CHAPTER 4

PROTOPLAST ISOLATION, CULTURE AND PLANT REGENERATION

4.1. INTRODUCTION

Genetic improvement of the cultivated groundnut (*Arachis hypogaea* L.) is a constant necessity due to the rapid development of the culture of this important oilseed crop in the world. Besides the classical methods of breeding, biotechnology seems to open other promising routes. Protoplasts provide an ideal experimental system for studies of genetic transformation, protoplast fusion, organelle transfer and somatic mutations (Parihar et al., 1995). Plant regeneration from protoplasts has long been considered one of the important *in vitro* tools for improving crop plants in general and development of plants resistant to diseases and pests in particular (Daub, 1986).

Plant regeneration from protoplasts in grain legumes is still quite recalcitrant, but immature tissues provide excellent material for protoplast isolation and culture (Wei and Xu, 1990). Regeneration of plants from protoplasts of groundnut has been regarded as rather difficult. Various tissue sources has been used for isolation of groundnut protoplasts, giving different results. Only a few successful achievements have been reported in cultured protoplasts isolated from immature and mature leaves, cotyledons and root tips of *A. hypogaea* (Bajaj and Gosal, 1988; Mhatre et al., 1985; Oelck et al., 1982; Rugman and Cocking, 1985). Some authors have tested the dividing capacity of isolated protoplasts and production of calli from young leaves, mature leaves and cotyledons (Mhatre et al., 1985; Oelck et al., 1982), but only mesophyll protoplasts divided with a high frequency. Other tissue types are thought to be relatively recalcitrant for further regeneration studies. Recently, Li et al. (1993) reported on the regeneration of fertile plants from leaf derived suspension protoplasts of *Arachis paraguariensis*. However, the frequency of regeneration was very low and few plants could be transferred to the greenhouse. In view of this, extensive work on isolation and culture of protoplasts needs to be taken up to develop the protoplast culture technology for cultivated groundnut.
There are no previous reports of sustained cell division of protoplasts and somatic embryogenesis in this oilseed crop. For this reason, we have started our investigations on isolation, culture, callus production and regeneration of plants via somatic embryogenesis from immature leaflet protoplasts and herein report a system of callus induction and subsequent plant regeneration through somatic embryogenesis in cultivated groundnut (*Arachis hypogaea* L.). In addition to this we have developed practically usable system for protoplast isolation and culture from hypocotyl derived cell suspensions.

### 4.2. MATERIALS AND METHODS

**4.2.1. Plant Material**

As described in Chapter 2.

**4.2.2. Methodology for Protoplast Isolation and Culture**

(i) **Germination of seeds**

As described in Chapter 2.

(ii) **Selection of explants**

As described in Chapter 3.

(iii) **Hypocotyl**

(a) **Callus initiation**

As described in Chapter 3.

(b) **Initiation and maintenance of cell suspension culture**

Suspension culture of hypocotyl callus and feeders cells were simultaneously initiated. The liquid medium used was MS modified with 1.0 mg/l NAA, 2.0 mg/l 2,4-D and 0.5 mg/l KIN. From the subcultured callus, friable and greenish calli were separated and inoculated in the conical flasks containing modified MS liquid medium which is given in Appendix 2. The steps involved are as follows:

i) 500 mg of the callus per 10 ml of the liquid medium was added and kept on shaker (80 rpm) in the dim light (30 μE m²s⁻¹) at 24±2°C.
ii) The medium was replaced once in seven days for the first three weeks. All the medium was removed with a sterile pipette and then 10 ml of the fresh MS liquid medium was added to each flask.

iii) The cultures were then subcultured two times per week from 2 weeks to one month in order to obtain a ‘fine’ cell suspension. Cells were selected using a 10 ml pipette. Finer cell clumps around the edge were picked up leaving the necrotic cells. The finer cells were transferred to a new flask containing fresh MS liquid medium. The volume of the medium was increased as the cell grew and packed cell volume increased.

iv) After one month, the well established hypocotyl suspension was subcultured once per week.

v) Growth curve of newly established cell line was recorded.

(c) Protoplast isolation

i) Preparation of hypocotyl cells for protoplast isolation: The hypocotyl cell suspensions were subcultured four days prior to protoplast isolation and maintained at 25°C in dim light at the same shaker speed of 80 rpm

ii) Cells prepared for feeder culture: The feeder cells were subcultured into fresh MS cell culture medium and maintained at 25°C in dim light at the same shaker of 80 rpm

iii) The media prepared were

- Protoplast isolation solution (See Appendix 3)
- Protoplast wash solution (See Appendix 4)
- Protoplast purification solution: 0.6M sucrose
- Protoplast culture a feeder layer medium

1) Best results in terms of protoplast yield and viability were obtained from suspension cultures which were in exponential phase of cell growth. This occurred 2 to 6 days after subculture of groundnut suspensions

2) The suspension culture was sieved through a 500 μm sieve by placing the screen on a petri plate of known weight. Gently stirred the tissue retained by the sieve with a spatula in order to release as many cell clumps smaller than 500 μm as possible
3) The suspension medium was discarded from each petri plate with a Pasteur pipette. This was reweighed in order to determine the fresh weight of the cells obtained.

4) Suspensions were pretreated with CPW medium (Frearson et al., 1973) containing 0.6 M mannitol solution prior to protoplast isolation. 

5) The CPW medium was replaced with protoplast isolation solution 20 ml per gram of cells, sealed with parafilm and placed on a slow shaker of 50 rpm for 2 hrs in darkness. This was followed by 8 hrs stationary incubation at 25°C.

6) The enzyme mixture consisted of 5 levels of cellulase and 4 levels of macerozyme.

7) The cells were observed periodically during incubation under a microscope. Critical time when maximum protoplast released was noted.

(d) Filtration of protoplasts

8) At the end of digestion, the contents were filtered through 30 μm nylon mesh stacked in a petri plate. After running the enzymes, the suspended protoplasts and undigested cell clumps adhering protoplasts, if any, were washed down with protoplast wash solution.

(e) Washing of protoplasts

9) The supernatant was discarded and protoplast resuspended pellet in 10 ml of protoplast was solution. Again centrifuged the supernatant was discarded and the protoplast pellet retained.

(f) Purification of protoplasts

10) The suspended protoplasts were layered on 5 ml of 0.6M sucrose in a 10 ml conical centrifuge tube and centrifuged at 40xg for 10 min.

11) The protoplasts floated on top of the sucrose cushion. Protoplasts were collected with a pasteur pipette and transferred to 5 ml of protoplast wash solution in a 10 ml conical centrifuge tubes and centrifuged at 100xg for 10 min.

12) The supernatant was discarded and suspended protoplasts were collected in CPW medium.

13) Protoplast viability test was then conducted.
(g) **Protoplast viability test**

14) Protoplast viability was determined using Evans blue dye (Gaff and Okong's o-Ogala, 1971). Evans blue stain solution of 0.2% (W/V) was prepared. One drop of protoplasts in suspension and one drop of the dye were mixed and after 5 minutes observed in a Nikon inverted microscope with white light illumination. 'Living' cells/protoplasts remained unstained and 'dead' cells/protoplasts were accumulated the stain.

\[
\text{Percentage of protoplasts} = \frac{\text{No. of viable protoplasts}}{\text{Total No. of protoplasts}} \times 100
\]

15) Using a haemocytometer, the total protoplast yield per gram of cells was determined. Protoplasts were diluted to 2.5x10^4 protoplasts per ml concentration with protoplast culture medium.

16) Protoplasts were plated on feeder layer and incubated in dark at 25°C

(h) **Preparation of feeder layer**

1) A 50 ml aliquot of 1.6% (W/V) agarose was melted and cooled to 40°C

2) Four days old feeder cells in MS cell culture medium were pelleted by centrifugation

3) 50 ml molten agarose and 50 ml of double strength protoplast culture medium were added in a conical flask with 10 ml of feeder cells (Packard Volume)

4) The flasks were swirled so that the cells were uniformly suspended in the medium and 5 ml medium was quickly pipetted out into each 60x15 mm petri plates

5) Then the flasks were allowed to cool and a sterile 0.8 μm millipore filter paper was placed into the top of the feeder layer.

(i) **Protoplast culture procedure**

1) Isolated protoplasts were cultured at a concentration of 0.8 to 4.5 x 10^4 protoplasts/ml in Kao’s medium (See Appendix 5) supplemented with NAA (0.5-2.5 mg/l) and BAP (1.0 mg/l) at pH 5.8

2) The protoplasts were pipetted onto the filter paper and carefully distributed them over 80% of the surface using an inoculation loop
3) The plates were allowed to stand in the hood until free standing liquid had evaporated. However, it was not allowed to dry completely.
4) The plates were wrapped with parafilm and placed in dark at 25°C.

(j) Plating efficiency
1) Plating efficiency was scored four weeks after initial plating date.
2) Microcolonies of callus, 0.1 mm diameter were seen under dissection microscope. They were counted to score plating efficiency.

\[
\text{Plating efficiency} = \frac{\text{No. of colonies observed}}{\text{No. of protoplasts plated}} \times 100
\]

(k) Culture of cell colonies and calli
1) After four weeks, there was sufficient amount of protoclonies on top of the filter. The mini callus was ready to be removed from the presence of feeder layer to protoplast callus culture medium (MS basal + NAA + 2,4-D + BAP + 3\% W/V sucrose+0.8\% W/V agar).
2) The entire filter containing protoplast callus of the feeder layer was lifted and callus was tapped gently, directly onto protoplast callus culture medium supplemented with NAA, 2,4-D and BAP (0.5 mg/l each).
3) Cultures were incubated under fluorescent light (30 \(\mu\)E \(m^2s^{-1}\)) at 25°C.
4) Well developed calli were transferred to shoot bud regeneration medium.

(iii) Immature leaflet
(a) Protoplast isolation

Seven day old seedlings were used for protoplast isolation. Seedlings were placed in dark for two days before protoplast isolation for starch degradation to occur. Tender and immature leaflets were chopped to small bits using a scalpel and preplasmolyzed in 5 ml of 0.6M mannitol solution prior to protoplast isolation. One gram of leaflet bits was incubated in 20 ml of enzyme mixture and digested for two hours on a 80 rpm shaker followed by overnight stationary digestion in dark at 25°C.
The enzyme mixture consisted of cellulase RS and macerozyme R-10. The levels of cellulase tried were 0.5, 1.0, 1.5, 2.0 and 2.5% (W/V), levels of macerozyme tried were 0.4, 0.6, 0.8 and 1.0 (W/V). Protoplast release was observed at timely intervals. Protoplast washing, purification and viability tests were done as mentioned before. The isolated protoplasts were cultured on callus induction medium directly without feeder layer. The protoplasts density were calculated using haemocytometer.

(b) Protoplast plating, cell division and colony formation

Isolated protoplasts were cultured at a concentration of 0.5 to $4.0 \times 10^4$ protoplasts/ml in Kao's medium supplemented with 3.0% (W/V) sucrose, NAA (0.5-2.5 mg/l) and BAP (1.0 mg/l) solidified with 0.4% (W/V) agarose at pH 5.8. Glass petri dishes (100 mmx15 mm) were used for culture, each with 0.5 ml of protoplast suspension. For the first 7-9 days, cultures were kept in the dark at 24±2°C. The plating efficiency (number of dividing protoplasts expressed as a percentage of the initial protoplasts population) was determined 15 days after culture. Later cultures at different developmental stages were gradually transferred to high intensity light conditions.

(c) Minicallus and greenish callus formation

Once small colonies (1-2 mm in size) could be observed visually they were picked up carefully from the beads or were released from the agarose matrix by applying pressure to the beads with a spatula. After 28 days, colonies picked up from the beads were further subcultured onto MS basal medium augmented with NAA, 2,4-D and BAP (0.5 mg/l each), 3% (W/V) sucrose and 0.8% (W/V) agar. These colonies were subcultured 3-4 times at 2 weeks intervals. When these colonies appeared green and compact they were transferred onto regeneration medium consisting 3.0% (W/V) sucrose and various growth regulators. Cultures were kept under fluorescent light (80 µE m$^{-2}$s$^{-1}$) at 24±2°C until nodular structures appeared on the periphery of calli.
After another three to four weeks, greenish calli were selected and transferred to MS liquid medium containing different concentrations of 2,4-D (1-5 mg/l) and in combination with BAP (1.0 mg/l) to evaluate callusing ability and to induce somatic embryogenesis. The cultures were kept in an rotary shaker and agitated at 80 rpm at 24±2°C in diffuse light (40 μE m²s⁻¹, 16h illumination, 8h darkness). One week later, the spent medium was replaced by 10 ml of fresh liquid MS medium. The cultures were maintained as described above for another week. After 21-28 days, globular, heart, torpedo and dicotyledonary staged embryos were observed in liquid culture and transferred to fresh medium for embryo maturation. Two weeks later the matured embryoids were transferred onto hormone free MS basal medium containing 0.3% (W/V) activated charcoal for germination. Embryoids germinated and converted into plantlets. The regenerated plants were transferred to the plastic cups containing red soil and sand mixture and were later established in the pots.

4.3. RESULTS

4.3.1. Protoplast Isolation and Culture

(i) Hypocotyl

(a) Initiation of callus

The details of this experiment have been described in Chapter 3.

(b) Growth studies on cell suspensions

Three week old calli was optimal for initiating cell suspension cultures. Groundnut cells in suspension were roughly spherical, about 50 μM in diameter and formed suspension of single cells and loose aggregates. When subcultured on weekly intervals, the cultures increased in fresh and dry weight as shown in Table 4.1. Typically, lag phase’ lasted for two days, when no growth was observed. This was followed by five to six days of rapid weight increase called ‘exponential phase’. For the next three days growth was steady showing ‘linear phase’. Maximum weight (3950 mg fresh wt./flask in VRI-2 and 3810 mg fresh wt./flask
Table 4.1. Growth of hypocotyl cell suspension cultures of groundnut

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>VRI-2 FW</th>
<th>VRI-2 DW</th>
<th>VRI-2 A 485nm</th>
<th>TMV-7 FW</th>
<th>TMV-7 DW</th>
<th>TMV-7 A 485nm</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>50</td>
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<td>500</td>
<td>50</td>
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<td>2</td>
<td>550</td>
<td>55</td>
<td>0.11</td>
<td>540</td>
<td>53</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>800</td>
<td>85</td>
<td>0.39</td>
<td>750</td>
<td>74</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>1450</td>
<td>155</td>
<td>0.70</td>
<td>1410</td>
<td>150</td>
<td>0.65</td>
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<tr>
<td>5</td>
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</tr>
<tr>
<td>6</td>
<td>3150</td>
<td>325</td>
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<td>3010</td>
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<td>2400</td>
<td>245</td>
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<td>230</td>
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<td>235</td>
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<td>2250</td>
<td>226</td>
<td>1.58</td>
</tr>
<tr>
<td>14</td>
<td>2150</td>
<td>220</td>
<td>1.65</td>
<td>2050</td>
<td>210</td>
<td>1.59</td>
</tr>
</tbody>
</table>

FW - Fresh weight (fresh tissues were taken from test tubes)
DW - Dry weight (after drying in oven at 80°C for overnight)
A - Absorbance
in TMV-7) was reached on ninth day, followed by a decline in growth called 'progressive deceleration phase'. Finally, there was no growth called 'stationary phase'. Between three and six days the doubling time of cells was 36 hrs. Same results were obtained by measuring the absorbance increase over the 12 days growth period at 485 nm in a spectrophotometer. Growth rate was measured with in

Table 4.1 shows the growth rate at varied subculturing intervals from 1 to 14 days. Optimum growth of cells (3950 mg fresh wt./flask) was recorded in cultures subcultured at weekly intervals. The maximum growth rate was observed in cultures subcultured every week. Cultures subcultured every 12 days showed poor growth. Viability of cells in culture declined after 9 days, and cells transferred to new medium after 12 days failed to grow. A fifteen day old groundnut suspension consisted of elongated tubular cells. A greater number of circular greenish cells were obtained after one month when subcultured at weekly intervals, with removal of dead clumps and debris.

(c) Isolation of protoplasts

Protoplasts were isolated from ninth day, after subculture of the cell suspension culture. There were 1.5x10⁶ cells/ml in the culture media. Before isolation of protoplasts, cells were preplasmolysed to increase the yield of protoplasts. The protoplast yield was affected due to the preplasmolysis period. Protoplast yield was increased (5.8x10⁶ protoplasts/g fresh wt.) with increase the preplasmolysis period from 0 to 4 hrs thereafter slightly decreased with further increase in both the cultivars (Table 4.2). Protoplasts were isolated enzymatically. Twenty enzyme combinations were tried. Concentrations of cellulase RS used were 0.5, 1.0, 1.5, 2.0 and 2.5% (W/V). Concentrations of macerozyme R-10 used were 0.4, 0.6, 0.8 and 1.0% (W/V). Protoplasts were reproducibly obtained from hypocotyl cell suspensions after 10 hrs treatment with the enzyme solution. About 75% protoplast isolation was obtained at the combination of 1.5% (W/V) cellulase RS + 0.8% (W/V) macerozyme R-10 (Table 4.3). One month old cell suspensions gave highest yields of protoplasts.
Table 4.2. Effect of preplasmolysis (0.6 M mannitol) period on yield and ratio of digested to undigested protoplasts using hypocotyl cell suspension cultures of groundnut

<table>
<thead>
<tr>
<th>Preplasmolysis period (hrs)</th>
<th>Yield of Protoplasts/ml x 10^4</th>
<th>Ratio of DP to UP % of protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VRI-2</td>
<td>TMV-7</td>
</tr>
<tr>
<td></td>
<td>DP</td>
<td>UP</td>
</tr>
<tr>
<td>0.0</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>1.0</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>2.0</td>
<td>4.5</td>
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</tr>
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<td>3.0</td>
<td>4.9</td>
<td>4.2</td>
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<td>5.8</td>
<td>5.1</td>
</tr>
<tr>
<td>5.0</td>
<td>5.4</td>
<td>4.8</td>
</tr>
</tbody>
</table>

DP - Digested protoplasts (cells without cell wall)

UP - Undigested protoplasts (cells with cell wall)
Table 4.3. Effect of enzyme concentration on viability, yield and ratio of digested to undigested protoplasts from hypocotyl cell suspension cultures of groundnut

<table>
<thead>
<tr>
<th>Enzyme Conc. (% W/V)</th>
<th>Viability of Protoplasts (%)</th>
<th>Yield of Protoplasts/mlx10^4</th>
<th>Ratio of DP to UP % of protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase Macerozyme</td>
<td>VRI-2</td>
<td>TMV-7</td>
<td>VRI-2</td>
</tr>
<tr>
<td>0.5</td>
<td>0.4</td>
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</tr>
<tr>
<td>2.5</td>
<td>1.0</td>
<td>66</td>
<td>65</td>
</tr>
</tbody>
</table>

DP - Digested protoplasts
UP - Undigested protoplasts
(d) Protoplast yield

The freshly isolated protoplasts were small, richly cytoplasmic and contained small starch grains. However, the yield varied with the genotype and ranged from $0.8 \text{ to } 5.8 \times 10^4$ protoplasts/g fresh wt. in VRI-2 and $0.6 \text{ to } 5.1 \times 10^4$ protoplasts/g fresh wt. in TMV-7 cultivar (Table 4.3). The highest yield of protoplasts $5.8 \times 10^4$ protoplasts/g fresh wt. was observed in VRI-2 whereas it was $5.1 \times 10^4$ protoplasts/g fresh wt. in TMV-7 cultivar.

(e) Protoplast viability

Cell and protoplast viability was determined using Evans blue dye method. The test showed 95% of the cells of the suspensions cultured were viable and 80% of the isolated protoplasts were viable. Viability of isolated protoplasts varied among experiments and genotypes, but was generally over 50% as indicated by Evans blue staining and protoplast morphology (Table 4.2).

(f) Protoplast culture

Protoplasts were cultured in the presence of different concentrations of NAA (0.5-2.5 mg/l) and BAP (1.0 mg/l) together with 2,4-D (0.5 mg/l) (Table 4.4). Isolated protoplasts were plated at a density of $2.5 \times 10^4$ protoplasts/ml onto protoplast culture and feeder layer medium. Within a week of incubation the protoplasts had regenerated a cell wall and entered cell division. In some cases, protoplasts had divided before cell wall formation. The protoplasts were highly cytoplasmic before division. During culture, the protoplasts increased as much as twice or more in diameter, and became highly vacuolated within 24-48 hrs. Cell wall regeneration occurred in about 5 to 10% of the protoplasts in patches around the periphery during the first 24 hrs and was completed in the next 24 hrs in all the viable protoplasts. Cell plate formation and the first division were first recorded after 60-70 hrs with 10% protoplasts which elongated and become dumbbell shaped. About 50% of the cells underwent their first cell division by the 3rd day of culture and 65 to 75% by the 5th day. The rapid cell divisions resulted in the prompt formation of multicellular colonies.
Table 4.4. Plating efficiency of hypocotyl cell suspensions derived protoplasts cultures of *Arachis hypogaea*

<table>
<thead>
<tr>
<th>Hormones Conc. (mg/l)</th>
<th>NAA+BAP</th>
<th>VRI-2</th>
<th>TMV-7</th>
<th>VRI-2</th>
<th>TMV-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA+BAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 1.0</td>
<td>14.2% (± 1.36)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.2% (± 1.10)</td>
<td>16.4% (± 1.86)</td>
<td>14.3% (± 1.42)</td>
<td></td>
</tr>
<tr>
<td>1.0 1.0</td>
<td>21.6% (± 2.44)</td>
<td>18.4% (± 1.15)</td>
<td>27.5% (± 2.59)</td>
<td>22.4% (± 2.27)</td>
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</tr>
<tr>
<td>1.5 1.0</td>
<td>37.8% (± 3.21)</td>
<td>33.5% (± 2.44)</td>
<td>42.4% (± 4.24)</td>
<td>39.1% (± 2.67)</td>
<td></td>
</tr>
<tr>
<td>2.0 1.0</td>
<td>58.2% (± 4.33)</td>
<td>51.7% (± 4.88)</td>
<td>67.3% (± 5.59)</td>
<td>61.9% (± 5.66)</td>
<td></td>
</tr>
<tr>
<td>2.5 1.0</td>
<td>46.6% (± 3.56)</td>
<td>42.8% (± 2.82)</td>
<td>61.7% (± 5.47)</td>
<td>53.2% (± 4.32)</td>
<td></td>
</tr>
</tbody>
</table>

a) Plating Efficiency: The percentage of protoplasts that had divided at least once in 7-10 days of culture in Kao's medium.

b) Data represents means of three independent experiments.
(g) **Plating efficiency**

Protoplasts of the two genotypes were cultured on Kao’s medium augmented with various concentration and combination of NAA (0.5-2.5 mg/l), and BAP (1.0 mg/l) (Table 4.4). Plating efficiency was measured 10-15 days after isolation. By this time cells had undergone several cycles of division and were typically at the small colony stage. The plating efficiency ranged from 14.2 to 58.2% in VRI-2 on Kao’s medium containing NAA 2.0 mg/l and BAP 1.0 mg/l while it ranged from 11.2 to 51.7% in TMV-7 cultivar. Indeed, the rise and subsequent fall in the division efficiency response of the protoplasts correlated well with an accompanying rise and fall of the mitotic index of the cell suspension. This is not surprising as donor tissue conditions and isolation conditions can dramatically affect both the yield and stability of isolated protoplasts as well as their subsequent metabolic activity and/or division efficiency. Protoplast plating density also influenced protoplast division. Presence of feeder cells in the protoplast culture medium enhanced protoplast division and growth. Moreover, microcalli were also visible within 15 days of culture.

Based on the preliminary experiments, plating efficiency slightly decreased with increasing NAA concentration beyond 2.0 mg/l. Interaction of initial auxin concentration and subsequent 2,4-D treatment (0.5 mg/l) was studied by setting up parallel cultures with and without 2,4-D treatment for all NAA concentration used. Data are summarized in Table 4.4. The frequency of colony formation was highest (67.3% in VRI-2 : 61.9% in TMV-7) at 2.0 mg/l initial NAA concentration with subsequent 2,4-D treatment in both the cultivars.

(h) **Callus culture**

On transfer to MS medium the colonies developed into rapidly growing, yellow-green calli, whose healthy growth and subsequent callus formation was observed. One month after transfer to the callus proliferation medium, protoplast derived calli were then transferred to the shoot regeneration medium in order to determine the regeneration ability. No shoot regeneration was observed from cell suspension derived protoplast calli. Root formation was observed in all the cultures.
(ii) Immature leaflet

(a) Protoplast isolation

Protoplasts were readily isolated from both the genotypes using different concentrations and combinations of cellulase and macerozyme after 14-16 hrs of incubation. Protoplasts could not be directly isolated from the explants because of incomplete maceration of cell walls and bursting of the isolated protoplasts. Therefore, the protoplasts were isolated after pretreatment of the leaflets in CPW medium containing 0.6 M mannitol solution. Protoplast releasing was increased (6.5x10⁴ protoplasts/g fresh wt.) with increase in the preplasmolysis period up to 4 hrs. If increase further, it was decreased substantially (Table 4.5).

(b) Protoplast yield

The maximum yield of protoplasts was 6.5 and 5.3x10⁴ protoplasts/g fresh wt in VRI-2 and TMV-7 respectively. Twenty combinations were tried. Enzyme mixture of 2.0% (W/V) cellulase and 1.0% (W/V) macerozyme gave the highest yield of 6.5x10⁴ protoplasts/g fresh wt. It was digested for two hours on a 80 rpm shaker followed by overnight stationary digestion in dark. However, the yield varied with the genotype and ranged from 1.3 to 6.5x10⁴ protoplasts/g fresh wt. in VRI-2 while it ranged from 1.1 to 6.0x10⁴ protoplasts/g fresh wt. in TMV-7 (Table 4.6).

(c) Protoplast viability

Protoplasts size ranged from 45-55 μM in diameter. 100 percent of the leaf cells were viable and 85% of protoplasts isolated from them were viable. Viability of isolated protoplasts varied among experiments and genotypes, but generally over 85% of the pretreated protoplasts showed high level of viability immediately after isolation (Table 4.6).

(d) Protoplast culture

In initial experiments protoplasts from both the cultivars were cultured (at a density 2x10⁴ protoplasts/ml) in Kao’s medium. Most protoplasts elongated and some began to divide 24 hrs after isolation. Further division led to the
Table 4.5. Effect of preplasmolysis (0.6 M mannitol) period on yield and ratio of digested to undigested protoplasts using immature leaflet explants of groundnut

<table>
<thead>
<tr>
<th>Preplasmolysis period (hrs)</th>
<th>Yield of Protoplasts/ml x 10^4</th>
<th>Ratio of DP to UP % of protoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VRI-2</td>
<td>TMV-7</td>
</tr>
<tr>
<td>0.0</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>1.0</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>2.0</td>
<td>4.7</td>
<td>4.0</td>
</tr>
<tr>
<td>3.0</td>
<td>5.2</td>
<td>4.6</td>
</tr>
<tr>
<td>4.0</td>
<td><strong>6.5</strong></td>
<td>5.3</td>
</tr>
<tr>
<td>5.0</td>
<td>6.1</td>
<td>5.1</td>
</tr>
</tbody>
</table>

DP - Digested protoplasts

UP - Undigested protoplasts
Table 4.6. Effect of enzyme concentration on viability, yield and ratio of digested to undigested protoplasts from immature leaflets of groundnut

| Enzyme Conc. (% W/V) | Viability of Protoplasts (%) | Yield of Protoplasts/mlx10^4 | Ratio of DP to UP % of protoplasts |  |
|----------------------|-------------------------------|------------------------------|----------------------------------|  |
|                      | VRI-2 | TMV-7 | VRI-2 | TMV-7 | VRI-2 | TMV-7 | DP | UP | DP | UP |  |
| 0.5                  | 55    | 52    | 1.3   | 1.1   | 18    | 82    | 15  | 85 |  |
| 1.0                  | 58    | 56    | 2.2   | 1.9   | 39    | 61    | 34  | 66 |  |
| 1.5                  | 65    | 63    | 3.4   | 3.2   | 48    | 52    | 45  | 55 |  |
| 2.0                  | 72    | 70    | 4.0   | 3.8   | 56    | 44    | 54  | 46 |  |
| 2.5                  | 70    | 69    | 3.7   | 3.5   | 54    | 46    | 50  | 50 |  |
| 0.5                  | 60    | 56    | 1.5   | 1.3   | 24    | 76    | 22  | 78 |  |
| 1.0                  | 64    | 60    | 2.7   | 2.5   | 43    | 57    | 40  | 60 |  |
| 1.5                  | 72    | 69    | 3.9   | 3.8   | 53    | 47    | 48  | 52 |  |
| 2.0                  | 76    | 74    | 5.1   | 4.8   | 65    | 35    | 62  | 38 |  |
| 2.5                  | 75    | 72    | 4.6   | 4.2   | 59    | 41    | 55  | 45 |  |
| 0.5                  | 62    | 57    | 2.3   | 2.1   | 27    | 73    | 24  | 76 |  |
| 1.0                  | 75    | 71    | 3.7   | 3.6   | 46    | 54    | 44  | 56 |  |
| 1.5                  | 78    | 76    | 4.5   | 4.4   | 69    | 31    | 65  | 35 |  |
| 2.0                  | 82    | 78    | 5.9   | 5.5   | 77    | 23    | 74  | 26 |  |
| 2.5                  | 77    | 73    | 5.7   | 5.0   | 72    | 28    | 69  | 31 |  |
| 0.5                  | 65    | 62    | 2.5   | 2.2   | 28    | 72    | 25  | 75 |  |
| 1.0                  | 70    | 67    | 3.8   | 3.6   | 49    | 51    | 42  | 58 |  |
| 1.5                  | 75    | 73    | 4.7   | 4.5   | 71    | 29    | 68  | 32 |  |
| 2.0                  | 85    | 81    | 6.5   | 6.0   | 85    | 15    | 76  | 24 |  |
| 2.5                  | 81    | 78    | 6.4   | 5.8   | 81    | 19    | 75  | 25 |  |

DP - Digested protoplasts
UP - Undigested protoplasts
formation of compact cell masses after 3 days of culture. Five combinations of growth regulators were chosen and their effect evaluated on the dividing activity of protoplasts and cell colonies.

(e) **Plating efficiency**

Protoplasts of the two genotypes were cultured on Kao’s medium containing different concentrations of NAA (0.5-2.5 mg/l) in combination with BAP (1.0 mg/l). On this medium, protoplasts of both genotypes gave plating efficiencies in the range of 36.5 to 85.6% in VRI-2 and 21.2 to 72.3% in TMV-7 after 10-15 days of culture. The highest frequency of protoplast division (85.6%) was observed in VRI-2 cultivar in the presence of NAA (2.0 mg/l) and BAP (1.0 mg/l) (Table 4.7). The other very important prerequisite for further dividing and growing activity of protoplast and cell colonies is suitable plating density. Protoplast plating efficiency was also influenced by the protoplast plating density. Higher or lower plating densities resulted in reduced protoplast plating efficiencies. The most effective initial plating density was 2.0x10⁴ protoplasts/ml.

When protoplasts were plated in 0.4% (W/V) agarose bead at 2.0x10⁴/ml protoplast density division was stimulated resulting in a plating efficiency of 85.6%. The use of the agarose bead culture technique minimized pigment production and release of phenolic compounds and also offered the possibilities of removing deleterious compounds, easily stabilizing the pH and osmotic pressure, since the medium could be changed readily.

(f) **Protocalli culture**

Various sizes of protocalli formed within three to four weeks, were visible to the naked eye. Protocalli were transferred to medium, grew and developed into undifferentiated and morphogenic callus. For further development and growth colonies of about 20 cells after 15 d were transferred to MS medium containing 3% (W/V) sucrose, BAP, NAA and solidified with 0.8% (W/V) agar. Different concentrations and combinations of BAP and NAA were tested for their efficacy in promoting shoot formation. None of the protoplast-derived callus colonies differentiated into shoots. Root formation was, however, predominant in all cultures (Plate 4.1).
Table 4.7. Plating efficiency of immature leaflet derived protoplast cultures of *Arachis hypogaea*

<table>
<thead>
<tr>
<th>Hormonal con. (mg/l)</th>
<th>Plating Efficiency(^a) (±SD)</th>
<th>VRI-2</th>
<th>TMV-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA + BAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>36.5% (± 3.13)</td>
<td>21.2% (± 2.36)(^b)</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>56.3% (± 4.09)</td>
<td>45.4% (± 3.41)</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>71.4% (± 3.85)</td>
<td>67.5% (± 3.59)</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>85.6% (± 4.78)</td>
<td>72.3% (± 4.07)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>72.3% (± 3.42)</td>
<td>64.1% (± 2.61)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Plating Efficiency: The percentage of protoplasts that had divided at least once in 10-15 days of culture in Kao’s medium

\(^b\) Data represents means of three independent experiments
(g) **Embryo induction and development**

Within two to three weeks embryos at different developmental stages (from globular to dicotyledonary shaped) could be observed in the cultures. Of the five growth regulator combinations tested (Table 4.8), the combination of 2,4-D (4.0 mg/l) and BAP (1.0 mg/l) gave the best results in terms of percent of dicotyledonary embryos (24.2%) within 21-28 days. Different stages of embryos viz. globular, heart, and torpedo were observed with varying frequency. Somatic embryo formation frequency was increased with increase in the 2,4-D concentration up to 4.0 mg/l thereafter slightly decreased with further increase. The combination of 4.0 mg/l 2,4-D and 1.0 mg/l BAP was found to be optimum level for maximum frequency of embryo production in both the cultivars. However, the frequency of somatic embryos was high in VRI-2 cultivar when compared with TMV-7 cultivar. The highest frequency of embryo was 24.2% in VRI-2 and it was 10.2% in TMV-7 cultivar (Table 4.8). In combination with 2,4-D, BAP was clearly a superior cytokinin for induction of cell division and cell colony formation. As well, the cytokinin supply appeared to be important during the time of embryo induction. The highest frequency of embryo formation occurred on MS medium supplemented with BAP (1.0 mg/l) in combination with 2,4-D (4.0 mg/l), although cells grew only moderately well on these media. Cells grown in the absence of 2,4-D were generally larger in size and tended to turn brown more readily compared to those grown in the presence of 2,4-D. Development of somatic embryos was synchronized; protoplasts grew and differentiated into proembryo, globular and heart shaped objects after 2 and 3 weeks, respectively, and after 4 weeks advanced stage of somatic embryos (dicotyledonary) were observed.

(h) **Development of plantlets**

Developmental stages characteristic of somatic embryogenesis could be observed in the cultures. However, the frequency of shoot formation was rather low. Further development of both shoot and root was observed only in less than 5% of embryos. Matured embryoids were germinated on hormone free MS medium containing 0.3% (W/V) activated charcoal. Few plants (4) of VRI-2 could readily be regenerated from embryogenic cultures on hormone free medium but only one plant could be regenerated in TMV-7 under these conditions. All these plants were eventually transplanted into plastic cups (Plate 4.2).
Table 4.8. Frequency of somatic embryogenesis from greenish calli derived from immature leaflet protoplasts of *Arachis hypogaea*

<table>
<thead>
<tr>
<th>Hormones Conc. (mg/l)</th>
<th>Percent of somatic embryos/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Globular</td>
</tr>
<tr>
<td></td>
<td>VRI-2</td>
</tr>
<tr>
<td>2,4-D BAP</td>
<td></td>
</tr>
<tr>
<td>1.0 1.0</td>
<td>14.4±2.13</td>
</tr>
<tr>
<td>2.0 1.0</td>
<td>20.6±2.54</td>
</tr>
<tr>
<td>3.0 1.0</td>
<td>36.8±3.12</td>
</tr>
<tr>
<td>4.0 1.0</td>
<td>43.6±3.44</td>
</tr>
<tr>
<td>5.0 1.0</td>
<td>41.2±3.09</td>
</tr>
</tbody>
</table>

a) Each value represents the mean of three replicates±SD
4.4. DISCUSSION

4.4.1. Protoplast Culture Technology

Protoplast culture technology has opened up an array of new opportunities for genetic manipulations in groundnut. The ability to regenerate plants from protoplasts is a prerequisite for the genetic manipulation of groundnut via somatic hybridization or cybridization and through the uptake of cell organelles, chromosomes or specific gene constructs. The immediate task for the groundnut tissue culturists, therefore, is to standardize reproducible and efficient protoplast culturing procedures. Improved and highly repeatable protoplast culture technique are bound to stimulate interest in molecular cloning and sequencing of groundnut genes and in turn in the study of their transformation and expression. With this in mind, the present study was conducted to develop a protocol for protoplast isolation and plant regeneration and to find out the best responding groundnut genotypes for isolation and culture of protoplasts (Fig. 4.1).

There are many factors controlling successful isolation and culture of groundnut protoplasts. The first factor is the genotype. It must be a highly responding genotype to tissue culture. The second factor is explants. The third factor is a successful, standardized protocol for the enzymatic procedure for protoplast isolation. The fourth factor is an effective procedure for efficient culturing of the viable protoplasts. The fifth factor is the media composition and growth regulators. All these factors which lead to successful protoplast isolation, culture and plant regeneration have been studied thoroughly and systematically in this present research.

4.4.2. Protoplast Isolation and Culture

(i) Hypocotyl cell suspensions

(a) Isolation of protoplasts

The suspension cultures used for these experiments had been established for one month when protoplast studies were first initiated. Protoplasts were readily isolated from both the cultivars in this study using twenty combinations
Fig 4.1. Protoplast isolation and culture protocol from hypocotyl cell suspensions and immature leaflets of groundnut showing enzyme combinations, culture media, growth regulators, timing of media changes and acclimatization

<table>
<thead>
<tr>
<th>Protoplast isolation and culture from hypocotyl cellsuspensions</th>
<th>Protoplast isolation and culture from immature leaflet explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypocotyl</td>
<td>Immature leaflet</td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MS + NAA (2.0 mg/l)</td>
<td>CPW + 0.6M mannitol</td>
</tr>
<tr>
<td>+ 2,4-D (2.0 mg/l)</td>
<td>↓</td>
</tr>
<tr>
<td>+ KIN (0.5 mg/l)</td>
<td>Preplasmolysis</td>
</tr>
<tr>
<td>↓</td>
<td>(4 hrs)</td>
</tr>
<tr>
<td>MS + NAA (1.0 mg/l)</td>
<td>CPW + Cellulase (0.5, 1.0, 1.5,</td>
</tr>
<tr>
<td>+ 2,4-D (2.0 mg/l)</td>
<td>2.0 and 2.5%) + Macerozyme</td>
</tr>
<tr>
<td>+ KIN (0.5 mg/l)</td>
<td>(0.4, 0.6, 0.8 and 1.0%)</td>
</tr>
<tr>
<td>↓</td>
<td>CPW + 0.6M sucrose</td>
</tr>
<tr>
<td>CPW + 0.6M mannitol</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>Enzyme treatment</td>
</tr>
<tr>
<td>CPW + Cellulase (0.5, 1.0, 1.5,</td>
<td></td>
</tr>
<tr>
<td>2.0 and 2.5%) + Macerozyme</td>
<td>(10 hrs)</td>
</tr>
<tr>
<td>(0.4, 0.6, 0.8 and 1.0%)</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>CPW + 0.6M sucrose</td>
</tr>
<tr>
<td>CPW + 0.6M sucrose</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>Protoplast washing and purification</td>
</tr>
<tr>
<td>Kao's medium + NAA (0.5-</td>
<td></td>
</tr>
<tr>
<td>2.5 mg/l) + BAP (1.0 mg/)</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>Protoplast culture</td>
</tr>
<tr>
<td>Kao's medium + NAA (0.5-</td>
<td></td>
</tr>
<tr>
<td>2.5 mg/l) + BAP (1.0 mg/)</td>
<td>(10-15 days)</td>
</tr>
<tr>
<td>↓</td>
<td>Minicalli culture</td>
</tr>
<tr>
<td>MS + NAA + 2,4-D + BAP</td>
<td>(21-28 days)</td>
</tr>
<tr>
<td>(0.5 mg/l each)</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>No shoot bud regeneration</td>
<td>(21-28 days)</td>
</tr>
<tr>
<td>↓</td>
<td>Acclimatization</td>
</tr>
<tr>
<td></td>
<td>(15-21 days)</td>
</tr>
</tbody>
</table>
of cellulase and macerozyme within 10 hrs of incubation. High yields of protoplasts were obtained using low levels of enzymes. Out of the twenty combinations investigated the highest yield \((5.8 \times 10^4\text{ protoplasts/g fresh wt.})\) of viable \((80\%)\) protoplasts was obtained when enzyme mixture of \(1.5\%\) Cellulase and \(0.8\%\) Macerozyme was used for isolation. The protoplasts were spherical, cytoplasmically rich and variable in size. Maximum yield of protoplasts were obtained from cell suspensions which were preplasmolysed in \(0.6\text{M mannitol}\) and digested with of the enzyme mixture for 10 hrs at \(25^\circ\text{C}\) temperature in dark.

(b) **Protoplast viability**

Among the several combinations tested, the combination of cellulase \((1.5\%\text{ W/V})\) and macerozyme \((0.8\%\text{ W/V})\) was found to be the optimum for protoplast isolation with VRI-2 because the viability of protoplasts was the highest \((80\%)\) and the yield adequate \((5.8\times10^4\text{ protoplasts/g fresh wt.})\). Viability of isolated protoplasts varied among experiments and genotypes but was generally over \(50\%)\) as indicated by Evans blue staining and protoplast morphology.

(c) **Protoplast culture**

When protoplasts were plated in \(0.4\%\) (W/V) agarose bead with feeders at \(2.5\times10^4/\text{ml protoplasts density},\) division was stimulated resulting in a plating efficiency of \(58\%).\) The use of agarose reduced protoplast lysis during the initial stage of culture and further increased the plating efficiency.\(^4\) The use of agarose bead culture technique minimized pigment production and release of phenolic compounds and also offered the possibilities of removing deleterious compounds, easily stabilizing the pH and osmotic pressure, since the medium could be changed readily. Embedding protoplasts in agarose medium produced better results, as more than \(80\%)\) of the initial protoplasts remained intact after plating which proved to be advantageous for inducing initial divisions, as well as for fast growth of the protoplast derived colonies. When the agarose bead method is used, it is also easier to change the medium without disturbing the developing colonies. The feeder cells promote the protoplast callusing.
(d) **Plating efficiency**

Protoplast plating efficiency was also influenced by the protoplast plating density. The highest plating efficiency was achieved when protoplast cultures were plated at a density of $2.5 \times 10^4$ protoplasts/ml. Higher or lower plating densities resulted in reduced protoplast plating efficiencies. Genotype differences were observed in plating efficiency with the highest rate (67%) for VRI-2 while TMV-7 exhibited the lowest rate (61%). This observation is in accordance with earlier reports on grain legumes (Dhir *et al.*, 1992).

(e) **Callus initiation and culture**

First division of the protoplasts was observed as early as the third day of culture. Further division lead to the formation of compact cell masses after 15 days of culture. Cell wall formation, as evidenced by the formation of elliptic cells, was observed 3-4 d after culture initiation. First cell divisions occurred about a week after exposure to the nurse culture. Culture density was observed to be an important determinant for protoplast division. Protoplast beads and plate cultures in a medium supplemented with nursing agent exhibited better division and colony formation. Prior to division, increase in protoplast size was common in cultures. The increase in protoplasts size may be caused by a change in the osmotic pressure. By increasing in size of the plasma membrane retains its functionality maintaining optimum osmotic potential in culture media. Some protoplasts exhibited abnormal divisions. The abnormal division of protoplasts may be caused by directional osmotic pressure at a particular site of the plasma membrane leading to swelling and subsequent elongation at that site. Similar observations were also reported by Konwar (1993) and Power and Chapman (1985). Protoplasts cultured in media containing 2,4-D or NAA either singly or in combination with BAP sustained division leading to colony formation. However, plating efficiency was higher in 2,4-D containing medium indicating that 2,4-D is more effective in sustaining protoplast division.

Transfer of small protoplast derived mini calli to a medium containing a reduced concentration of sucrose promoted cell division and resulted in colonies of about 20 cells after 21 days. Various sizes of protocalli formed within three
to four weeks, were visible to the naked eye. Callus colonies reached a size of about 1 mm in diameter two week after transfer to callus induction medium. Protoplast derived callus colonies were transferred to medium augmented with various combinations of growth regulators. A variety of plant growth regulators; alone or in combination, failed to induce any morphogenesis in the calli. Protoplasts isolated from suspensions of both the cultivars gave similar results. Root formation was, however, predominant in all the cultures. Protoplasts isolated from cell suspensions, however, failed to produce shoot buds. The micro-calli became green in colour. Their individual colony was visually verified shortly after plating. Regular subculturing helped the growth of colonies which otherwise turned brown, presumably due to accumulation of phenolic compounds, thereby affecting subsequent viability of the colonies. The apparent rapid loss of regeneration is probably due to the accumulation of deleterious compounds which leads to the loss of regeneration ability.

(ii) Immature leaflet

(a) Protoplast isolation

Leaflets were cut into 1-5 mm pieces, and preplasmolysed in 0.6 M mannitol solution for 4 hrs. Protoplast release was enhanced following pretreatment of explants in CPW medium containing 0.6 M mannitol. Among the 20 combinations, cellulase 2.0 % (W/V) and macerozyme 1.0 % (W/V) combination was found to be best for highest frequency of protoplast isolation in both the cultivars. However, the protoplast frequency was high (85%) in VRI-2. Similar results were also reported in groundnut by Mhatre et al. (1985).

(b) Protoplast viability

Protoplast viability was also affected by enzyme concentrations. When increase the concentration of enzymes, the viability as well as yield of protoplasts were decreased. Cellulase (2.0% W/V) and macerozyme (1.0% W/V) was found to be optimum level for maximum yield with more viable protoplasts in both the cultivars. The maximum yield was 6.5x10^4 protoplasts/ml in VRI-2 and it was 6.0x10^4 protoplasts/ml in TMV-7. Protoplast viability was also varied. Highest
protoplast viability was observed (85%) in VRI-2. The viability depends on osmoticum used, concentration of enzymes. Preplasmolysis increased protoplast viability. Wei and Xu (1990) reported that the 90% of the pretreated protoplasts showed strong and high level of viability.

(c) Protoplast culture

Protoplasts were cultured on Kao’s medium containing different concentrations of NAA (0.5-2.5 mg/l) and BAP (1.0 mg/l) for colony formation. The plated protoplasts showed swelling and first division was witnessed on 48 hrs and on the 7th day protoplasts had divided into multicellular colonies. Microcalli were observed within 21-28 days of culture. Similar results were also reported by Parihar et al. (1995) in Brassica napus and Sarangi et al. (1992) in Cajanus cajan.

(d) Plating efficiency

Plating efficiency was increased with increase in the concentration of NAA and 2.0 mg/l was found to be best for highest plating efficiency (85.6%) within 15 days. The other very important prerequisite for further dividing and growing activity of protoplasts and cell colonies is suitable plating density. The most effective plating density was 2x10^4 protoplast/ml. The use of 0.4% (W/V) agarose reduced protoplast lysis during the initial stage of culture and further increased the plating efficiency up to 85.6%. Of the two cultivars, the plating efficiency was high (85.6%) in VRI-2 and it was 72.3% in TMV-7.

(e) Protocalli culture

Minicalli were transferred onto the regeneration medium on which they enlarged and became greenish, yellowish or white. Protoclones of groundnut did not differentiate shoot buds and plantlets despite using various concentrations and combinations of auxins and cytokinins. Colonies proliferated into actively growing calli. The protoplast derived callus was yellowish green and compact. No regeneration was obtained when protoplast callus was subjected to different hormonal treatments. Protoplasts isolated from shoot cultures underwent divisions and formed colonies and callus in peanut (Mhatre et al., 1985).
In general, large-seeded legumes have been difficult to regenerate from undifferentiated tissues, especially protoplasts. In groundnut, regeneration from multicellular explants can be accomplished by appropriate choice of an explant source and media. Whereas protoplasts from the organogenic systems retained their regeneration competency, there is only one report of protoplasts from immature leaf suspensions showing plant regeneration (Li et al., 1993). Previous researchers (Bajaj and Gosal, 1988; Mhatre et al., 1985; Oelck et al., 1982; Rugman and Cocking, 1985) using similar protocols showed that it was possible to grow protoplast derived callus with high efficiency, but impossible to regenerate plants.

(f) Plant regeneration via somatic embryogenesis

Alternative method for shoot regeneration from protoplast-derived callus was tried through somatic embryogenesis. The present study clearly shows that plants can be regenerated from immature leaflet protoplasts of Arachis hypogaea via somatic embryogenesis. In addition, the system developed in this study is simple and efficient; only one medium is required for growth and differentiation of embryos up to plant regeneration. The highest frequency of somatic embryos was observed on a medium containing 4.0 mg/l 2,4-D in combination with 1.0 mg/l BAP within 28 days. In the present work, auxin appears to be more important for induction of somatic embryogenesis, and results suggest that both auxin and cytokinin are very essential to give a better response.

The present study shows variation in the capacity for somatic embryogenesis and subsequent plant regeneration between VRI-2 and TMV-7. Although both cultivars exhibit a similar pattern of protoplast growth and differentiation, embryo production in TMV-7 is generally low in comparison with VRI-2, which produced 2 times as many embryos. In TMV-7, a low frequency of embryo formation may have contributed in part to poor plant regeneration on hormone-free medium. Eapen et al. (1989) also reported a considerable variation in embryo production and subsequent plant regeneration between two Brassica juncea cultivars in which cv. Rai produced 3-4 fold more abundant somatic embryos of which 2-3% was regenerative, in comparison with cv. TM4 of which all embryos were not regenerative. Nevertheless, somatic embryogenesis of Arachis hypogaea in this study is also markedly influenced by the hormonal composition.
of the culture medium in which the presence of 2,4-D (4.0 mg/l) and BAP (1.0 mg/l) is most effective for maximum frequency of embryo formation. It was essential to transfer the embryos onto hormone free MS basal medium as soon as shoot elongation had started. Plantlets with well developed roots could be transplanted into plastic cups and later established in pots.

4.4.3. Response of Genotypes

In the present study, two groundnut varieties were screened. Higher yield protoplast isolation, viability, callusing, somatic embryogenesis and plant regeneration was noticed in VRI-2 only. Thus genotype influence governing protoplast isolation, culture, callus induction and plant regeneration in groundnut is evident from the present investigation.

Similarly as far as protoplast culture is concerned the genotype and culture media were the major factors influencing the rate of protoplast division and callus formation. In an earlier report (Oelck et al., 1982) on Arachis protoplast only one local variety had been investigated whereas in the present study protoplast isolation and culture has been extended to two important commercial cultivars. The failure to get plant regeneration in protoplast callus from six varieties of peanut (Mhatre et al., 1985) as well as previous report on Arachis protoplast (Oelck et al., 1982) points out the of peanut callus inability to undergo differentiation. Similar observations were made in the present study.

4.4.4. Explants Response

In the present study, pretreated hypocotyl cell suspensions and immature leaflets were used for protoplast isolation. Fast growing cell suspension cultures were used as the source material for protoplast isolation by Amudha (1991). The suspensions consisted of small, densely cytoplasmic, thin walled cells and loose free flowing aggregates. Suspension of such nature required only low concentration of enzymes for digestion in a shorter digestion period.

Immature leaflet explant was also used for protoplast isolation. Before enzymatic treatment, explants were pretreated with with 0.6 M mannitol. High frequency of protoplasts with good viability was isolated from immature leaflets. Of the two explant sources, high frequency of protoplasts with more viable was observed using immature leaflet explant whereas the protoplast frequency and viability was low using suspension culture. From this study, immature leaflet explant was found to be best source for protoplast isolation and culture in groundnut.
4.5. CONCLUSION

1. One month old hypocotyl suspension cells yielded high yield of protoplasts.
2. Preplasmolysis was essential for getting maximum yield as well as viable protoplasts in both hypocotyl cell suspensions and immature leaflet explants.
3. Among the 20 combinations, cellulase (1.5% W/V) and macerozyme (0.8% W/V) combination was found to be best for highest percentage of protoplasts from hypocotyl cell suspensions in both the cultivars.
4. Maximum yield of protoplasts was obtained from immature leaflets using cellulase 2.0% (W/V) and macerozyme 1.0% (W/V) in both the cultivars.
5. Of the two explants tested, immature leaflet was found to be efficient for maximum yield of viable protoplasts in both the cultivars.
6. Both protoplast viability and plating efficiency were found to be high in immature leaflet than hypocotyl cell suspensions in both the cultivars.
7. The plating efficiency was found to be high in Kao's medium supplemented with NAA (2.0 mg/l) and BAP (1.0 mg/l) in both the explants within 15 days.
8. When NAA concentration was increased, the plating efficiency was also increased up to 2.0 mg/l.
9. Efficient callus initiation was observed on MS medium containing NAA, 2,4-D, and BAP (0.5 mg/l each) within 28 days of culture.
10. Protoplast derived colonies proliferated into actively growing calli. Further attempts to regenerate plants from such calli were not successful. Root formation was, however, predominant in all the cultures.
11. An alternative method for plant regeneration from immature leaflet protoplast derived callus by via somatic embryogenesis was achieved.
12. Among the different concentrations of 2,4-D, 4.0 mg/l in combination with 1.0 mg/l BAP induced maximum frequency of embryos in both the cultivars.
13. Of the two genotypes tested, VRI-2 responded better.