Chapter - 5

DISCUSSION

The technique of plant tissue culture has been applied to agricultural problems for more than 50 years. Cell and tissue culture technology provide a source to regenerate the genetically modified single cell into whole plant. In vitro technique has, thereby, contributed to the advancement of agricultural successes. Availability of efficient and reproducible regeneration system is a prerequisite for all biotechnological approaches. Presently solanaceous species are the plant model systems with which many of the techniques of tissue culture and genetic transformation have been developed. This is primarily because of the ease in regeneration and manipulation.

Most of the commercial and agricultural crops are difficult for in vitro manipulation. Hence, they lag behind in biotechnological applications. The knowledge gained from model plant systems has been made applicable to most plant species. Soybean is probably the first major crop to reach commercialization of transgenic lines and cotton will be very close behind (Stewart, 1991).
5.1. Studies on callus induction and proliferation

The present study is aimed at establishing a successful in vitro culture system in cotton. The study was conducted with four explants using modified MS medium supplemented with two auxins (2,4-D and NAA) along with three cytokinins (Kin, 2 iP and BAP). Experiments were conducted to investigate the effect of explant, media compositions, aminoacids and organic supplements. In addition, suspension culture was established to obtaining somatic embryos. Protoplast culture and transgenic calli by Agroinfection were attempted. The results obtained in these experiments are discussed below.

5.1.1. Effect of 2,4-D and Kinetin

In this study, four sources of explants, namely, hypocotyl, cotyledon, leaf and immature embryo were evaluated for their callus induction ability. The highest per cent of callus induction was recorded in cotyledons (88.3%) in MS medium supplemented with 2,4-D and kin, each of 0.1 mg 1⁻¹ (Table 9). Leaf explant needed a little more concentration of 2,4-D and kin each of 0.5 mg 1⁻¹. Hypocotyl responded less in 2,4-D, (0.1 mg 1⁻¹) along with medium dose of kin (0.5 mg 1⁻¹).
The immature embryos recorded 60 per cent of callus induction at 0.1 mg l\(^{-1}\) concentration of 2,4-D and 1.0 mg l\(^{-1}\) of kin. This revealed that the explants, and hormone level greatly influenced the callus induction ability. The explants, in particular, play a very critical role in development of \textit{in vitro} cultures (Vasil, 1987). Similar results were observed by Trolinder and Goodin (1988 a).

Morphology of callus differed with explants and growth regulator combinations. In all the explants, low concentration of 2,4-D and kinetin resulted in vigorous growing callus with medium friability. But, low concentration of 2,4-D with a little higher concentration of Kinetin was found to be better for getting friable calli. This may be due to the endogenous auxin and cytokinin levels present in the explant. Trolinder and Goodin (1988 a) also recorded the effect of 2,4-D and Kinetin on the morphology of callus of \textit{G. hirsutum Var. Coker 312}.

\section*{5.1.2. Effect of 2,4-D and 2 iP}

The auxin 2,4-D and cytokinin 2 iP were used to get the best friable calli. Among the four explants, cotyledons resulted in the maximum (73.3\%) per cent in
MS medium supplemented with 2,4-D of 0.1 mg l\(^{-1}\) and 2 iP of 3.0 mg l\(^{-1}\). This was followed by leaf, hypocotyl and immature embryo. All the explants other than the immature embryos resulted in the production of better friable calli when 2 or 3 mg l\(^{-1}\) of 2 iP was used. This was in agreement with the results of Zimmerman and Robacker (1988). On the contrary, Price and Smith (1979) reported, that a high concentration of 2 iP (10 mg l\(^{-1}\)) was necessary for callus induction and embryoid formation.

5.1.3. Effect of 2,4-D and BAP

As compared to the other explants tried, the leaf explant exhibited maximum of 66 per cent callus induction in MS medium supplemented with 0.3 mg l\(^{-1}\) of 2,4-D and 2 mg l\(^{-1}\) of BAP. When compared with other cytokinins (Kin and 2 iP), BAP was less active in terms of callus induction and proliferation. Trolinder and Goodin (1988 a) also tried the same combination but with an admixture of 2,4-D and BAP each with 0.5 mg l\(^{-1}\) concentration. They reported this combination to be an optimum level for all explants. Moreover, they also recorded the less effectiveness of BAP in callus proliferation. The present study revealed that 2,4-D and BAP combinations were well suited for only leaf explants.
5.1.4. Effect of NAA and kinetin

MS medium supplemented with 2 mg l⁻¹ NAA and 0.5 mg l⁻¹ kin, resulted in highest per cent (82.7) of callus induction in hypocotyl. This explant exhibited more induction ability than other explants. The callus was less friable and its proliferation rate was lower when compared with 2,4-D treatment. This suggested that the auxin NAA is less effective for callusing in cotton. Similar results were also observed by Smith et al. (1977), Trolinder and Goodin (1988 a) and Gawel et al. (1986).

5.1.5. Effect of NAA and 2 iP

Leaf explant recorded maximum of 85.7 per cent callusing in MS medium supplemented with NAA (2 mg l⁻¹) and 2 iP (3 mg l⁻¹). The callus initiation was observed from both leaf midrib as well as cut ends. Callus induction need not be always from the cut end of explants (Dodds and Roberts, 1985). This result holds good wherein callus induction occurred from a wound free portion of an explant viz., midrib. This proved the influence of hormone rather than the injury.

Kaminek (1992) reported that the regulation of plant growth and development including regeneration of
plants from isolated cells and tissue to be under the influence of growth hormone. The callus formed from the edges of the leaf discs was hard and white, while the callus from midrib was friable and light yellow. These were the characteristic features of embryogenic calli in cotton. Price and Smith (1979) also found MS medium supplemented with NAA (1 mg l\(^{-1}\)) and 2 iP (10 mg l\(^{-1}\)) as the best for the induction of such calli. Gawel et al. (1986) reported the midrib calli to be greyish white and friable. The same type of callus was found in cultivar Coker 312 by Trolinder and Xhixian (1989) from several combinations of NAA.

5.1.6. Effect of NAA and BAP

MS medium supplemented with NAA and BAP, each 2 mg l\(^{-1}\), resulted in maximum (68.7%) callusing in leaf explant. The callus was found to be nonembryogenic and most of the combinations resulted in root initiation. These results are in accordance with the results of Trolinder and Goodin (1988 a). In all the explants evaluated, the calli derived from hypocotyl were more embryogenic than other explants. Similar results were obtained by Shoemaker et al. (1986), Zimmerman and Robacker (1988), Trolinder and Xhixian (1989) and Voo et al. (1991).
5.2. Time taken from callus induction to embryoid formation in various explants

The time taken from the callus induction to embryoid formation in different explants was calculated. Among the four explants, hypocotyl took a maximum of 75 days for embryoid formation, while, immature embryo took 107 days. Cousins et al. (1991) reported hypocotyl as the only explant to produce embryoid within a short period of 9 to 12 months from the explant isolation to plant formation. Voo et al. (1991) summarised the requirement of 135 to 170 days in Gossypium hirsutum cv. Coker 312 for plant recovery.

5.3. Dosage of auxin and cytokinin for callus induction

It was found that NAA 2.0 mg l\(^{-1}\) along with 2 iP 3.0 mg l\(^{-1}\) was the best for callus induction. However, the combination and dosage varied with the explants.

The young leaves had maximum callus induction in NAA 2.0 mg l\(^{-1}\) along with 2 iP 3.0 mg l\(^{-1}\). In hypocotyl, NAA 2.0 mg l\(^{-1}\) along with Kin 0.5 mg l\(^{-1}\) recorded the maximum. In cotyledon, 2,4-D and Kin (0.1 mg l\(^{-1}\) each) had maximum callus induction and 2,4-D (0.1
Fig 3. Optimum dose of Auxin and Cytokinin for callus induction
mg l⁻¹) and Kin (1.0 mg l⁻¹) were the best for immature embryos.

The differential response of the explants towards callus induction revealed the influence of explant, its age, growth regulators and their concentration. Such response might indicate the general tendency of Gossypium towards callus induction. There were no significant differences with the auxins tried. However, the two auxins, 2,4-D and NAA, had individual effect when used along with different cytokinins. This apparent difference in callus induction might be the different explants used in the present study. Similar results were also recorded by Larkin and Scowcroft (1981).

When 2,4-D was used as auxin, along with three different cytokinins Kin, 2 iP and BAP, for callus induction, here again cotyledonary explants recorded maximum (83.3%) frequency of callusing with 2,4-D and BAP (0.3 and 2.0 mg l⁻¹, respectively). A dose of 2,4-D and 2 iP (0.1 and 3.0 mg l⁻¹ respectively) was better for callus induction from cotyledon.

Trolinder and Goodin (1988 a) too reported differential performance when 2,4-D was used with no
Table 15 Optimum dosage of auxin and cytokinin for induction of callus in explants

<table>
<thead>
<tr>
<th>Explant</th>
<th>Hypocotyl</th>
<th>Cotyledon</th>
<th>Young leaf</th>
<th>Immature embryo</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth hormone concentration mg/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-D : Kin</td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>2,4-D : 2 iP</td>
<td>0.1</td>
<td>2.0</td>
<td>0.1</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2,4-D : BAP</td>
<td>0.1</td>
<td>1.5</td>
<td>0.1</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>NAA : Kin</td>
<td>2.0</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>NAA : 2 iP</td>
<td>1.0</td>
<td>2.0</td>
<td>1.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>NAA : BAP</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mean</td>
<td>75.07</td>
<td>69.57</td>
<td>69.27</td>
<td>54.32</td>
<td>67.05</td>
</tr>
</tbody>
</table>

Values in parentheses indicates maximum induction of callus.
callus induction in cotyledon explants of Coker 312. This contradicts the present results wherein maximum callus induction with cotyledon was recorded with 2,4-D as auxin (83.3%).

When NAA was used as auxin along with three different cytokinins, NAA (2.0 mg l\(^{-1}\)) with 2 iP (3.0 mg l\(^{-1}\)) had higher callus induction (85.7%) for leaf tissue. With NAA and Kin, at 2.0 and 0.5 mg l\(^{-1}\), respectively, hypocotyl recorded higher callus induction. For NAA and BAP combination, young leaves responded well at 2.0 mg l\(^{-1}\) each.

It was suggested that auxin NAA along with cytokinin 2 iP combination was the best for leaf explant to form calli. Trolinder and Xhixian (1989) also reported NAA with 2 iP to be the best suited for hypocotyl callusing.

5.4. Regeneration studies in solid culture

Plant regeneration is a critical step in success of any crop improvement programme involving tissue culture techniques. Plant regeneration can be achieved in two ways through organogenesis or through somatic embryogenesis. The latter is the preferred method and is easily manipulated than plants derived
through organogenesis (Haccius, 1978). In general, cotton is regenerable only through somatic embryogenic pathway. In dicots, for somatic embryogenesis, a low auxin to cytokinin ratio of 0.5:1.0 was found to be essential (Murashige, 1982). Auxin to cytokinin ratio of 0.5:1.0 and addition of organic and inorganic constituents for the regeneration of plants have been found to vary with species, genotype and explant.

In the present study, MS medium supplemented with 2 iP (1, 2 and 3 mg l⁻¹) recorded friable light brown calli. This was not accompanied by embryogenesis. Price and Smith (1979) found embryogenesis after preculture of callus on a medium containing 10 mg l⁻¹ of 2 iP as cytokinin. The embryoids thus formed failed to regenerate into seedling. The callus when subjected to NAA (1.5 mg l⁻¹) along with BAP and kinetin, each of 0.5 mg l⁻¹, resulted in browning followed by rooting. This was in agreement with Trolinder and Goodin (1988 a).

In an effort to regenerate calli in modified MS medium supplemented with coconut milk (100 ml l⁻¹), biotin (0.25 mg l⁻¹), ascorbic acid (5 mg l⁻¹), serine (25 mg l⁻¹), arginine (25 mg l⁻¹), glutamine (365 mg l⁻¹), CH (0.5 g l⁻¹), calcium D pantothenate (50
mg 1\(^{-1}\), NAA (1.5 mg 1\(^{-1}\)), Kin (0.5 mg 1\(^{-1}\)) and GA\(_3\) (0.5 mg 1\(^{-1}\)) with gelrite (0.2%) as gelling agent, friable light green to light brown calli were obtained throughout the culture period. There were some stray incidence of rhizogenesis alone, but shoot formation was not observed. The increase in the quantum of friable calli and its retention for longer periods may be due to the influence of CH as was observed by Everett et al. (1985), Power (1987) and Knittel et al. (1991) in sunflower hypocotyl, zygotic embryo and cotyledon culture.

According to Pattabhi (1990), coconut milk contains active fractions of inositol, diphenylurea and sorbitol, which have kinin-like activity in the mild form. The addition of coconut water (100 ml 1\(^{-1}\)) could have also increased greening.

In the present study, gelrite 0.2 per cent was used as gelling agent in the place of agar. Gelrite is an exocellular heteropolysaccharide produced as fermentation by-product from a species of Pseudomonas (Kang et al., 1982). Even though gelrite has better gelling impact than agar, it did not aid in shoot initiation. On the contrary, Zimmerman and Robacker (1988) could obtain better regeneration in gelrite media.
Glucose, at the rate of 3 per cent, was found to be better than sucrose. Smith et al. (1977) found that glucose containing media inhibited browning of calli. On the other hand, Shoemaker et al. (1986) reported that the embryogenicity was lost in embryogenic callus on media containing 3 per cent glucose, while it got occasionally lost in calli subcultured on media containing 3 per cent sucrose. Although the specific role of sucrose in cellular differentiation remains obscure, the mechanism of action may be comparable to that of inducer-repressor system (Jeffs and Northcote, 1966).

In the present study, the callus subjected to ABA and 2 iP treatments (2 mg l⁻¹ each) along with glutamine (15 mM), resulted in somatic embryoid formation. In suspension culture, globular (Plate 6A) and heart shaped (Plate 6B) embryoids with shoot and root poles were well distinguishable. In solid culture the pro-embryoids gave rise to a single cotyledon (Plate 7) and it appeared to be an abnormal development as compared with the usual development of seedling. In the same way, with ABA (50 um), Voo et al. (1991) identified six types of mature embryos viz., tulip, trumpet, fused, jar, umbvella and irregular shaped.
Increased concentration of KNO$_3$ and addition of MgCl$_2$ was not found to be significant for embryoid formation. Trolinder and Goodin (1988 a, b), Shoemaker et al. (1986) and Gawel et al. (1986) recorded high frequency of somatic embryos when $2X$ KNO$_3$ and MgCl$_2$ were added. This might be due to the influence of the genotype used by them.

5.5. Regeneration studies in suspension culture

Tissue culture methods for the improvement of cotton has lagged seriously as compared to other crops. For regeneration in cotton, a morphogenetically competent cell suspension seems to be needed which would facilitate cell level gene manipulation and plantlet regeneration. Establishing a fine cell suspension is a source material for protoplast culture as well as for the induction of somatic embryos. In the present study, hypocotyl derived embryogenic calli were used for cell growth studies. In the first two days in suspension culture, there was no cell growth indicating the lag phase. At the 4th and 5th days, the quantum of cells got doubled both in fresh weight and dry weight. The exponential phase then followed and was represented by a linear cell growth upto the 9th day. This suggests the need for weekly subculture to establish a fine
suspension. In cotton, cell growth study in suspension has not been reported earlier. This is mainly due to the poor establishment of suspension culture and also due to the secretion of secondary metabolites which will be toxic to growing callus in suspension. Recently Trolinder and Shange (1991) regenerated cotton plants for high temperature stress through in vitro cell selection.

In the present study it was established that development somatic embryos of cotton in hormone free liquid medium was faster than in solid medium. In a solid support system, calli or embryogenic tissues are only in partial contact with the medium. The liquid system helps in faster growth responsiveness due to its medium-tissue contact. In the present study, friable hypocotyl calli proliferated for 45-50 days were subjected to suspension culture. Trolinder and Goodin (1987) reported that, one month old calli rarely contained embryoids whereas, 6 weeks old calli frequently contained numerous globular embryos.

MS basal medium along with 2,4-D (0.1, 0.2 and 0.3 mg l⁻¹) and 2 iP (3 and 5 mg l⁻¹) with various concentrations of glutamine (5, 10, 15 and 20 mM) was included in suspension for cell maintenance and
proliferation. Trolinder and Goodin (1987 and 1988) reported MS medium with 2,4-D and kinetin, each of 0.1 mg l\(^{-1}\) concentration, was the optimum dosage combination for getting embryogenic suspension.

Glucose 3 per cent was better than sucrose 3 per cent. When sucrose was used in the medium, gradual darkening occurred within two weeks of culture. Beasley and Ting (1973) and Smith et al. (1977) also reported glucose medium to inhibit browning in cotton tissue culture.

Glutamine (15 mM) with 2,4-D (0.1 mg l\(^{-1}\)) in MS basal medium resulted in formation of somatic embryoids. Price and Smith (1979), Finer and Smith (1984) and Trolinder and Goodin (1987) encountered somatic embryo in glutamine containing media. When glutamine alone was used, the embryogenic cells produced less frequency of embryoids and sometimes darkening of the medium was observed. This suggested the influence of glutamine to enhance embryo development in liquid medium. Optimum 2,4-D levels kept the tissue in proliferative state. Under conditions when both glutamine and 2,4-D are available, and when the auxin level used was low during liquid culture, embryogenic
tissues were pushed towards proliferation and development of somatic embryoids. This is in agreement with Finer (1988).

5.6. Somatic embryo and plant recovery

In the present study, globular and heart shaped (Plate 6 A & B) somatic embryos were obtained in suspension culture. This might have originated from single cells, because of the typical size of the embryoids like zygotic embryo. In the semisolid culture, abnormal embryos viz., embryoid without or with one cotyledon (Plate 3) were obtained. The embryoids obtained were transferred to regeneration media containing MS macro-and micro-salts along with B5 vitamins, myoinositol (100 mg l\(^{-1}\)) and 2x KNO\(_3\) with the elimination of NH\(_4\) NO\(_3\). Glucose 3 per cent was used and media was soldified with gelrite 2 per cent and MgCl\(_2\) (750 mg l\(^{-1}\)). Embryoids developed into leaf like structures but failed to develop further. Finer (1988) regenerated somatic embryos from suspension cultures in the absence of MgCl\(_2\). However, Cousins et al. (1991) used the same medium for germinating Australian cultivar siokera 1-3.
5.7. Isozyme studies

In any crop improvement programme, it would be advantageous to have biochemical markers to identify the unknown events. Such markers can be conveniently used to discriminate recalcitrant morphogenetic pathways. Isozymes and restriction fragment length polymorphism (RFLP) have proved to be efficient markers (Beckmann and Soller, 1986). They have been successfully used to initiate and advance genetic research (Torres et al., 1988).

Isozymes often reflect more than one gene product. Almost every gene in a higher organism is differentially regulated in each tissue and developmental stage. Isozymes have attributed vital roles in regulation of cell growth and differentiation and, therefore, are useful in the differentiation process.

In this study, isozymes were utilized-

i) to study the differences during developmental stages through banding pattern and

ii) to identify markers that would help in discrimination of embryogenic calli from non-embryogenic calli.
5.7.1. Peroxidase

The peroxidase activity was high at the callus stage which progressively reduced in the subsequent stages. Coppens and Girlls (1987) reported reduced activity of peroxidase during callus differentiation and embryoid formation. Similarly Veera Raghavan (1988) reported that peroxidase activity got reduced during callus proliferation.

In the present study, isozyme patterns of peroxidase (Fig 6, lane 1c and 2c) were similar in all samples, except in embryogenic calli. The appearance of new single band in proliferating calli indicated embryoid formation. This suggested the possibility of integration between endogenous hormone levels and enzyme activity in the regulation, development and subsequent morphogenesis of calli. The relation between peroxidase activity and the metabolism of auxin in different tissues was also reported by Swarnakar et al. (1986), Coppens and Girlls (1987) and Thorpe and Gasper (1978).

The embryogenic calli of hypocotyl showed an extra band (Fig 6, lane 2c) which was fast moving (PER 7 Re. 0.649). This might be due to the embryogenic nature of the explant. Xiaolizhon et al. (1992) observed in
culture that embryogenic calli showed much higher peroxidase activity than nonembryogenic calli; embryogenic callus was found to possess an extra peroxidase band. Similarly, Maheswaran and Sree-Rangasamy (1992) reported in rice that among the three groups of isoperoxidases, a group of fast migrating bands was noticed during the process of differentiation. The appearance of these fast migrating isoperoxidase PER 7 (Re.0.649) can be used as marker to identify embryogenic calli.

5.7.2. Esterase

All the explants were similar in EST 1 (Re. 0.165) activity. Similarly EST 5 (Re. 0.415) appeared in all the hypocotyl explants except for the leaf. The hypocotyl explant showed a distinct band EST 3 (Re. 0.330) which was absent in all other explants experimented. This reveals the differential enzyme pattern. The hypocotyl explant showed two extra bands - EST 3 and EST 4 (Re. 0.330 and Re. 0.385). From this study, it can be inferred that hypocotyl may be more embryogenic than the other explants and this can be used as marker for embryogenicity.

5.8. Protoplast culture studies

Genes can be transferred into plants by a number of routes, of which protoplast mediated gene
transfer is the one, which is well established. The expression of genes by direct gene transfer was used by Paszkowski et al. (1984), which refers to the uptake of DNA into plant protoplasts without the mediation of Agrobacterium or its Ti plasmid. Even then, the crop improvement through protoplast culture technology was limited to the crops of Solanaceae and Cruciferae, the drawback of application of protoplast technology in major crop species was due to the poor plating efficiency and difficulties in regeneration. In the present study, in cotton, several studies were made to standardize the techniques of protoplast isolation, purification and culturing of microcalli from all sources of explants, and to attempt for their regeneration. The results obtained are discussed below:

5.8.1. Protoplast isolation

Effect of different enzyme concentrations on protoplast yield in different explants

Twenty different enzyme concentrations with five explants, namely, hypocotyl, cotyledon, young leaf, hypocotyl callus and cell suspension were tried. Among the five explants digested, cell suspension yielded a maximum of $1.65 \times 10^6/gFW$ in 0.5 per cent cellulase and 0.3 per cent macerozyme, followed by cotyledon, callus,
young leaf and hypocotyl explants. Finer and Smith (1982) used 14 day old subcultured calli of *G. klotzschianum* by using 1 per cent cellulase and 0.5 per cent macerozyme. The cell suspension had high frequency of finely dividing, isodiametric and thin walled embryogenic cells with dense cytoplasm. Moreover, enzyme concentration used was much reduced when protoplasts were isolated from cell suspension. This may be due to the lower content of pectin in the cell wall. Latif et al. (1993) used tomato cell suspensions for protoplast isolation and 70 per cent of protoplast derived calli regenerated shoots. In cotton, none of the authors used cell suspension for protoplast study. This may due to the practical difficulty of maintaining cotton cell suspension in the manner needed for protoplast isolation. In the present study, calli at proliferative stage yielded a maximum of $1.45 \times 10^6$/gFW protoplast digested in 1.0 per cent cellulase and 0.5 per cent macerozyme for six hours. Finer and Smith (1982) and Firoozabady and DeBoer (1986) used proliferating calli for protoplast isolation. They recorded microcolony formation which failed to divide further.

Seedling tissues, viz., hypocotyl, cotyledon and young leaf were used in the present study. Among
these, the cotyledon exhibited a maximum of $1.55 \times 10^6$/gFW protoplasts at 1.5 per cent cellulase and 0.5 per cent macerozyme for ten hours. This suggests that seedling tissues needed a little more concentration of cellulase enzyme since it contains more amount of cellulose and pectin. Ei-Shily and Evans (1983) used cotyledons of *G. barbadense*, and recorded comparatively high protoplast yield ($1.95 \times 10^6$/gFW). This might be due to the influence of the genotype. Leaf mesophyll yielded a maximum of $1.35 \times 10^6$/gFW protoplasts. Most of the mesophyll protoplasts had their chloroplast either clustered at one pole or were scattered throughout. Some of the protoplasts had a pink or red pigmentation (Plate 9) and had no chloroplast. The red pigmented protoplasts might have originated from vascular cells; Firoozabady and DeBoer (1986) also observed similar type of protoplasts while digesting mesophyll tissues.

**Effect of enzyme concentration on protoplast conversion**

Protoplast conversion was measured by calculating the number of cells before digestion and after protoplast release. A maximum of 62.5 per cent protoplast conversion in cell suspension with 0.5 per
cent cellulase and 0.1 per cent macerozyme was observed. But subcultured calli resulted in 57.5 per cent protoplast conversion. Bhojwani et al. (1977) observed very low (15%) conversion rate using 6-9 day old subcultured calli. In the meantime, Finer and Smith (1982) recorded 20.5 per cent conversion by using 14 day old calli. The protoplast conversion from calli mainly depends on its degree of friability. The light brown calli derived from hypocotyl was embryogenic and resulted in a high frequency of protoplast conversion.

In this study, amongst the seedling tissues, young leaf (2 to 3 week old) resulted in a maximum of 63.5 per cent conversion with 2.0 per cent cellulase and 0.3 per cent macerozyme mixture, thereby suggesting that the mesophyll tissues contained more uniform cells than the hypocotyl and cotyledon. In the same way, Khasanov and Butenko (1979) observed 68.5 per cent conversion from cotyledonary tissues, but the dividing protoplast failed to organize macroscopic colonies.

Effect of pre-plasmolysis on yield and viability of protoplast

The pre-plasmolysis period was an important factor to increase protoplast yield and viability. Hypocotyl, cotyledon and young leaf were pre-treated in
CPW salts with 9 per cent mannitol for different time durations (0 to 7 h). Among these, the hypocotyl resulted in a maximum of $0.97 \times 10^6 / \text{g FW}$ protoplast with 63.3 per cent viability when pre-treated for 4 h. At the same time, cotyledon and young leaf also resulted in maximum yield ($1.73$ and $1.97 \times 10^6 / \text{g FW}$, respectively) at 4 h plasmolysed period but the viability of protoplast was found to decrease (73.3 and 69.3%). This suggested that a period of 3 h for pre-plasmolysis was optimum to get both viable protoplasts and maximum yield. Ei-Shily and Evans (1983) recorded 4 h pre-plasmolysis period to enhance the total yield and viability of protoplasts from cotyledon tissue. Firoozabady and DeBoer (1986) found preplasmolysis of the cotyledons on agar medium rather than liquid medium.

**Effect of age of seedlings on protoplast yield and viability**

Hypocotyl from 5-7 day old seedlings, cotyledon from 8-14 day old, and young leaf from 15-45 day old seedlings were used to finding out the tissue suitability. Hypocotyl accounted maximum ($1.03 \times 10^6 / \text{g FW}$) protoplasts from 5 day old seedlings. At the same time viability was maximum (65%) in 4 day old seedlings. This suggests that the 4 day old seedlings
need to be used to obtain maximum viable protoplast. Narasimhulu et al. (1993) recorded seven day old hypocotyls to result in higher yield of protoplasts when compared to ten day old seedlings in Brassica.

The cotyledons from 5 day old seedlings resulted in a maximum of $1.80 \times 10^6$/gFW, while the viability was maximum (65%) on 4 day old seedlings. Mesophyll tissue of 25 day old seedlings was the best suited and yielded $1.93 \times 10^6$/gFW. Here again the viability was maximum in 30 day old seedlings (70%). Firoozabady and DeBoer (1986) revealed that the genotype and age of the plants were important factors influencing protoplast yield and viability. They recorded optimum protoplast yield from 10 to 12 day old cotyledons and mesophyll leaves from 3 week old plants.

Effect of incubation on protoplast yield and viability

After pre-plasmolysis period, the tissues were incubated in the shaker (60 rpm) under dark. Various tissues exhibited different duration for the best yield and viability of the protoplasts. Hypocotyl and cotyledon resulted in maximum yield of protoplasts 6 h after incubation (1.53 and $1.80 \times 10^6$/gFW, respectively). Hypocotyl showed 45 per cent viability. The cotyledons after 8 h incubation gave 74.7 per cent
viability. Mesophyll leaves required comparatively more time (10 h) to put forth maximum yield (1.87 x 10^6/gFW). Here again, the viability was higher at 8 h after incubation (66.7%). This suggests, that the viability and yield are closely linked.

In respect of callus source, 6 hour centrifugation was optimum for both yield (1.43 x 10^6/gFW) and viability (51.7%). At the same time, cells from suspension culture needed a lesser time of centrifugation (4 h) to exhibit higher yield (1.73 x 10^6/gFW) and viability (61.0%).

**Size of the protoplast**

Among the various tissue sources, hypocotyl and cotyledon protoplasts were relatively larger (30-40 μm) and more uniform than others. This reveals that within a genotype, the size of the protoplast was influenced by the tissue source. Firoozabady and DeBore (1986) measured the cotyledon protoplasts to be 30-35 μm and mesophyll leaf to be 20-25 μm size.

**5.8.2. Protoplast culture**

Purified protoplasts were cultured on both Ko liquid and agarose solid medium as followed by Saka et al. (1987), supplemented with NAA (1 mg l⁻¹) and 2 iP (2
mg l⁻¹). After 48 h the protoplasts lost their spherical shape, and started dividing. Firoozabady and DeBoer (1986) observed the cultured protoplasts lost their spherical shape, elongated and grew in volume after 48 h. Ei-Shily and Evans (1983) also observed the same phenomenon. According to Bajaj et al. (1977), the protoplast wall regeneration revealed that the wall materials were progressively deposited on the surface of the plasmalemma. Takebe et al. (1968) reported a multilamellar wall material deposition preceded the formation of a cellulosic wall. It was observed in this study that, while culturing the protoplast, the cell wall formation lead to simultaneous division. First division was apparent after 3 days in liquid K₃ medium and 5 days in solid agarose medium (Plate 15). Cell colony formation occurred after one week. Saka et al. (1987) noticed cell colony formation after 8 days of culture. Bhojwani et al. (1977) reported that divided protoplast underwent 25-30 celled colonies after 5 weeks of culture. Finer and Smith (1982) noticed that protoplasts isolated from callus formed colonies consisting of 40-50 cells after 4 weeks.

In the present study, two types of cell colonies were observed after one month, a larger colony of 20-30 cells and smaller colony of 3-5 cells. Amongst
the various explants tried, hypocotyl, young leaf and callus protoplast organised both types of cell colonies but protoplasts from cell suspension formed only smaller colonies. However, those from cotyledonary source failed to organise cell colonies beyond budding. The budded protoplasts never divided beyond the second division but degenerated afterwards (7-10 days). Similar process was noticed by Firoozabady and DeBoer (1986). The differential behaviour of protoplasts from various tissue sources implies the importance of donor source of tissue for protoplast culture, and that various tissues of the same genotype might respond differently in culture.

The plating density was an important factor influencing colony formation leading to callus development. Here, in seedling tissues, viz., hypocotyl, cotyledon and young leaf, the maximum plating efficiencies of 12.60, 10.60, and 6.25 per cent, respectively were observed at an optimum of 5 x 10⁶/ml protoplasts plated. In case of higher (6 x 10⁶/ml) and lower (4 x 10⁶/ml, 3 x 10⁶/ml and 2 x 10⁶/ml) protoplast density, initial cell division was noticed and in later stages, colony formation leading to growth was not sustained. This indicates that an optimum protoplast
population is necessary for proper growth and development.

Firoozabady and DeBoer (1986) reported the optimum culture density for *G. hirsutum* cultivars as $4-6 \times 10^4$ protoplast/ml and those for *G. barbadense* cultivars around $2.5 \times 10^4$ protoplast/ml. In the same way, Saka et al. (1987) also reported a culture density of $5 \times 10^5$ protoplast/ml was optimum both in liquid and solid medium. When colonies were transferred to Kao agarose medium after 40-50 days, the culture formed microcalli (1-2 mm). The microcalli were further attempted for regeneration but failed to develop seedlings. Saka et al. (1987) also obtained only such a result.

5.9. Genetic transformation studies

Genetic engineering is the most important component of plant biotechnology, which will have implications in cotton improvement. With this method, traits such as resistance to herbicides, insects, fungi, nematodes, and bacteria can be transferred to commercial cultivars of cotton. Among the various methods of gene transfer, *Agrobacterium*-mediated transformation has dominated the reports to date, specific to Coker cultivars. The present study was conducted to finding out the optimum dosage of kanamycin that can be used to
get the transformed calli which could be confirmed by the GUS activity. The hypocotyl segments of 4-5 day old seedlings were infected with binary vector *Agrobacterium tumefaciens* strain LBA 4404. Firoozabady *et al.* (1987 a) used 12 day old cotyledon segments, but Umbeck *et al.* (1987) and Perlak (1990) experimented with hypocotyl explants. Hypocotyl derived calli were more embryogenic than the other explants.

Prior to transformation experiments, studies were conducted to understanding the sensitivity of hypocotyl explants to kanamycin using six doses, namely, 0, 10, 20, 30, 40 and 50 µg/ml.

Callusing frequency with leaf explant was reduced in kanamycin treated over control (85%). It was 50 per cent in 10 µg/ml kanamycin which further got reduced to 30 per cent in 20 µg/ml concentration, and hence this dose was taken for selection. However, in rice 10 µg/ml of kanamycin was the best (Amutha, 1991). Firoozabady *et al.* (1987 b) used 25 µg/ml of kanamycin as selection regime. At the same time, Finer and McMullen (1990) standardized 100 µg/ml of hygromycin for screening transferred embryogenic suspension cells. In the present study, no callus formation was observed beyond 20 µg/ml kanamycin and indicated the toxic level.
The callusing frequency was also significantly reduced to 0.430 g when treated with 10 μg/ml over control (1.600 g). Similar results were recorded by Rajeswari (1992) in tomato.

The transformed calli were subjected to flurometric assay using MUG (4-methyl umbelliferyl glucuronide). Blue fluorescence was observed in this GUS assay which indicated transformation. Graham et al. (1990) reported GUS gene expression in Rubus using MUG confirming transformation.