DEVELOPMENT OF AN IN VITRO PROTOPLAST CULTURE SYSTEM
FOR ALBIZIA LEBEK (L.) BENTH. - AN ECONOMICALLY
IMPORTANT LEGUMINOUS TREE.

THESIS

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By

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CHAPTER 1

INTRODUCTION

*Albizia lebbeck* (L.) Benth. – an endangered, economically important tree species

Forests originally covered an estimated 45 to 50 percent of the earth’s land surface before the advent of agricultural and industrial revolutions. Now they have been reduced 30 to 50 percent according to different estimates, to cover approximately 30 percent of the earth’s land surface. Forests perform many vital functions in all the biogeochemical cycles, in maintenance of air and water quality, in soil formation, in the development and preservation of biodiversity, and in the moderation of weather and climate (1). A 1979 publication by the American National Academy of Sciences on legumes (2) outlined some of the urgent research needs for a great variety of economically important woody species whose remaining genetic resources need urgently to be collected and conserved. The conventional method of seed propagation is restricted in these woody legumes because of an acceptably low rate of germination and a high death rate from exuded polyphenols from the seedling of seeds that do germinate. Propagation by cuttings is not uniformly adaptable because it is slow, labor intensive, and some of the woody legumes are difficult or impossible to root from...
cuttings (3, 4). Some of the species most threatened at the present time are and have a high value as timber crops. Tissue culture propagation using different explants is now gaining increasing recognition as a viable alternative in tree propagation and improvement (5, 6, 7, 8).

*Albizia lebbeck* (L.) Benth., a leguminous tree belonging to the family Leguminaceae and sub-family Mimosoideae, is a native of the Andaman Islands, Burma, Ceylon, and India. This tree is usually found throughout the warmer regions of the world. It reached the United States via Egypt sometime around twentieth century and is nowadays limited to lower Florida and the Florida keys. This tree is commonly known as Kokko and has various uses like browse for domestic and wild animals, screen for young plantations, as fuel wood and as a fodder crop. The insect *Tachardia lacca*, whose resinous secretions are the source of commercial shellac, is commonly cultured on this tree. It is also used in paneling and for fixtures in large public buildings. In India and other Asian countries it is used in the manufacture of furniture, gun stocks, casks, tennis racquet frames, paneling, wheels, carvings and railway carriages (9). A water soluble gum is exuded if the trunk is damaged, and their barks also contain tanning compounds. At various times these gums and bark have commercial uses.

In *Albizia*, the hard seed coat is often a barrier for seed germination. Propagation by cuttings is virtually impossible since there is no viable method of vegetative propagation. Therefore, a new method of mass propagation is vital to the survival of this tree species. In this context we propose to develop an *in vitro*
system of protoplast generation (10, 11, 12) and subsequent plant regeneration from those protoplasts to improve and multiply the species in a novel way. The basic steps involved in this venture are:

1. Establishment of callus.
2. Establishment of cell suspension culture.
3. Protoplast isolation and culture.
4. Protoplast viability testing.
5. Plant regeneration from the isolated protoplasts.

The protoplast thus generated can be used in further research efforts for the improvement of this tree species. Some of the research work envisaged are:

1. Regeneration of the whole tree from the protoplast culture thus, bypassing the seed germination stage.
2. Protoplast fusion experiments to produce interspecific, intraspecific or intergeneric hybrids.
3. Transformation of the protoplasts with the help of Agrobacterium plasmid to do away with the harmful effect of polyphenolic activity in seed germination or to introduce other desirable traits.

In vitro techniques of regeneration of plants offer a safe, effective and an economical way by which plants can be produced, maintained, multiplied and transported as disease free propagules or regenerants. In vitro cultural studies of Albizia lebek have been reported previously by several workers (Gharyal and Maheshwari, 1981, 1982, 1983; Upadhyaya and Chandra, 1983; Varghese and
Kaur, 1988; Tomar and Gupta, 1988) (13, 14, 15, 16). However, I found no reports on the protoplast culture of this economically important tree species. The present investigation is to set up a viable protoplast culture system that can be used for further plant improvement techniques.

Callus cultures

A callus consists of an amorphous mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue (2, 17). Callus has no organizational pattern, although localized areas of meristematic activities are present. Using tissue culture techniques, callus formation can be induced in numerous plant tissues and organs that do not usually develop callus in response to injury (Street, 1969) (18). All multicellular plants are potential sources of explants for callus initiation (Yeoman and Macleod, 1977) (19). The general growth characteristics of a callus involve a complex relationship between the plant material used to initiate the callus, the composition of the medium, and the environmental conditions during the incubation period. Establishment of the callus from the explant can be divided roughly into three developmental stages: induction, cell division, and differentiation. During the initial induction phase metabolism is stimulated as the cells prepare for division. The length of this phase depends mainly on the physiological status of the explant cells as well as the cultural conditions. Subsequently, there is a phase of active cell division as the explant cells revert to a meristematic state. A third phase involves the appearance of cellular differentiation and the expression of certain metabolic pathways that
lead to the formation of products. Some callus growths are heavily lignified and hard in texture, whereas others break easily into small fragments and are “frangible”.

Callus cultures are dependent upon exogenous sugar for continued growth even if they are derived from chlorophyll-containing organs (Hildebrandt et al, 1963) (20). The nutritional requirements for the initiation of callus cultures varies considerably for primary explants of different origin. The majority of the excised tissues, however, require the addition of one or more growth factors to the medium in order to stimulate callus development (Yeoman and Macleod, 1977). After the callus has been grown for a period of time in association with the original tissue, it is necessary to subculture the callus to a fresh medium. Growth on the same medium for an extended period of time leads to depletion of essential nutrients and to gradual dessication of the agar because of water loss. Metabolites secreted by the growing callus also accumulate to toxic levels in the medium. The transferred callus must be of sufficient size to assure renewed growth on the fresh medium. If the transferred inoculum is too small, it may exhibit a very slow rate of growth or none at all. Street (1969) suggested that the inoculum be at least 100 mg. Successive sub-cultures should be performed every 28 days but again this depends on the rate of callus growth. A typical growth curve for callus cultures resembles the growth curve for bacterial cultures (FIG. 1) (5). The callus is transferred to the surface of a sterile petridish and sliced into fragments with a sterile scalpel and then transferred onto fresh medium. Brown or necrotic tissue is discarded.
Suspension cultures

According to King (1980), the term “suspension culture” has no clear-cut biological definition, and these tissue culture systems are more than simple aggregates of cells suspended in liquid medium. A suspension culture originates with a random critical event occurring during the early exposure of the plant cells to the liquid medium. Cells undergoing this transition in growth rate and metabolic products and produce “cell lines”. Some of the characteristics of cell lines include (a) a high degree of cell separation, (b) homogenous cell morphology, (c) distinct nuclei and dense cytoplasm, (d) starch granules, (e) doubling times of 24-48 hours, (g) loss of totipotency, (h) increased ploidy levels (King, 1980) (21). Cell suspension cultures are generally initiated by transferring fragments of undifferentiated callus to a liquid medium, which is then agitated during the culture period. Suspension culture can also be started by inoculating the liquid medium with an explant of differentiated plant material (e.g., a fragment of a hypocotyl or cotyledon). The dividing cells gradually free themselves from the inoculum because of the swirling action of the liquid. No suspension culture has been shown to be composed entirely of single cells (Butcher and Ingram, 1976) (22). After a short time the culture will be composed of single cells, cellular aggregates of various sizes, residual pieces of inoculum, and the remains of cells. The degree of cell separation of established cultures already having the characteristics of high friability can be modified by changing the composition of the nutrient medium. Increasing the auxin: cytokinin ratio,
Figure 1: Growth response of a typical callus culture. This particular callus should be subcultured approximately at the time indicated by X.
in some cases, produces a more friable culture.

The initiation of a cell suspension culture requires a relatively large amount of callus to serve as the inoculum, approximately 2-3 g for 100 ml (Helgeson, 1979) (23). When the plant material is first placed in the medium, there is an initial lag phase prior to any sign of cell division (FIG. 2) (5). This is followed by an exponential rise in cell number and a linear increase in the cell population. Then there is a gradual deceleration in the division rate. In order to maintain the viability of the culture, the cells should be subcultured early during this stationary phase. Because cells from different plant material vary in the length of time they remain viable during the stationary phase, it is prudent to subculture during the period of progressive deceleration.

Nutritional requirements of the callus and the suspension culture

Because there is a division of labor by the different organs of the plants in the biosynthesis of essential organic metabolites, less is known about the nutritional requirements of the various organs and tissues of the plant than is known of the whole plant. In 1934, White (24) discovered that isolated tomato roots had the potential for unlimited growth, if they were provided with a liquid medium containing a mixture of inorganic salts, sucrose, thiamine, pyridoxine, nicotinic acid, and glycine. In addition to the somewhat selective biosynthetic activity of certain isolated tissues and organs, cultured systems may exhibit changes in their metabolic pathways over a period of time. These changes in
Figure 2: Growth curve of a cell suspension grown under batch conditions relating total cell number per unit volume to time.
metabolism require corresponding changes in nutritional requirements. The requirement for a particular organic supplement could be due either to the inability of the cultured tissue to produce it or to new requirement resulting from a shift in metabolism.

Isolated protoplast – a tool for tree improvement practices

The aseptic culture of plant cells has emerged in recent years as a powerful technique for plant improvement and agriculture. During the past few years, the potential of plant cell culture has vastly improved due to the fast emerging technology of isolation, cultivation, and fusion, of protoplasts. The term "protoplast" refers to the spherical plasmolysed contents of a plant cell enclosed in the plasmalemma and set free of the covering wall by a suitable experimental method (11). The naked cells so obtained constitute an ideal developmental system because protoplasts are separate entities capable of reforming cell walls and regenerating whole plants. This fact is of considerable advantage as propagation of desired plants becoming more efficient. For a purposeful application of protoplast technology for crop improvement of any species, first the protoplasts have to be isolated and then the plant regenerated from those protoplasts. Protoplasts are rapidly gaining recognition as an important research tool in a variety of developmental, physiological, and biochemical investigations (25). These include studies on permeability and transport of ions and solutes (e.g. Akerman et al., 1983; Cornel et al., 1983; Rahat and Reinold, 1983),
photosynthesis (e.g. Heber, 1982; Chapman and Hatch, 1983; Kaiser and Heber, 1983), the mechanism of action of plant hormones (e.g. Hooley, 1982; Chang et al, 1983), and of phytochrome (Kim and Song, 1981) to cite a few.

The idea that cells could contain all the information necessary for the regeneration of a whole organism is implied in the cell theory proposed by Schwann (1839)(26). Haberlandt (1902) originated the concept of cell culture and was the first to attempt to cultivate isolated plant cells in vitro on an artificial medium. The regeneration of carrot plantlets from cultured secondary phloem cells of the taproot clearly demonstrated the 'totipotency' of plant cells (27). Totipotency is the ability to regenerate a whole plant from a single cell. The list of plants from which whole plants have been regenerated from single cells or protoplasts is continuing to grow and is now well over 100 species. Some caution should be exercised in believing that the concept of totipotency can be applied to all the plants. In species where it has been difficult to regenerate whole plant from single cells, often it is concluded that the right medium was not encountered, although in many cases it may so because some cells may have lost some critical genetic material during differentiation and those cells simply could not differentiate into a whole plant. Regeneration of plants from isolated cells or protoplasts is normally by one of two different pathways, either by organogenesis or embryogenesis.

Organogenesis takes place from a callus and not directly from a single cell. When isolated protoplasts are put into culture under appropriate
conditions they go through a series of events like (1) wall regeneration (2) early mitotic division and callus formation and (3) organogenesis. The first indication that *in vitro* organogenesis could be hormonaly regulated was given by Skoog (28). He found that the addition of auxin to the culture medium stimulated the formation of roots while inhibiting shoot formation. Later, it was found that adenine sulfate (cytokinin) promoted shoot interaction and that this compound reverses the inhibitory effect of auxin.

Isolated protoplasts are 'naked' plant cells in which the cell wall has been experimentally removed. The isolated protoplast is unusual because the outer plasma membrane is fully exposed and is the only barrier between the external environment and the interior of the living cell (Cocking, 1972; Evans and Cocking, 1977) (29, 30, 31). Protoplasts nowadays are being utilized in several areas of study:

1. Fusion of two or more protoplasts to produce hybrid variety of plant. In some cases, when hybrids cannot be produced by conventional plant breeding techniques because of sexual or physical incompatibility, somatic cell fusion can come to the rescue.

2. The isolated protoplast is capable of ingesting foreign materials like nuclei, chloroplasts, mitochondria, DNA, plasmids, bacteria, viruses, etc. into it's cytoplasm.

3. The cultured protoplast can rapidly generate a new cell wall, which offers a novel system for studying cell wall biosynthesis and deposition.
Protoplasts can be isolated by either a mechanical or enzymatic process. The mechanical approach involves cutting a plasmolyzed tissue in which the protoplasts have shrunk and pulled away from the cell wall. Subsequent deplasmolysis results in expansion and release of the protoplasts from the cut ends of the cells. By this method, the yield of protoplasts is meager. Since the early 1960’s nearly all of the protoplast isolation work has been performed with enzymatic procedure. The basic techniques consist of (1) surface sterilization of the starting material, (2) sequential or mixed enzyme treatment, (3) purification of the isolated protoplasts by removal of enzymes and cellular debris, (4) transfer of the protoplasts to a suitable medium with the appropriate cultural conditions (FIG. 3a and 3b) (11).

The plant cell wall consists of a complex mixture of cellulose, hemicellulose, pectin, and lesser amounts of protein and lipid. Because of the chemical bonding of these diverse constituents, a mixture of enzymes is necessary to effectively degrade the cell wall. Cellulose is a polymer consisting of subunits of D-glucose. The bulk of hemicellulose is formed of xylans (Northcote, 1972) (32). These polymers consist, however, of several monosaccharides in addition to xylose. Pectins are polysaccharides containing the sugars galactose, arabinose, and the galactose derivative galacturonic acid (Northcote, 1974). Hence, protoplast isolation is achieved by using a combination of cellulase, pectinase and hemicellulase. Most commercial preparation of these enzymes are isolated from microorganisms and so they often exhibit a variety of enzymatic activities like
those of nucleases, proteases, and several toxic enzymes. It is essential that these enzymes be purified before use in protoplast isolation procedures. A list of commercial preparations indicating the major enzymatic activity is given in TABLE 1.
TABLE 1: Some enzyme preparations exhibiting cell wall degrading activity classified according to their major function.

<table>
<thead>
<tr>
<th>Cellulases</th>
<th>Hemicellulases</th>
<th>Pectinases</th>
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<tr>
<td>1. Cellulase, Onuzuka (R-10, RS)</td>
<td>1. Rhozyme HP-150</td>
<td>1. Macerase</td>
</tr>
<tr>
<td>2. Cellulysin</td>
<td>2. Hemicellulase (Sigma)</td>
<td>2. Macerozyme R-10</td>
</tr>
<tr>
<td>3. Meicelase (CESB, CMB)</td>
<td></td>
<td>3. Pectolyase Y-23</td>
</tr>
<tr>
<td>4. Driselase</td>
<td></td>
<td>4. Pectinase (Sigma)</td>
</tr>
<tr>
<td>5. Cellulase (Sigma)</td>
<td></td>
<td></td>
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Figure 3a: Basic technique for the isolation of protoplasts from an excised leaf.

The leaf surface is sterilized, rinsed repeatedly in sterile distilled water, and the cells are plasmolyzed in a solution of mannitol. The lower epidermal layer is stripped from the leaf to enhance enzyme penetration into the mesophyll tissue. Following treatment with one or more wall degrading enzymes, a crude suspension of mesophyll protoplasts is obtained.
Figure 3b: Purification procedure for isolated protoplasts.

Crude protoplast preparation

Transfer to culture medium + agar

45 μm mesh

75 x g centrifugation

Supernatant + debris

Protoplasts

Remove supernatant and resuspend in fresh medium twice

Resuspend at appropriate protoplast concentration

Protoplasts
CHAPTER 2

MATERIALS AND METHODS

Plant materials and establishment of callus

The starting material for protoplast establishment was the seeds of *Albizia lebbeck* (L.). Viable seeds of *Albizia lebbeck* (L.) Benth. were obtained from a seed supplier, SHIDH Sales Corporation of Dehradun (UP), India, and was imported to the U. S. A by permission from the USDA (permit no. 77437). Only dry, mature and healthy seeds were used for the experiment. The seeds were thoroughly washed in tap water and submerged in 5% Tween-20 for 15-20 minutes. Then the detergent was decanted and the seeds were washed in distilled water several times before keeping them in distilled water for 2-3 hours. After this treatment, the seeds were treated with (0.1% w/v) mercuric chloride solution for 8 minutes. Then they were again washed in distilled water to remove all traces of mercuric chloride.

The seeds were germinated on half strength hormone and vitamin free MS medium (Murashige and Skoog, 1962) (TABLE 2) (33) with 0.6% w/v agar (Fisher Scientific, A360-500). After 3-4 weeks, seedlings came out of the germinated seeds. The leaves from these *in vitro* plants were excised and
TABLE 2: Murashige and Skoog Salt w/o agar.

<table>
<thead>
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<th>Components</th>
<th>MM-100 Mg/L</th>
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<tbody>
<tr>
<td>NH₄NO₃</td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900.000</td>
</tr>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>333.000</td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>181.000</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170.000</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>36.700</td>
</tr>
<tr>
<td>H₃BO₄</td>
<td>6.200</td>
</tr>
<tr>
<td>MnSO₄. H₂O</td>
<td>16.900</td>
</tr>
<tr>
<td>ZnSO₄. 7H₂O</td>
<td>8.600</td>
</tr>
<tr>
<td>KI</td>
<td>.830</td>
</tr>
<tr>
<td>Na₂MoO₄. 2H₂O</td>
<td>2.500</td>
</tr>
<tr>
<td>CuSO₄. 5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl. 6H₂O</td>
<td>0.025</td>
</tr>
</tbody>
</table>

inoculated aseptically on modified MS medium. Young leaflets were selected for initiating the callus because in vitro propagation of adult or mature woody plants is adversely affected by characteristics accompanying maturation such as reduced growth rate, reduced or total lack of rooting ability (Thompson et al., 1984; Hutchinson et al., 1988).

The *in vitro* Albizia plant, the leaves of which were used to carry out this research work, was a kind gift from Dr. Ghosh, University of North Texas, Denton.

The leaf explants were cultured on MS medium modified with growth hormones like kinetin (0.5 mg L\(^{-1}\)) and 2, 4 D (2 mg L\(^{-1}\)) and growth factors like coconut milk (10% v/v) and casein hydrolysate (0.1% w/v). Agar (0.8% w/v) was used to gel the medium. The pH of the media was adjusted to 5.8 before autoclaving at 138 KPa and 121\(^0\)C for 15 minutes. Culture vessels were maintained in the tissue culture room at 25± 1°C with 60% relative humidity under a 16 hour photoperiod with 35 uE/cm\(^2\)/sec from cool fluorescent light (FIG. 4). Throughout the subculture procedure the instruments used for transferring callus were flamed and aseptic techniques maintained. The whole procedure was carried out in the laminar flow cabinet (FIG. 5)

Seven weeks old callus obtained was further subcultured on MS medium modified with kinetin (0.5 mg L\(^{-1}\)), 2, 4 dichlorophenoxy acetic acid (2 mg L\(^{-1}\)), casein hydrolysate (1 g L\(^{-1}\)), PVP (0.5% w/v), vitamins (1ml of 1000X Murashige and Skoog vitamin soln.), coconut milk (10%), thiamine HCL (1 ml of 1000X),
sucrose (30 g L⁻¹), glycine (1.5 g L⁻¹) and agar (8 g L⁻¹). After 3-4 months of subculturing mainly two types of callus cell lines was established.

Establishment of suspension cell culture

The next step was to establish suspension cell lines. Approximately 3 gm. of callus was transferred into 200 ml flasks containing 40 ml liquid MS medium (Murashige and Skoog, 1962) modified with 2 mg L⁻¹ 2, 4-D, 0.5 mg L⁻¹ kinetin, thiamine HCl (1 ml of 1000X), vitamins (1 ml of 1000X Murashige and Skoog vitamin solution) and sucrose (3 g L⁻¹). The flasks were incubated on a shaker at 120 rpm in the dark at 27° C ± 2° C and were subcultured every 5 days. In the original subcultures, the bigger clumps of tissue were gradually removed. After 3 months in culture, a suspension cell line was established.
Figure 4: Culture vessels maintained in the tissue culture room at 25± 1°C.

Figure 5: A laminar flow cabinet.
Protoplast isolation and culture

About 1 g of the cell suspension culture was digested from 8-10 hours with 20 ml of the enzyme solution taken in a 9 cm petridish, agitated at 30 rpm, in the dark at 26 ± 2°C. Mainly two types of callus were used to generate protoplast cultures, (1) the friable white callus and (2) the green compact type, to observe which one gave the more number of protoplasts per gm of callus digested (TABLE 4). The enzyme solutions used contained pectinase, cellulase and pectolyase in different concentrations (TABLE 3) apart from containing 1470 mg L\(^{-1}\) CaCl\(_2\), 95 mg L\(^{-1}\) KH\(_2\)PO\(_4\), and 0.6 mg L\(^{-1}\) glycine, d-mannitol (13%), PVP (2%), sucrose (0.2M), pH 5.8. Following digestion, the crude protoplasts were passed through 100 um and 53 um nylon meshes and collected into centrifuge tubes. The mixture was centrifuge at 500 rpm for 5 minutes. The protoplasts were washed twice with enzyme free isolation solution (TABLE 5) and once with medium M1 (TABLE 6). Protoplasts were mainly cultured by two methods;

(a) in liquid improved MS media (TABLE 7) in 9 cm petridishes containing 10 ml of the medium M1. The cultures were maintained at 25 ± 2 °C in the dark.

(b) in agarose embedment culture; protoplasts were plated in solid medium by mixing 1.5 ml of the protoplast mixture with an equal volume of molten agarose medium with 1.6% agarose.

After a week the cultures were gradually diluted with corresponding lower osmotic M2 medium (TABLE 6).
Protoplast viability testing

Protoplast viability testing was done with the use of fluorescein diacetate (FDA). Protoplast preparation was treated with 0.01% FDA solution and observed under fluorescent microscopy within 5-15 minutes.
TABLE 3: Enzymes of isolation solutions.

<table>
<thead>
<tr>
<th>Isolation Solutions</th>
<th>Cellulase (%) (Sigma)</th>
<th>Pectinase (%) (Sigma)</th>
<th>Pectolyase (%) (Sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution I</td>
<td>3</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>Solution II</td>
<td>2</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Solution III</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

TABLE 4: Comparison of the amount of protoplasts obtained from (a) the friable white callus and (b) the green compact type after enzyme treatment of various concentrations.

<table>
<thead>
<tr>
<th>Callus type →</th>
<th>(a) friable white callus</th>
<th>(b) green compact callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solution I</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Solution II</td>
<td>++++++</td>
<td>+++</td>
</tr>
<tr>
<td>Solution III</td>
<td>++++++++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 5: Enzyme free wash solution

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration per Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>1470 mg</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>95 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.6 mg</td>
</tr>
<tr>
<td>d-mannitol</td>
<td>130 gm</td>
</tr>
<tr>
<td>PVP</td>
<td>29 gm</td>
</tr>
<tr>
<td>Sucrose</td>
<td>68.46 gm</td>
</tr>
</tbody>
</table>
TABLE 6: Protoplast culture media

<table>
<thead>
<tr>
<th>Media* →</th>
<th>Compound</th>
<th>M1 Improved MS</th>
<th>M2 Improved MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sucrose (%)</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Glucose (%)</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>KT (mg L⁻¹)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>2,4-D (mg L⁻¹)</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*M1 and M2 filter sterilized
TABLE 7: Organic elements added in improved MS medium$^a$ (mg L$^{-1}$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content</th>
<th>Compound</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sucrose</td>
<td>20,000</td>
<td>7. Vitamin B1</td>
<td>10</td>
</tr>
<tr>
<td>2. Glucose</td>
<td>90,000</td>
<td>8. Vitamin B2</td>
<td>1.0</td>
</tr>
<tr>
<td>3. Fructose</td>
<td>200</td>
<td>9. Vitamin C</td>
<td>1.0</td>
</tr>
<tr>
<td>4. Ribose</td>
<td>200</td>
<td>10. Pantothenic</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acid</td>
<td></td>
</tr>
<tr>
<td>5. Inositol</td>
<td>100</td>
<td>12. Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>6. CH</td>
<td>300</td>
<td>13. Folic acid</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$^a$ Inorganic elements were half-strength MS salts (without NH$_4$NO$_3$)
CHAPTER 3

RESULTS AND DISCUSSION

Choice of nutritional components
of the culture media

The seeds that were germinated on half strength, vitamin free MS medium
gave rise to green healthy seedlings in three to four weeks (FIG. 6). Young
leaflets were chosen to establish callus. Health and physiological condition of the
explant was the prime condition for choosing it to establish callus these two
characteristics are important factors for the success of an in vitro culture of a
woody legume. In most of the tissue cultural studies it has been found that
explants from mature trees possess very little regenerative potential while those
from the juvenile portions of a plant show a higher degree of regenerative
capacity (34, 35). Also, in vitro plantlets must be aseptic and with younger leaves
there is a lower chance of developing infection in the culture medium.

Cultured plant tissues require a continuous supply of certain inorganic
chemicals like carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium,
calcium, magnesium, and sulfur. The Murashige and Skoog medium (1962) used
in our experiments (TABLE 1) contains most of these macronutrients. Nitrogen is
present in the form of nitrate (NH₄NO₃). Magnesium sulfate anhydrous (MgSO₄).
2H₂O) satisfies both the magnesium and sulfur requirements. Phosphorus and potassium requirements are fulfilled by KH₂PO₄. CaCl₂ is present as a calcium requirement. Micronutrient elements, iron, manganese, zinc, boron, copper, molybdenum, and chlorine are also present. For the iron requirement usually a stock solution was prepared separately because of the problem of iron solubility. The iron stock was prepared in a chelated form as the sodium salt of ferric ethylenediamine tetracetate (NaFeEDTA). This media also contained traces of boron and molybdenum. Agar was also the source of some mineral elements.

The growth regulator requirements for most callus cultures are auxin and cytokinin (FIG. 7) (5). Auxins stimulate shoot elongation and cytokinins promote cell division in plant tissues. Cytokinins are mainly N⁶-substituted aminopurine derivatives, although there are some exceptions. Auxin - cytokinin supplements are instrumental in the regulation of cell division, cell differentiation, and organ formation. The auxins most commonly employed are IAA, α-IAA, and 2,4-D. Callus cultures can also be stimulated by 2,4,5-T, indole-3-butyric acid (IBA) and ρ-chlorophenoxyacetic acid (4-CPA). The most effective auxin for callus proliferation of most cultures is 2,4-D (10⁻² – 10⁻⁵), often in the absence of any exogenous cytokinin. In our investigation, 2, 4 dichlorophenoxyacetic acid was used to initiate callus cultures from the leaf explants and it proved to be an effective inductor of callus formation. This herbicide is a powerful suppressant of organogenesis and should not be used in experiments involving root and shoot initiation (Gamborg et al., 1976).
The most widely used cytokinins in culture media are usually kinetin, benzyladenine, and zeatin. Kinetin and benzyladenine are synthetic compounds whereas zeatin occurs naturally. Another naturally occurring cytokinin is 6-\([\gamma, \gamma\text{-dimethylallylamino}]\) purine. Diphenylurea, a growth factor reported present in coconut milk (Jacobs, 1979) elicits cytokinin-like responses in some bioassays. In our investigations, kinetin was added at a concentration of 0.1-0.5 mg L\(^{-1}\) for the induction of callus. Coconut milk was also added to the culture media as a cytokinin source for a final concentration in the medium of 10-15% v/v.

Vitamins have a catalytic function in enzyme systems and are required in trace amounts. Some consider that thiamine (vitamin B\(_1\)) may be the only essential vitamin for nearly all plant cultures. Thiamine was added to the culture medium as thiamine-HCl in amounts varying from 0.1-30 mg L\(^{-1}\). Ascorbic acid, may be employed to alleviate tissue browning (Reynolds and Murashige, 1979).

With the exception of glycine (aminoacetic acid), which is a component of several media, amino acids are not usually added to the plant culture media. If a mixture of organic nitrogen is considered necessary, the medium can be enriched either with casein hydrolysate or casamino acids (0.05-0.1% w/v). It is not uncommon to observe growth inhibition following the addition of a combination of amino acids, a phenomenon that has been attributed to competitive interactions between the various amino acids (Street, 1969).

Some media require the presence of a carbon and energy source. Sucrose or D-glucose was usually added in concentrations of 2-3% w/v. Nearly all
cultures appeared to give the optimal growth response in the presence of the disaccharide sucrose. Sucrose is heat labile and the result is a combination of sucrose, D-glucose, and D-fructose, so that an autoclaved medium may give completely different results from one containing filter-sterilized sucrose (Ball, 1953). The cyclitol myo-inositol was added to culture media as a growth factor at the concentration of 1g L⁻¹. The choice and concentration of the sugar to be used depends mainly on the plant tissue to be cultured and the purpose of the experiment.

The water employed in all tissue culture media, including the water used during culture procedure, was double distilled or demineralized. Cultures were usually grown on a semisolid or solid matrix. Contaminants, released by the matrix, may contribute to the nutrition of the cultured tissue. All the cultures were grown on an agar base, in a concentration of about 0.8% w/v.

Establishment of callus

Two different types of calli were obtained after culturing and subculturing the callus from the leaf explants. Based on the color, morphology, and texture of the different calli obtained they could be grouped into:

1. Compact dark green type: the callus was dark green in color with a hard texture (FIG. 8).

2. Friable white callus: the callus was white in color, loose and friable (FIG. 9).

A number of cultures showed browning of the culture medium. Browning usually results from the oxidation of phenolic compounds released from the cut
ends of the explants and oxidised by polyphenol oxidases (Mayer and Harel, 1979), oxidases (Loornis and Battaile, 1966; Vaughn and Duke, 1984) or air (Robinson, 1983). The oxidized products, quinones, are highly reactive (36). They inhibit enzyme activity and ultimately lead to the death of the explants (Hu and Wang, 1983). The addition of PVP (polyvinylpyrrolidine) inhibited browning (37). This compound probably acts by adsorption of the inhibiting compound or phenol complexes (Tyagi et al., 1981, Babbar and Gupta, 1982).

Choice of starting material for protoplast isolation

Actively growing young cell suspension cultures were chosen for isolation of protoplasts (FIG. 10). Normally the cell suspension was filtered prior to the enzymatic treatment to remove large cell clumps which were not easy to digest. Old or stationary phase-culture was avoided as they had a tendency to form dense clumps of cells with thick walls that were difficult to digest with the enzymatic mixture (38, 39, 40).

Choice of enzymes and osmotica

The structural composition of the cell wall dictates that the digestion mixture should be able to degrade cellulose, hemicellulose, pectin and in some cases callose. Most of the commercial enzyme preparation are contaminated with a wide range of biologically active impurities like nucleases, lipases and proteases, which have a degrading effect on the plasma membrane of the isolated
protoplasts (41). So the enzyme preparation was sterile filtered with 0.2 um disposable nylon filters (Fischer) before using it for digestion purposes.

Protoplast isolation and culture

Enzyme concentration and callus type had an influence on the yield of the protoplasts (TABLE 4). The enzyme mixture was dissolved in a necessary component, calcium chloride dihydrate and potassium phosphate to stabilize the isolated protoplasts. Once the cell wall has been digested away the isolated protoplasts is subject to a lot of osmotic stress. If an osmotic stabilizing agent is not included in the medium the isolated protoplasts would take in water and eventually burst because there is no cell wall to constrain the cells. Thus, osmotic stabilizers like mannitol and sucrose were used to adjust the osmotic potential of the bathing incubation medium and make it almost equal to the osmotic potential of the isolated protoplasts' cytoplasm. Mannitol at a concentration of 13% worked as a good osmotic stabilizer. Sucrose was also added to the incubation medium as an osmotic stabilizer but the downside of adding sucrose to the medium is that the isolated protoplasts use up the sucrose as a metabolizable carbon source and the concentration of the sucrose in the culture media falls as the protoplasts are maintained in the culture. Care was also taken as not to have too high a concentration of osmoticum, as it causes the protoplasts to shrink; when this happens cell wall regeneration is inhibited. Also, too low a concentration of osmoticum leads to multinucleate protoplasts owing to spontaneous fusion of two or more protoplasts during the isolation procedure (41, 42, 43).
The 'one-step' enzymatic isolation procedure was used to isolate the protoplasts. In this procedure, all the three different types of enzymes (pectinases, cellulases and pectolyases) were mixed in a solution containing an osmotic stabilizer. Protoplasts isolated from solution I (TABLE 3) disintegrated after 5 days. So in order to reduce the destructive effect of higher concentrations of enzyme on protoplast viability, the concentrations were reduced in subsequent solutions used for protoplast generation.

The freshly isolated protoplasts were bright, small in size, spherical and densely cytoplasmic (FIG. 11).

**Purification of isolated protoplasts**

The isolated protoplasts in the incubation mixtures are present in the media together with a range of cell debris and broken cell organelles. The crude protoplast suspension was decanted into a conical tip centrifuge tube and centrifuged at low speed of 100 g for 5 minutes. Under these conditions, the intact protoplasts form a soft pellet in the tip of the tube. The supernatant, containing the broken cells and debris can be pippetted off. The pellet was washed several times in enzyme free wash solution to get a relatively clean protoplast preparation.
Figure 6: Seeds germinated on half-strength MS media gave rise to seedlings in 3-4 weeks.

(a) 4 week old seedling

(b) 6 week old seedling
Figure 7: Structural formulae of some auxins and cytokinins. Auxins include
(a) indole-3yl-acetic acid, (b) α-naphthalaeneacetic acid, (c) 2, 4-
(b) dichlorophtenoyxacetic acid. Cytokinin activity is shown by (d) adenine, (e)
kinetin, (f) trans-zeatin.

(a) \[ \text{Auxin: } \text{CH}_2\text{COOH} \]

(b) \[ \text{Auxin: } \text{CH}_2\text{COOH} \]

(c) \[ \text{Auxin: } \text{OCH}_2\text{COOH} \]

(d) \[ \text{Cytokinin: } \text{NH}_2 \]

(e) \[ \text{Cytokinin: } \text{HN-CH=CH=CH}_2\text{COOH} \]

(f) \[ \text{Cytokinin: } \text{HN-CH}_2\text{-CH}=\text{CCH}_3\text{CH}_2\text{OH} \]
Figure 8: Dark green, compact type callus.
Figure 9: Friable white callus.
Figure 10: Actively growing young cell suspension cultures chosen for protoplast isolation.
Figure 11: Freshly isolated protoplasts. These are small in size, spherical and densely cytoplasmic.

Figure 12: Protoplasts after a 24 hour culture period in the liquid.
Figure 13: First divisions in the cultured protoplast seen three to ten days after plating.

Figure 14: Four celled clumps found after the seventh day.
Figure 15: Two week protoplast colonies in the culture medium.
Protoplast viability testing

Protoplast viability testing was done 0.01% FDA. As the FDA accumulates within the plasma membrane viable protoplasts fluoresce green/white (FIG. 9). Protoplast preparation treated with FDA must be observed within 5-15 minutes because after this time the FDA dissociates from the membrane.

Protoplasts culture

The protoplasts were allowed to regenerate a new cell wall either in liquid culture before embedding or were directly embedded after isolation. The agarose embedding method is the classical method of Nagate and Takebe (1971) in which isolated protoplasts were mixed with 1.0% agar/culture medium maintained at 40-45 °C. Small amounts of the agar (liquid)/protoplasts mixture were then poured into sterile petriplates. The isolated protoplasts thus became fixed in a position in the solidified agar and could be routinely monitored by microscopic analysis. The protoplasts in culture began to show new wall synthesis and deposition within a few hours. Complete synthesis of the wall required two days to two weeks.

After twenty hour culture in liquid, some protoplasts became elongated (FIG. 12), indicating formation of cell wall. First divisions were seen 3-10 days after plating (FIG. 13). By the seventh day, 4 cell clumps were found (FIG. 14). By two weeks in culture, small colonies were formed (FIG. 15), many of which were visible to the naked eye.
The regeneration of the cell wall is the first of a series of critical events that need to take place if the whole totipotential sequence of plant regeneration from a single cell is to be achieved. Without regeneration of the wall it is not possible for the cell to undergo some of the next steps in the chain of events; that is, synthesis of nucleic acid, replication of chromosomes, mitosis and cell division. In some systems DNA synthesis and mitosis may take place without division, thus forming multinucleate protoplasts; but, these multinucleate cells never been regenerate a whole plant (44).

The protoplast generation system developed from our experiments was stable and can be used for transformation experiments of Albizia lebbeck (L.) Benth.
REFERENCES


44. Bhargava, S., Upadyaya, S., Garg, K., Chandra, N., Differentiation of shoot buds in hypocotyl explants and callus cultures of some legumes. 431-433.
