka R10 + 8% mannitol	24 h at 25°C; 53 rpm in dark	1.5 x 10^5	40	0.027
Ginger					
Leaf	0.5% macerozyme R10 + 3% hemicellulase + 5% cellulase Onozuka R10 + 7% mannitol	16 h; at 15°C for 10 h, 30°C for 6 h; 53 rpm in dark	2.5 x 10^5	55	0.021
Callus	1% macerozyme R10 + 3% hemicellulase + 6% cellulase Onozuka R10 + 7% mannitol	18 h; at 15°C for 10 h, 30°C for 8 h; 53 rpm in dark	1.0 x 10^5	72	0.039

solution except the one with 1% macerozyme R10 and 2% cellulase Onozuka R10 with 8% mannitol was sufficient to liberate protoplasts after 24 hours of incubation (Fig. 1B). A protoplast yield of 1.5 x 10^5 per g of tissue with 40% viability was observed. In ginger, observations after 18 h indicated that only in enzyme solution containing 1% macerozyme, 3% hemicellulase and 6% cellulase Onozuka R10, the protoplasts were released from round cells with dense cytoplasm. The yield of protoplast from ginger callus was 1 x 10^5 per g of tissue. The protoplasts were round with little or no chloroplasts inside (Fig. 1E). The viability assessed by Evan's blue staining was 72%.

Protoplast culture

Isolated protoplasts were cultured in petridishes. The protoplasts were re-suspended in culture medium I (Table 2). Five drops of culture medium containing protoplasts were placed in the bottom half of the petridish, covered with lid and then sealed with parafilm to reduce the loss of water from the culture medium. The preparation was covered with aluminium foil and kept in dark at 25°C. Fresh culture medium with low osmoticum was added every 7 days, to replenish the nutrients. The samples were observed periodically for cell wall regeneration, division and microcalli formation. The protoplast derived from cardamom leaf tissue was more or less of the same size (0.015 mm) and contained numerous chloroplasts. The protoplasts from cell suspension cultures were heterogeneous in size. Wall formation was observed in 90% of the cardamom protoplasts after 2 days incubation in culture medium I (Table 3). After 20 days of culture, 50% of the cells showed further division. At this stage, the protoplast suspension was plated on agar solidified culture medium II. Within 30 days of culture, 30% of the dividing cells produced microcalli.

The protoplasts derived from ginger leaf tissue were heterogeneous and comprised of protoplasts of different sizes (0.015 mm to 0.021 mm). The protoplasts derived from cell suspension cultures were mostly of uniform size (0.039 mm). They were cultured as droplet cultures in MS liquid medium supplemented with 3% sucrose and 7% mannitol (culture medium) up to 20 days. The cell contents became dense by 1 week and fresh medium need to be added at 7 days interval. More than 90% of the protoplasts started regenerating the cell wall within 2–3 days. Within

3–4 weeks of culture, cells started dividing (Fig. 1C). Division was noticed in 70% of protoplast after 4 days in culture. Protoplasts plated on liquid as well as agarose media after 20 days gave similar results. The dividing cells developed into microcalli in 50–70 days (Fig. 1F) in MS medium with 1 mgl^{-1} NAA and 1 mgl^{-1} BAP (culture medium II).

Since the first report on the regeneration of complete plants from isolated protoplasts (Takabe *et al.* 1971), tremendous progress has been made and a number of crops of commercial importance such as cereals, potato, tomato, tobacco, rice, linseed, alfalfa, cucumber, eggplant, lettuce, *Brassica* sp. etc., have been regenerated paving the way for their genetic manipulation. Refinement in technology has also enabled the successful culture of somatic hybrids/cybrids at interspecific and intergeneric levels with far reaching implications in crop improvement programmes for widening the pool of genetic resources. Another aspect of considerable interest is the storage of protoplasts through immobilization and cryopreservation, which are of great importance especially in the pharmaceutical industry (Bajaj 1989a; 1989b). The successful isolation of protoplasts, their culture and subsequent regeneration into complete plants depend on a number of factors, the important ones being genotype, tissues from which protoplasts are isolated, the physiological conditions under which the plant cultures are raised, purity of enzymes and plasmolyticum, period of incubation, culture media and growth regulators, milieu of protoplasts, plating density, method of culture (liquid/solid) and incubation conditions.

Enzymatic isolation of protoplasts was first reported in tomato root tips by Cocking (1960). The easy availability of purified commercial enzymes such as cellulase, cellulysin, pectinase, macerozyme, driselase, rhozyme and hemicellulase have now increased the yield, viability of protoplasts and their subsequent response in the culture medium. The period of treatment is reduced and thus deleterious effects on plasma membrane are avoided. Usually a combination of pectinase and cellulase is used to macerate cells and also liberate protoplasts in a single cell (Power & Cocking 1970). The concentration and the combination of enzymes required depend upon age, genotype and stage of differentiation of the tissue from which the protoplasts are to be isolated. In rice, young embryogenic suspension cultures easily liberate protoplasts while order cultures yield very little protoplasts (Abdullah *et al.* 1986). Though protoplasts can be isolated from a variety of tissues, young *in vitro* grown plants (Bajaj 1972), tissues and explants such as root tips (Xu *et al.* 1982), hypocotyl (Glimelius 1984), cotyledons (Hammatt *et al.* 1987) and shoots (Russell & McCown 1986) require low concentrations of enzymes and relatively short period of treatment as compared to leaves from older mature plants. Cells and tissues grown *in vitro* particularly suspension and callus cultures in exponential growth stage, are also important sources of protoplasts (Wallin & Eriksson 1973). For cereals, a mixture of cellulase, pectinase, driselase and rhozyme is necessary to obtain optimum yield of viable protoplasts. Though an incubation temperature of 25–30°C is suitable for release of protoplasts in most plants, a low incubation temperature (14°C) followed by short period at 30°C was beneficial in cereals (Vasil & Vasil 1980).

Manipulation of young tissue, pure enzyme and reduction in period of incubation often result in higher viability and plating efficiency. The present study indicated the requirement of higher concentration of macerozyme R10 and cellulase Onozuka R10 and longer incubation period for release of ginger protoplasts. This may be due to the relative age of the cell suspension cultures. Similar observations were also made by Abdullah *et al.* (1986) in rice where the embryogenic callus cultures released protoplasts easily while practically no protoplasts were released from older cells. The two step incubation condition of lower and higher incubation temperatures agrees with the protocol of Vasil & Vasil (1980) in cereals. This is the first report of protoplast isolation from cardamom. Protoplast isolation, culture and development of microcalli from ginger tissue were reported by Babu (1997), using the same procedure. These protoplasts form the basis for the utilization of advanced techniques such as somatic cell hybridization and genetic manipulation using direct or *Agrobacterium*-mediated gene transfer for crop improvement in cardamom and ginger.

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