ONE

INTRODUCTION
General Introduction to Plant Biotechnology

Since the evolution of mankind plants have been known for food, flavor, fragrance and therapeutic significance. Plants generate many secondary metabolites through biosynthetic routes enabling the assembly of wide range of complex compounds, which are used as drug molecule, or plant derived compound for synthesis of drugs in pharmaceutical, perfumery, insecticide and flavoring industries. Table- 1 shows different species studied so far for phytochemical and biological significance.¹

Table -1: Showing phytochemical and biological importance of plants.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Number based on Biological activity</th>
<th>Number based on phytochemical entity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocots</td>
<td>1283</td>
<td>3721</td>
</tr>
<tr>
<td>Dicots</td>
<td>11924</td>
<td>31126</td>
</tr>
<tr>
<td>Gymnosperms</td>
<td>239</td>
<td>638</td>
</tr>
<tr>
<td>Pteridophytes</td>
<td>349</td>
<td>961</td>
</tr>
<tr>
<td>Bryophytes</td>
<td>39</td>
<td>457</td>
</tr>
<tr>
<td>Lichens</td>
<td>118</td>
<td>625</td>
</tr>
</tbody>
</table>

Plant biotechnology gives an alternative route for production of therapeutically significant secondary plant metabolites by exploiting biosynthetic pathways and other techniques like embryogenesis, plant regeneration, micropropogation, immobilization elicitation, differentiation and more recently transgenic cultures.

Biotechnology is the name given to a very wide range of agricultural, industrial and medical technologies that make use of living organisms or there parts for modification and improvement of these biological systems be it a plant, animal, micro organism or cell in culture.

Plants being the primarily positioned in all food chain and secondarily the only renewable energy source play a key role to life on earth. Plant biotechnology can play a major role to cope up with all requirements.
Introduction

Plant tissue culture is the cultivation of plant cells and tissues aseptically on specially formulated growth medium in appropriate conditions to regenerate a plant or biomass of undifferentiated tissues. These can be initiated from parenchymatous tissues of shoots, root and other plant structures. They are broadly divided into static cultures and suspension cultures.2

Static culture: Cell or tissue of a plant are cultured on solid basal medium (Murashiage and Skoog, Gamborg's (B5) medium, Wood and Braun, Nitsh and Nitsh, Whites medium etc.), supplemented with various growth hormones (Auxins: Indole acetic acid, indole benzyl adenine, naphthyl acetic acid, 2- 4, dichlorophenoxy acetic acid, Cytokinins: Kinetin, BAP, zeatin, adenine, Gibberelins: GA3, Abscissic acid and ethylene). These phytohormones in various concentrations and combination are used to initiate callus, shoot, root or whole regenerated plant production.

Suspension culture: Here the division of cell is encouraged in liquid growth media. Several form of suspension culture commonly used are:

Batch suspension: This system is a closed system where growth of cells directly depends on medium left. First cells inoculated in the medium undergo lag phase then they follow exponential growth and in the last stationary phase is reached at which component of medium necessary for growth are reached. The growth recommences when cells are transferred to a fresh medium or medium is replaced.

Semi continuous cultures
This is an open system here medium is replaced periodically with a fresh medium and cultures are removed simultaneously by which growth of culture is continuously maintained.

Continuous cultures
There are two types of open continuous cultures

- Chemostat: The volume of culture remains constant and steady state is maintained by limiting the nutrient particularly necessary for growth.
Turbostat: The volume of culture is maintained by withdrawing culture continuously to maintain certain level of turbidity.

Use of Plant Tissue Culture Techniques for Production of Secondary Metabolites

Plants produce a wide range of secondary metabolites which are of great importance for production of color, fragrance, antioxidant, flavor, dyes, insecticide, pheromones and above all therapeutically active constituents. Each individual cell has its inherent capacity to produce secondary metabolites this can be manipulated to produce high yielding or new metabolites of great importance to the mankind. High yielding plant cell lines can be established and preserved. Plant cells in culture can also give higher yields as compared to natural plants. High yielding plant cell culture and new secondary metabolites produced in cell cultures are shown in Table- 2 and Table- 3.

Table -2: High yielding plant cell cultures

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Metabolites</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberis wilsonia</td>
<td>Lattrohizin</td>
<td>10.0</td>
</tr>
<tr>
<td>Coellis blumei</td>
<td>Rosemarinic Acid</td>
<td>21.4</td>
</tr>
<tr>
<td>Coptis Japonica</td>
<td>Berberine</td>
<td>15.0</td>
</tr>
<tr>
<td>Lithospermum erythrorhizon</td>
<td>Shikonin</td>
<td>12.4</td>
</tr>
<tr>
<td>Morinda Citrifolia</td>
<td>Berberine</td>
<td>10.0</td>
</tr>
<tr>
<td>Dioscorea deltoide</td>
<td>Diosgenin</td>
<td>7.0</td>
</tr>
<tr>
<td>Nicotiana tobaccum</td>
<td>Nicotine</td>
<td>50.0</td>
</tr>
<tr>
<td>Catharensus roseus</td>
<td>Serpentine</td>
<td>2.2</td>
</tr>
</tbody>
</table>
The purpose of immobilization is to bring together as many cells as possible in production units to create a continuous process, characterized by decoupling growth and secondary metabolite production without loss of biomass. This fixation on surface allows recycling, protection of cells from mechanical stress and better control of microenvironment. Table -4 shows increased production of secondary metabolites in some plant cells on immobilization.

Types of immobilizations

- Direct intercellular binding due to adhesion, adsorption and agglutination
- Covalent coupling in otherwise inert matrices
- Intercellular connection via crosslinking
- Mixing with suitable materials changing their consistency with temperature
- Purely physical retention within the framework of diverse pore size.
Introduction

Table - 4: Shows enhanced secondary products released upon immobilization.

<table>
<thead>
<tr>
<th>Species</th>
<th>Biotransformation Reaction</th>
<th>Matrix</th>
<th>Supplied</th>
<th>Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daucus carota</td>
<td></td>
<td>Alginate</td>
<td>Gitoxigenin</td>
<td>5β hydroxy gitoxigenin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Digitoxigenin</td>
<td>5β hydroxyl digitoxigenin</td>
</tr>
<tr>
<td>Digitalis lanata</td>
<td></td>
<td>Alginate</td>
<td>β methyl digitoxin</td>
<td>β methyl digoxin</td>
</tr>
<tr>
<td>Mucuna pruriens</td>
<td></td>
<td>Alginate</td>
<td>L tyrosin</td>
<td>L Dopa</td>
</tr>
<tr>
<td>P. Somniferum</td>
<td></td>
<td>Alginate</td>
<td>Codeinone</td>
<td>Codeine</td>
</tr>
</tbody>
</table>

De Nova Synthesis

<table>
<thead>
<tr>
<th>Species</th>
<th>Matrix</th>
<th>Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaranthus tricolor</td>
<td>Chitosan</td>
<td>Oxalate</td>
</tr>
<tr>
<td>Asclepias syriaca</td>
<td>Chitosan</td>
<td>Protease</td>
</tr>
<tr>
<td>Capsicum frutescens</td>
<td>Polyurethane</td>
<td>Capsacin</td>
</tr>
<tr>
<td>Glycyrrhiza echinata</td>
<td>Alginate</td>
<td>Echinatin</td>
</tr>
<tr>
<td>Thalictrum minus</td>
<td>Alginate</td>
<td>Berberine</td>
</tr>
</tbody>
</table>

Biotransformation

The production of valuable plant secondary metabolites from cheap precursors by biotransformation is another useful technique in plant tissue culture. Some commercially viable biochemical conversions are shown in Table- 5.

Table- 5: Commercially viable biotransformations

<table>
<thead>
<tr>
<th>Species</th>
<th>Reaction</th>
<th>Precursors</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anethum graveolens</td>
<td>Hydroxylation</td>
<td>Agroclavine</td>
<td>C-8 compounds</td>
</tr>
<tr>
<td>Cannabis sativa</td>
<td>Oxidation</td>
<td>Geraniol</td>
<td>Nerol</td>
</tr>
<tr>
<td>Datura spp</td>
<td>Esterification</td>
<td>Tropine</td>
<td>acetyltropine</td>
</tr>
<tr>
<td>Datura innoxia</td>
<td>β- D glucosylation</td>
<td>Hydroxyquinone</td>
<td>Arbutin</td>
</tr>
<tr>
<td>Digitalis</td>
<td>Hydroxylation</td>
<td>β methyl digitoxin</td>
<td>β methyl digoxin</td>
</tr>
<tr>
<td>Digitalis lanata</td>
<td>Glucosylation</td>
<td>Gitoxigenin</td>
<td>Gitoxin</td>
</tr>
</tbody>
</table>
**Introduction**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Reaction</th>
<th>Product</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Menthe spp</em></td>
<td>Reduction</td>
<td>(-) Menthone</td>
<td>(+) neomenthone</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Reduction</td>
<td>Testosterone</td>
<td>Androsterone</td>
</tr>
<tr>
<td><em>Ruta graveolens</em></td>
<td>Epoxidation</td>
<td>Hydroxyl coumarin</td>
<td>Furano coumarin</td>
</tr>
</tbody>
</table>

**Induced Secondary Plant metabolite production in cell cultures**

Secondary plant metabolite production can be induced by the use of elicitors or genetic modification.

**Elicitors**

It is now known that large number of secondary plant metabolites are phytoalexins and are produced when plant cells are subjected to stress conditions like physical, chemical stimuli and microbiological infections. When plant cell cultures are subjected to such elicitors some genes are depressed resulting in production of secondary metabolites that are found in entire plant. Following are some elicitors:

- **Colchicine** colchicines acts as elicitor when induced in medium of cell suspension culture of *Valerina Wallichii*. There is six folds increment in production of valepotriates.
- **Yeast, yeast extracts and carbohydrates** induced four fold increment in berberine in *Thalictrum rugosum*. Rosmarinic acid production was stimulated in *Orthosiphon aristatus*.
- **Copper sulphate** shikoin production is increased by the use of copper sulphate in *lithospermum erythrosinum*.
- **Fungal Mycelia** coumarin production is stimulated in *Pimpinella anisum*.
- **Thiosalicylic acid** promotes biosynthesis of sterols in *Panax ginseng*.

**Genetic Engineering**

The controlled modification of genetic material by artificial means is genetic engineering. This technique relies on the ability to isolate specific stretches of DNA at precise locations using various enzymes. Selected DNA fragments can then be transferred into plant cells this can be done in several ways:
**Introduction**

*Agrobacterium* mediated transformation:

The best way of gene transfer is the use of gram-negative soil bacterium *Agrobacterium*. These bacterium have tendency to introduce their plasmids portions or T- DNA into plant cell and use of plant cells for production of carbon and nitrogen source for *Agrobacterium*. This kind of host- parasite relationship is described as genetic colonization. They have natural ability to alter the genetic material of plant cells so that outgrowths like hairy roots and crown galls are formed. Biologists have adapted the mechanism of *Agrobacterium* to transfer desired genetic information into plant cells by the use of these Ti and Ri plasmids in *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. These plasmids can be used to transfer desired information into plant cells for better and relevant production of plant metabolites.

**Biolistic impregnation**

An unlikely sounding method that has achieved some success with cereals and other crops. It involves sticking the DNA to be introduced into the plant onto minute gold and tungsten particles, then firing these like bullets into plant tissues. A portion of the plant cells treated in this way take up the DNA from metal pellets these can be separated from rest by the use of marker genes.

**Protoplasts**

These are the plant cells from which cell wall has been removed this makes it easier to get DNA into them, either by treatment of chemicals or use of high voltages as:

1. **Electroporation**: Micro to milliseconds pulses of strong electric field causes minute pores to appear momentarily in plant cells allowing DNA to enter from surroundings.

2. **Microscopic Crystals**: A more recent method uses microscopic crystals to puncture holes in plant cells allowing DNA to enter them.
3. **Microinjections**: microinjection involves the direct injection of DNA material into host cell using finely drawn micropipette needle. DNA can be injected in chloroplasts which have their own DNA. Chloroplast DNA is usually found in female parts of plant and not in pollen so plants transformed by this way cannot transfer their introduced genes through pollens.

With all current gene transfer techniques only a small proportion of treated cells successfully incorporate the novel DNA. Therefore so-called marker genes are usually linked with the genes to be transferred. These are classified as:

**Reporter Genes:**
A reporter gene codes for enzyme or other protein which can be quantified using a biological assay. The most widely used reporter gene in *E. coli* transfer is *gus* gene coding for β-glucuronidase. The spectrofluorimetric assay and histochemical assay be used for determination of GUS activity. Similarly *cat* and *luc* genes coding for chloramphenicol acetyltransferase and firefly luciferase respectively, can also be used in plants for accurate assays of gene expressions.

**Selectable Markers:**
This gene gives an ability to transformant to grow in presence of selective agent or selection media which is otherwise toxic to untransformed cells. Most widely used selectable marker are Kanamycin resistance gene, sulfonamide resistance gene, bialaphos resistance gene, streptomycin resistance gene etc.

**Phenotypic Markers:**
Phenotypic marker give transformant distinct features which can be identified phenotypically for example *thaumatin II* gene has been used as simple marker to confirm transformed genotype by intensely sweet taste in potatoes. Others are anthocyanin regulatory genes giving pigmented phenotype to transformed cells.
Introduction

By the use of these genes transformed cells can be separated from untransformed. A population of transformed cells can thus be raised separating them from untransformed ones.

Genetic modification of plant using various techniques of biotechnology involves:

- Inserting individual genes that have been isolated in plant laboratories inside plant genome
- Separating the transformed population of cells
- Checking that inserted gene work as expected

Antisense Technology:
It is used to neutralize the action of undesirable genes. The technique can be used to combat the activity of plant viruses providing a means of controlling viral infections. Many of the current applications of plant biotechnology depend on antisense genes.

Techniques for Genetic Transformation of Plant Cell Tissues and Their Cultures

*Agrobacterium* induced gene transformation:
*Agrobacterium* are gram negative rods that belong to bacterial family *Rhizobaceae*. They are classified according to their phytopathogenic characteristics as *A. tumefaciens* which induce crown gall disease, *A. rhizogenes* which induces hairy root disease and *A. radiobacter* which is a virulent. *A. tumefaciens* is capable of transferring a defined piece of DNA (T- DNA) containing tumorigenic loci from its tumour inducing Ti plasmid into the genome of larger number of angiosperms and gymnosperms. This process requires cis acting T- DNA border sequence and trans acting (*vir*) function encoded by Ti plasmid and *Agrobacterium* chromosome. The transfer process is fully active when the *vir* function and the T-DNA are located on separate compatible replicons in *Agrobacterium*. Similarly in *A. rhizogenes* instead of Ti plasmid there is Ri plasmid that causes copious rooting. In nature these soil harboring bacteria cause crown gall and hairy root disease.
however the Ti and Ri plasmids can be genetically modified to add desired characteristics to the genes of the plants this is achieved as

- Introduction of plasmid into *Agrobacterium*
- Infecting the plant cells with these *Agrobacterium*.

The Ti plasmid

Ti (T-DNA) contains a number of genes:

- two of these are *tms 1* and *tms 2* involved in synthesis of auxins
- one gene *tmr* involved in synthesis of cytokinins

These are under control of eukaryote-like gene promoter and gene control sequences. The result of transformation with Ti plasmid is 'crown gall disease' a rapid proliferation of callus at the point of infection due to increased auxin and cytokinin levels produced by pant cells in response to introduced T-DNA.

The Ri Plasmid

The Ri plasmid is similar to Ti plasmid

- Loci that are homologous to *tms 1* and *tms 2*
- Second set of genes called *rol* genes.

These genes seem to increase the sensitivity of plant to auxin’s. This results in rapid growth of roots and gives rise to ‘hairy root disease.’ Some advantages of Ri mediated *Agrobacterium* transformations are:

1. Ri transformed roots of many plant species grow faster than untransformed roots and yield greater concentration of product than untransformed roots.
2. Recent research suggests that tropane alkaloids are produced at growing region of roots hence the number of root meristem will influence the productivity of cultures
3. Hairy root tend to be highly branched unlike untransformed roots.
4. There is increased rate of hairy root formation.
5. The roots are generally good materials for biochemical experimentation. They take up labeled compound and their biochemistry is relatively simple as they do not photosynthesize.

6. The function of foreign gene can be studied in roots it is sufficient to introduce the gene into root.

7. Ri mediated transformed plants show phenotypical visual evidence for the presence of Ri T- DNA in them so selection and screening are built in.

8. The transformed cells producing roots can be used to study biogenetic pathways.

Some internationally important strains of *Agrobacterium* used for transformation are given in Table- 6.
Table - 6: Shows some international strains of *Agrobacterium* used for transformation

<table>
<thead>
<tr>
<th><em>Agrobacterium</em> strain</th>
<th>Plasmid content</th>
<th>Type strain</th>
<th>source*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. rhizogenes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>pArA4a, pArA4b, pArA4c</td>
<td>Agropine</td>
<td>L</td>
</tr>
<tr>
<td>15834</td>
<td>pAr15834a, pAr15834b, pAr15834c</td>
<td>Agropine</td>
<td>J</td>
</tr>
<tr>
<td>15834</td>
<td>Par15834</td>
<td>Contains only one plasmid</td>
<td>P</td>
</tr>
<tr>
<td>8196</td>
<td>pAr8196a, pAr8196b, pAr8196c</td>
<td>Mannopine</td>
<td>J</td>
</tr>
<tr>
<td>TR7</td>
<td>pArTR7a, pArTR7b, pArTR7c</td>
<td>Mannopine</td>
<td>J</td>
</tr>
<tr>
<td>TR101</td>
<td>pArTR101a, pArTR101b, pArTR101c</td>
<td>Mannopine</td>
<td>J</td>
</tr>
<tr>
<td>11325</td>
<td>Not characterized</td>
<td>Nopaline</td>
<td>J</td>
</tr>
<tr>
<td>HR1</td>
<td>pArHR1</td>
<td>Agropine</td>
<td>O</td>
</tr>
<tr>
<td><strong>A. tumefaciens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C58</td>
<td>pAtC58</td>
<td>Nopaline</td>
<td>JS</td>
</tr>
<tr>
<td>C58C1</td>
<td>pAtC58</td>
<td>Derivative of C58 resistant to rifampicin and streptomycin</td>
<td>JS</td>
</tr>
</tbody>
</table>

*L; L.W Moore, Plant pathology department, Oregon state university, Corvallis, Ore.97331, J; J.A.Lippincott, Dept. of Biological science, Northwestern university, evaston III, 60201, USA. JS; J. Schell Max Plank Institute, D- 5000 Koln*
Introduction of Plasmid into \textit{Agrobacterium}

Plasmids can be introduced into \textit{Agrobacterium} either by conjugation or by transformation. Transformation is limited to only few strains and is less efficient technique. Conjugation process is most commonly used to transfer plasmids into \textit{Agrobacterium}. So far only plasmids of \textit{E.coli} group are known to be stably maintained by \textit{Agrobacterium}. These plasmids which are transferred into \textit{Agrobacterium} are normally carrying marker genes also to identify transformations. Many \textit{Agrobacterium} show only a very low or no spontaneous mutation frequency towards resistance for rifampicin, streptomycin, kanamycin and carbenecillin. Spontaneous streptomycine and tetracycline resistant mutants are often generated at a higher frequency. Therefore markers of \( \text{Sp}^R \) and \( \text{Km}^R \) are most suitable for selection of plasmid transfer to \textit{Agrobacterium}.

In contrast to direct DNA uptake methods of plant transformation that require plant cell protoplasts, \textit{Agrobacterium} is capable of infecting intact cells. As a result tissue culture limitations are much less of a problem in \textit{Agrobacterium} based transformation systems. Transformed cells can be regenerated more rapidly and more often when plant tissues are transformed directly without protoplast isolation. \textit{Agrobacterium} is also capable of transferring large fragments of DNA very efficiently without substantial rearrangement direct DNA uptake methods tend to induce rearrangements of introduced DNA within transformed plants. The strategy for development of Ti plasmid rests on two factors of T- DNA that is transferred from \textit{Agrobacterium} and stably inserted into plant genome.

1. The T- DNA is bounded by two directly repeating sequence of 25 base pair, called border sequence, that are essential for the transfer to occur.
2. None of the gene located on T- DNA are responsible for transfer of this DNA.

However many plasmid vectors cannot replicate in \textit{Agrobacterium}. They can be maintained in \textit{Agrobacterium} after co integration with \textit{Agrobacterium} replicon.
Gene Replacement

In many cases it is desirable to introduce particular mutation gene into *Agrobacterium* genome or a region of foreign DNA into the genome. Here the gene is first cloned in *E. Coli*.

- The mutation of choice is introduced into cloned sequence of DNA
- Transfer the mutated clone to *Agrobacterium*
- Screen for introduction of mutation into *Agrobacterium* genome.

Construction of plasmid vectors

- Binary vector

In such type of system, the *Agrobacterium* host strain contains a wild type Ti plasmid or disarmed Ti plasmid (from which tumor gene / hairy root has been depleted) that carries vir functions and serves as a helper. The T DNA borders are located on compatible replicons that are stable and functional in both *Agrobacterium* and *E.coli*. This system is always a system of choice because the in-vitro manipulation of small binary vector is much simpler than in vivo or vitro manipulation of intact Ti mega plasmid whereas integrative vector require more complicated in vivo manipulation of binary vector step that is time consuming and laborious.

Following the in vitro manipulations of binary vector with *E. coli* as host, the desired *Agrobacterium* host is is simply transformed with crude DNA preparation. The structure of plasmid is verified by alkaline lysis quick screen procedure.

As the properties of one vector may be quite different from the other so one must be cautious while choosing binary vector. The most important feature of binary vector is its host range, one can readily test a number of bacterial chromosomal background and helper Ti plasmid combinations to determine best one for infection in particular plant.
Vectors

Cosmid vector: 11
In addition to normal vector they contain, col El origin of replication and a fragment containing the bacteriophage lamda cos site required for in vitro packaging. Upto about 140 kb of DNA can be inserted into this cosmid vector.

Promoter analysis vector: 10
In addition to general features of binary vector system the promoter analysis vector contains the coding sequence for the tn9 chloromphenicol acetyltransferase gene (cat) upstream of T- DNA 6b transcript terminator of octopine- type Ti plasmid. Upstream of the coding region is the 39 bp polylinker containing 7 unique cloning sites. Immediately next to it the promoter of lac operon from E. coli
The useful plant promoters can be inserted into the cloning sites within the polylinker between the lac promoter and cat coding sequence. Sensitivity to chloramphenicol is used as screen for insertion during cloning in E. coli.

Upstream element analysis vector: 12
This vector is useful for functional analysis of upstream elements of plant promoters. The plasmid contains 5' truncated fragments of the constitutive nos promoter. These promoter fragments are linked in functional orientation with respect to reporter gene.

Expression vectors: 12
This is a vector which contains in addition to general element in binary vector, the DNA fragment carrying the transcript 7 and 5 terminators of octopine type Ti plasmid fragment containing fully functional nos promoter.
The polylinker sequence containing 6 restriction sites is located between the plant promoter and terminator.
Cointegrating vectors

This system requires intermediate plasmids that are used for cloning and analysis in *E. coli* and are subsequently introduced into *Agrobacterium* cells where they recombine with resident Ti plasmid to give a co-integrate that contains hybrid T-DNA.

Leaf disc transformation

This system presents efficient gene transfer, selection and regeneration to be coupled together in a simple process. Surface sterilized leaf discs of plants are inoculated with *Agrobacterium* for 48 hours. The leaf discs develop infection they are then transferred into selection medium.

Triparental mating

It involves the use of *A. tumefaciens*, which contains the Ti plasmid harbouring the virulence gene responsible for plant transformation. The recombinant plasmid is present in one *E. coli* strain and a conjugation-proficient ‘helper’ plasmid in another. When the three strains are mixed the helper plasmid transfers to the strain carrying the recombinant plasmid which is then transferred to *Agrobacterium*.

Freeze thaw method

Once a desired molecule is constructed in *E. coli*, the molecule can be transferred into *Agrobacterium* by freeze and thaw method. The transformation frequency is low by this method (approximately 10^3 transformant / μg DNA). Compared to triparental mating the technique is reliable and very rapid plus it also eliminates much of plasmid rearrangement that occurs during triparental mating.
**Introduction**

*Agrobacterium* plasmid quick screen

This technique is based on alkaline lysis procedure and allows the structure of many *Agrobacterium* transformants to be verified quite rapidly.

**Plant transformations**

Many co-cultivation procedures are now available for plant transformation whether protoplast, suspension cell, tissue pieces or even imbibed seeds are employed depends largely on transformation efficiency and tissue culture capabilities of plant under study.

**Suspension cell transformation**

This is an efficient technique and has been reliably demonstrated in a *N. tabacum* suspension cell line. A related procedure has also been successfully developed to transform *N. plumbaginifolia* and *P. hybrida* cell clumps quite efficiently. Preinduction of *Agrobacterium vir* genes with acetylsyringone and initial culture of transformed tissues under non-selective condition may improve transformation efficiency in most cell lines.

**Co-cultivation with protoplast**

Freshly isolated protoplasts are cocultivated with *Agrobacterium* washed and then cultured in an antibiotic-containing medium.

**Disadvantages with *Agrobacterium* induced transformation**

- There must be a reasonable match between plant genome and that of bacterium both for DNA transfer to occur and for Gene encoded on T-DNA to function.
- The plant should always be in receptive state which is very difficult variable to control.
- *Agrobacterium* are known to infect mostly dicots and to bring infection to monocots is rather difficult.
Selectable and Screenable Markers in *Agrobacterium* Mediated Transformations

**Selectable markers**

**Neomycin phosphotransferase:**
The gene responsible for this enzyme is *nptII*. Neomycin phosphotransferase inactivates aminoglycoside antibiotics like kanamycin, geneticin etc by phosphorylation. These genes are useful as selectable markers in various plant species including Solanaceae (*Nicotiana* species, *lycopersicum esculustum, solanum tuberosum*), *Medicago sativa*, Brassicca spp., *Zea mays*, *Triticum monococcum*, *Secale cereale*, *Glycine max* and *Oryza sativa.*

**Hygromicin phosphotransferase:**
Hygromycin Phosphotransferase (*hpt*) is a suitable selectable marker in both plants and animals. Only disadvantage in using hygromycin in selection medium is that leaf material turns dark brown.

**Hormone biosynthetic gene derived from T- DNA:**
Three genes of T- DNA are responsible for hormone autotrophic growth gene 2 codes for an enzyme that converts indol-3- acetamide (IAM) to indol-3- acetic acid (IAA). So a, medium containing IAM or naphthalene-2- acetamide (NAM) can be used for positive selection.

**Screenable markers**

**Chloramphenicol acetyl transferase:**
This marker has been extensively used to analyze and regulate the activity of plant promoters. In most plant cells CAT activity is easy to detect and identify.
Opine synthesis genes

The opines are complex amino acids that are coded by T-DNA of Ri and Ti plasmids.\textsuperscript{19, 20, 21} These opines are used as energy source by \textit{Agrobacterium}. These can however be detected by paper electrophoresis method.

Opine biosynthetic genes encoded by different Ri and Ti plasmids are as;

\textit{oos}: octopine synthase, that catalyses the condensation between several amino acids (e.g., arginine, lysine, histidine and ornithine) and pyruvate.

\textit{nos}: nopaline synthase, that catalyses the condensation between arginine or ornithine and a-ketoglutarate.

\textit{mas}: mannopine synthase, that catalyses the reductive conjugation between glutamine and mannose.

\textit{ags}: agropine synthase, that catalyses lactonisation of mannopine

\textit{acs}: agrocinopine synthase, responsible for production of phosphorylated sugar derivatives.

The assays for opines are rather simple and detectable in all plant tissues in which the genes have been introduced.

General layout for \textit{Agrobacterium} infection induction in plant cells

\begin{itemize}
  \item First the explants are surface sterilised
  \item Normally wound attracts the \textit{Agrobacterium} infections so leaf discs are made or protoplasts are used. Acetosyringone is released during wounding a plant this can however be added in medium.
  \item The wounded explants are added to liquid \textit{Agrobacterium} medium or inoculum is added on the wounded surface.
  \item The explant is left for contact for contact time of 2-48 hours for effective infection.
  \item This explant is then transferred into antibiotic (carbenecillin) containing medium.
  \item The callus is raised and maintained on antibiotic medium.
  \item The callus cells are transferred in selection medium to kill untransformed cells.
  \item The selected transformed cells are then maintained on antibiotic free selection medium.
\end{itemize}
Use of Plant viruses for transformation

Most plants are subjected to viral infections, so viral infection can be exploited for genetic transformation of plants. This can simply be achieved by rubbing virus directly on the leaf surface. There are wide classes of viruses that infect the plant but only a few are of concern since most of the viruses have RNA as genomes rather than DNA. RNA viruses are not so useful because manipulations with RNA are very difficult to carry out.

Two classes of viruses that are used for transformation purpose are

- Caulimovirus vector
- Germiniviruses

Caulimovirus vectors:
The caulimovirus was used in 1984 for the first time to clone a new gene into turnip plant. However the use of this virus as cloning vectors has some limitations

- The total size of Caulimovirus genome is very small even after the removal of non-essential sections of the virus genome the capacity to carry the inserted DNA is less.
- The host range of this virus is very narrow. This restricts the cloning experiments to only few plants mainly brassicas.

The use of cauliflower mosaic virus (CaMV) genome that lacks several essential genome, and provides more space for large inserted genomes can be used to solve the first limitation problem. CaMV cannot by itself cause infection. Plants are inoculated with the vector DNA along with normal CaMV genome. The normal viral genome provides the information for cloning vector to be packaged.

Caulimovirus are used as highly active promoters to obtain expression of gene introduced by Ti plasmid cloning or direct gene transfer experiments.
Geminivirus vectors:
The natural hosts of these plants are maize and wheat they can be used to transform monocots. Only limitation that makes it an unsuitable vehicle for transformation is

- The genome of geminivirus undergoes rearrangement and deletions during the infection cycle. This can scramble the inserted DNA of interest.

Direct Gene Transfer
This gene transfer makes use of super coiled plasmid DNA possibly a simple bacterial plasmid pBR322, into which an appropriate selectable marker and gene to be cloned has been inserted. Biolistics is frequently used to introduce the plasmid DNA into plant embryos

When species that are being engineered can be regenerated from plant protoplasts or plant cells more efficient techniques other than biolistics can be used

- One method is suspending protoplast in viscous solution of polyethylene glycol, a negatively charged polymeric compound that is thought to precipitate DNA onto the surface of protoplasm and to induce uptake by endocytosis
- Protoplast can also be fused with DNA containing liposomes
- Intact cell can be vigorously shaken with DNA-coated silica needles, which penetrate the cell walls and transfer DNA into the interior.

Boillistic impregnation
This has become the second most widely used vehicle for plant genetic transformation after Agrobacterium system of genetic transformation. The microscopic metal particles coated with genetically engineered DNA are explosively accelerated into plant cells. The gene gun consists of specimen holder, vacuum chamber, apparatus positioner and vacuum pump

The plasmid particle mixture: The plasmid particle mixture is first made for this; metal particles are first autoclaved and suspended in ethanol and mixed well by vortex, centrifuged and to this DNA solution of specified amount is added. To this solution CaCl₂ and spermidine are added and incubated at cold temperature with wet ice. Supernatant of this solution is taken to bombard the plant cells.
The tissue is first clamped in specimen holder with magnets. A calculated amount of DNA / particle mixture is screened into a vacuum jar through plastic filter holder. The vacuum jar is then placed on the base and evacuated at 90KPa.

Vacuum Infiltration and Floral Dip

In this technique plants are uprooted at early flowering stage and *A. tumafaciens* are applied to intact plant by using vacuum. This is achieved by immersion of whole plant in liquid growth medium containing *Agrobacterium*, sucrose and surfactant. The assessment of transformant in the progeny is made by selection using antibiotic. This procedure has so far been successfully carried out in *Arabidopsis thaliana*. This technique is successful with only few species. Recently with *medicago sp.* the repeated dipping of plant and covering infected plant has shown two folds increase in induction of transformation.

Protoplast Fusion

Mechanical or enzymatic removal of cell wall leaving protoplast bounded with cell membrane at suitable conditions regenerate the entire plant or cell cultures.

Two operations that facilitate protoplast isolation are

- Subjecting the plant to slight drying to induce incipient leaf wilting
- Plasmolysing detached leaves in 13 % (w / v) mannitol for about 4- 5 minutes prior to enzymatic treatment

A variety of enzymes are available for isolation of single cell and protoplast from plants examples are cellulose, pectinase macerozyme, pectinase and hemicellulase. After removal of cell wall protoplast must be kept in a solution of right osmolality to prevent bursting of protoplasm.

Different approaches for insertion of transgenes into protoplast can be widely classified as

- Chemical technique
- Electrical technique
- Micro injections
**Chemical techniques**

1. Mixing the two protoplasts in 5.5% sodium nitrate in 10% sucrose solution.
2. Treating the protoplast with 0.05 M CaCl$_2$ at pH 10.5 for few minutes and then centrifuging them gently.
3. By exposing protoplast to 60% polyethylene glycol and shaking the mixture for few minutes.
4. Fusion induction by means of poly-L-ornithine, concanavalin A and cytochalasin B.

**Electrical technique:**
Micro to milliseconds pulses of strong electric field causes minute pores to appear momentarily in plant cells allowing DNA to enter from surroundings.

**Micro injection:**
Microinjection involves the direct injection of DNA material into host cell using finely drawn micropipette needle. DNA can be injected in chloroplasts which have their own DNA. Chloroplast DNA is usually found in female parts of plant and not in pollens so plants transformed by this way cannot transfer their introduced genes through pollens.

**Antisense Technology**
The gene to be cloned is ligated into vector in reverse orientation. The cloned gene is transcribed the RNA that is synthesized is reverse complement of mRNA produced from normal version of gene. This reverse complement is referred to as antisense RNA (asRNA). An antisense RNA can prevent the synthesis of the product of the gene it is directed against. It is an effective way of gene subtraction. The possible mechanism of action of antisense technology is that these asRNA hybridise with mRNA and this double stranded RNA is rapidly degraded with cellular ribonuclease. A best example of antisense technology at present is that of tomatoes and other soft fruits. These fruits are normally picked before they are fully ripe to be transported to market before they are fully ripe. The immature fruits do not develop their flavour till they are fully ripe so the results are fruits with bland taste. Polygalacturonase enzyme slowly breaks down
polygalacturonic acid component of cell wall in the fruit pericarp leading to softening of fruit. This is where the use of antisense technology can be done. This is done by cloning the antisense polygalacturonase gene. The result showed that less enzyme was formed in transformed plant hence less spoilage when they are transported fully ripe. Other examples are as shown in Table-7.

**Table- 7: Example where antisense technology is useful**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Modified characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polygalacturonase</td>
<td>Delays fruit ripening</td>
</tr>
<tr>
<td>Aminocyclopropane carboxylic acid synthase</td>
<td>Modified fruit ripening in tomatoes</td>
</tr>
<tr>
<td>Polyphenol oxidase</td>
<td>Discoloration prevention in fruits and vegetables</td>
</tr>
<tr>
<td>Starch synthase</td>
<td>Reduction of starch content in vegetables</td>
</tr>
<tr>
<td>Delta-12 desaturase</td>
<td>High oleic acid content in soybean</td>
</tr>
<tr>
<td>Chalcone synthase</td>
<td>Modification of flower color in various decorative plants.</td>
</tr>
</tbody>
</table>
Literature review on *Agrobacterium rhizogenes* transformed cultures

The literature review is divided into three parts:

- Showing metabolites with enhanced production of secondary metabolites
- Hairy root cultures showing new compound production
- Hairy root cultures used for various significant studies

Reports showing increased production of secondary plant metabolites

*Agrobacterium rhizogenes* is able to induce hairy root symptoms in large variety of dicot species. These hairy roots can be excised and cultivated indefinitely under sterile conditions and constitute interesting material for production of secondary plant metabolites of pharmaceutical value. They are usually able to produce same amount of compound that can be found in normal roots of the parent plants, without the loss of concentration which can be frequently observed in callus or cell suspension cultures. Compared to de differentiated cells hairy roots grow faster and are genetically stable. Many strategies can be employed to increase the production of secondary metabolites by hairy root cultures in bioreactors, including elicitation, use of permeabilization agents and a two liquid phase system.

Increase in production of valepotriates in *Valeriana wallichii*.\(^{25}\)

The hairy root cultures were tried on *Valeriana wallichii* using A4 and LBA 9402 strains. A4 appeared to be better strain than LBA 9402 in terms of rate and growth of hairy root formation. The valepotriate content from roots of 24 week old wild plant from the field was compared with the 12, 15 and 20 week old hairy root lines. The quantitative HPLC estimation of homodidrovalarate, didrovalarate, isovaleroxyhydroxydidrovaltrate and acevaltrate contents individually and totally (in terms of total valepotriates) showed 3.3 times increase in content for LBA 9402 and 2.0 times higher for A4 induced hairy root lines, than that of control roots.
Artemisinin\textsuperscript{26, 27}\n
Artemisinin is an antimalarial isolated from leaves of \textit{Artemisia annua}. Using a medium containing sucrose (70 g / l), nitrates (30 mM), inorganic phosphate (1.5 mM), giberrelic acid (5 mg / l) and a ratio of 1:5 \( \text{NH}_4^+ : \text{NO}_3^- \) and use of \textit{Aspergillus oryzae} 3762 showed an increase upto 550 mg / l of artemisinin. Hairy root cultures of \textit{A. annua} in modified inner loop airlift bioreactor to promote artimisin production. Under suitable conditions highest production reached upto 577.5 mg / l

Diosgenin\textsuperscript{28}

The hairy root lines were established for \textit{Trigonella foenum-graceum} using A4 \textit{Agrobacterium rhizogenes} strain in different medium. The best results were obtained in half McCown's woody plant medium with 1 % sucrose (0.04 %) which was twice the amount detected in 8- months old non transformed roots (0.024 %). The addition of 40 mg / L chitosan elevated diosgenin level to three times in hairy root cultures.

Cycloartane saponins in \textit{Astragalus mongolicus}\textsuperscript{29}

The sterile grown seedlings of \textit{A. mongolicus} were transformed using different strains of \textit{Agrobacterium rhizogenes} (LBA 9402, ATCC 15834, R 1601 And TR 105). The transformed roots grew best in Murashiaige and Skoog medim without ammonium nitrate and plant growth regulators. Saponin production was increased when sitosterol was added in culture medium.

Saponins from \textit{Gynostemma pentaphyllum}\textsuperscript{30}

\textit{A. rhizogenes} strain R 1600 was used to produce hairy roots from \textit{G. pentaphyllum}. Saponin content in hairy roots was twice that in the roots.

Scopolamine from \textit{Dubosia leichhardtii}\textsuperscript{31}

Hairy roots were induced and inoculated in leaf disc and stem segments of \textit{Dubosia leichhardtii} with \textit{A. Rhizogene} strain 15834 and A4. Fourty five hairy root clones derived from individual meristems were established by subculturing hairy roots for at least five passages in growth regulator modified Heller's (HF) medium. Clone DL- 34 which
produced the most scopolamine was established from selection of number of hairy root clones. After 4 week of culture in double strength HF medium at 25° C in dark, its weight had increased 64 folds and scopolamine content was 1.8 % DW twice the amount present in leaves of D. leichhardtii. The double strength HF medium was best for growth of DL-34 and single strength was best for scopolamine production.

Scopolamine from *Datura candida*[^12]

Hairy root culture were obtained following inoculation of stems of sterile plantlet with *A. rhizogenes*. The scopolamine and hyoscyamine contents were quantified by HPLC and compared with non transformed plants. Total alkaloid content in hairy roots was 2.6 times the root of parent plant. The scopolamine / hyoscyamine ratio was 5:1 which makes it worth of consideration as a source of scopolamine.

Ginsenosides from *Panax ginseng*[^33]

The *P. ginseng* plant was infected with *A. rhizogenes* to produce crown galls. The hairy roots generated from the crown gall when crown gall were incubated on solid MS medium. The hairy root contained higher amounts of adenosine and guanosine than in intact plant.

Glycyrrhizin from *Glycyrrhiza uralensis*[^33]

Transformed hairy root cultures of *G. uralensis* were obtained by inoculation with *A. rhizogenes* either using aseptically grown plantlets or excised plant stems. One of the liquid culture line produced glycyrrhizin with an yield of 4.7 %.

**Tropane alkaloids**[^34, 35, 36, 37, 38, 39, 40]

Six clones of *D. stramonium* hairy roots were obtained by transformation of plant by *A. rhizogenes*. Hairy roots grown in 1 % sucrose became green and showed higher amount of tropane alkaloids than clones grown in dark.

In another report hairy root cultures were established following infection of *D. stramonium* with *A. rhizogenes*. The transformed cultures grown in absence of growth
regulators showed 55 fold increases in cell mass in 28 days. The hyocyamine production showed growth during the first 15 days of incubation.

Hairy roots were also produced by infecting seedlings of *D. stramonium* and cultured on MS medium without growth regulators. Several strains of *D. stramonium* were tried which varied in scopolamine production from nil to 0.56 %, they produced no hyocyamine, only a single strain of *Hyoscyamus niger* produced hyocyamine at a level (0.07%) comparable to found in vivo.

Another study where *Duboisia leichhardtii* was transformed with *A. rhizogenes* strain HRI, one month after the inoculation many roots were seen at the point of inoculation. These roots were designated as AR-4. The scopolamine content of AR-4 was lower whereas hyocyamine content was higher than the normal plant.

Hairy root clones of *Scopolia japonica* were established by selecting adventitious root segments inoculated with *A. rhizogenes* strain 15834. Two clones S 1 and S 22 were selected for their higher contents of scopolamine and hyoscyamine respectively. The weight of root tissues increased 40 folds in heller medium supplemented with 0.078mM Fe-NaEDTA instead of ferric chloride. Maximum scopolamine content was obtained after 4 weeks of Heller's medium supplemented with casamino acid instead of NaNO₃, with pH 7.0. For S 22, Schenk and Hildebrandt medium with pH 7.0 instead of 5.8 gave an increase of 102 fold in weight after 4 weeks. Hyocyanin production was optimum in Knop medium reaching 1.3 % after 4 weeks.

A comparative study of alkaloid production was done for adventitious and hairy root cultures of various species. The highest content of tropane alkaloid was obtained in adventitious roots of *Hyocyamus albus* cultured in hormone free half strength MS medium. On the other hand the hairy root of *H. albus* transformed with MAFF 03-01724 *A. rhizogene* strain grew very fast and yielded 5 mg hyoscyamine / 100 ml flask within 22 days.

The growth and hyocyamine production of transformed roots were also examined in modified 14 litre stirred tank reactor in both batch and fermentation on media containing half and full strength Gamborg's B5 salt and at three different temperatures (25°, 30° and 35° C). The roots grown on half strength B5 salt with 3% sucrose had higher dry matter content and higher hyocyamine content up to 0.52 mg / g wet weight than roots grown in
full strength B5 with same level of sucrose. Growth at 30° C was initially faster than any other temperatures, the drained weight of roots in fermenters was about four folds greater than at 25° C and twice that at 35° C. The ultimate hyocyamus level attained were similar at both 25° and 30° but some 40% lower at 35°. In continuous fermentation at 25°, the release of hyocyanine into culture media was low but was 7 folds higher in fermentors operated at 30° or 35°.

Cardenolides in *Digitalis lanata* ⁴¹
The five clones of *D. lanata* were established using *A. rhizogenes* A4 strain. Hairy root cultures in dark accumulated very small amount of cardenolides whereas the content of green hairy roots cultured in light was about 600 folds higher (16.5 µg/g DW). The cardenolide estimation was done by competitive ELISA technique using digoxin specific antibody.

Polyphenol production in *Fragaria x ananassa* ⁴²
Hairy roots of strawberry induced with *A. rhizogenes* ATCC 15834, grew well in hormone free MS medium and Gamborg B5 liquid media. In MS medium hairy roots showed best growth and produced high concentration of polyphenols especially (+)-catechin and procyanidin B-3.

Anthocyanins from root cultures of *Leontopodium alpinum* ⁴³
Five hairy root lines of *L. alpinum* were induced by infection with *A. rhizogenes*. The transformed roots were grown as batch cultures in phytohormone free modified MS medium. A time course experiment with most productive cell lines showed that a culture period of 6 weeks was optimum for biomass production yielding a 70 fold increase in fresh weight. A 70% enhancement of anthocyanin formation was induced by addition of Benzyl adenine to culture media 14 days before harvest.

Plumbagin from *Plumbago zeylanicum* ⁴⁴
A maximum of 3.5 ± 0.5 shoots were produced from a single nodal segment of a four year old field grown plant after four week of transfer to MS basal medium supplemented
with 8.87 m mol/ L BAP 0.49 m mol/ L IBA. Optimum root induction response was achieved upon transferring to half strength MS medium containing 0.49 mmol / L IBA. Hairy roots generated at 0.9 ± 0.05 relative transformation frequencies with the A4 strain of *A. rhizogenes* containing 4 % sucrose. Growth kinetic studies revealed a maximum of 21 fold increase in biomass yield after 6 weeks of culture. The fresh hairy roots produced 2.5 times higher amount of plumbagin than the fresh untransformed control roots of the same age.

**Naphthaquinone from *Sessamum indicum***

2- Isopropenynaphthazarin-2, 3-epoxide was isolated from invitro cultures of hairy roots of *Sessamum indicum*. The content of compound in roots was over 50 times that found in mother plant.

Table - 8, Shows some important phytoconstituents which are reported with increased production through hairy root cultures.

**Table- 8: Enhanced production of secondary metabolites in hairy root cultures**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Family</th>
<th>Increased Metabolite</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Valeriana wallichii</em></td>
<td>Valirianaceae</td>
<td>Valepotriates</td>
<td>25</td>
</tr>
<tr>
<td><em>Artemisia annua</em></td>
<td>Asteracea</td>
<td>Artemisin</td>
<td>26,27</td>
</tr>
<tr>
<td><em>Trigonella foenumgraceum</em></td>
<td>Papilionoideae</td>
<td>Diosgenin</td>
<td>28</td>
</tr>
<tr>
<td><em>Astragalus mongolicus</em></td>
<td>Papilionoideae</td>
<td>Saponins</td>
<td>30</td>
</tr>
<tr>
<td><em>Dubosia leichhardtii</em></td>
<td>Orchidacea</td>
<td>Scopolamine</td>
<td>31</td>
</tr>
<tr>
<td><em>Datura candida</em></td>
<td>Solanaceae</td>
<td>Scopolamine</td>
<td>32</td>
</tr>
<tr>
<td><em>Panax ginseng</em></td>
<td>Araliaceae</td>
<td>Adenosine, Guanamine</td>
<td>33</td>
</tr>
<tr>
<td><em>Glycyrrhiza uralensis</em></td>
<td>Leguminaseae</td>
<td>Glycyrrhin</td>
<td>33</td>
</tr>
<tr>
<td><em>Datura stramonium</em></td>
<td>Solanaceae</td>
<td>Tropane alkaloids</td>
<td>34,35</td>
</tr>
<tr>
<td><em>Scopolia japonica</em></td>
<td>Solanaceae</td>
<td>Scopolamine</td>
<td>38</td>
</tr>
</tbody>
</table>
New Compounds from Hairy Root Cultures

Two new flavonoids from *Glycyrrhiza glabra*\(^{46}\)

Two new prenylated flavonoids, licogrochalcone -A and licoagrocarpin were isolated from the hairy root cultures of *G. glabra* along with 8 known flavonoids. On the basis of spectroscopic evidence new compounds elucidated were 3- prenyl-2', 4, 4' - trihydroxylchalcone and (6aR, 11aR) - 4- prenyl -3- hydroxyl -9- methoxypterocarpan.

A new prenylated biauroline\(^ {46}\), licoagrone along with five known flavonoids Kan zonol, D,afrormosin, odoratin, phaeoseol and echinatin were also isolated from hairy root cultures.

Five new indole alkaloids from *Catharanthus trichophyllus*\(^ {47}\)

Five new indole alkaloids of yohimbine type base were isolated from hairy root cultures of *C. trichophyllus*. Anthranerspine and dimethoxyanthranerspine were found to contain the 11 methoxy epiallo- yohimbine skeleton, esterified at C-18 with 2- acetaaminobenzoic zcid and 2- acetaamido-4, 5- dimethoxybenzoic acid. Three other congeneres of the same series were isolated in trace amount.
Phenyl glucoside from *Swertia japonicum*\(^{48}\)

Two new phenyl glucosides 5- (3'-glucosyl) benzoyloxygentisic acid and 2,6-dimethoxy-4-hydroxyphenol 1-glucoside were isolated together with 1-sinapoyl glucoside from the hairy root cultures of *S. japonicum*.

**Polyacetylene compounds from *Lobelia inflate*\(^{49}\)**

The hairy roots of *L. inflate* were induced by direct infection with *A. rhizogenes*. Two new polyacetylene compounds, lobetyolin and lobetyol were isolated from lyophilized cells which had been cultured in hormone free MS liquid medium for 6 weeks in dark.

**Five new compounds from *Panax pseudoginseng*\(^{50}\)**

Five new compounds: three esters and two glycosides were isolated as biotransformation products of digitoxigenin by hairy root cultures of *P. pseudoginseng* transformed with *A. rhizogenes*. Biotransformation involving esterification of stearic acid, palmitic acid, myristic acid and lauric acid and glycosylation of gentibiose and sophrose were reported. The new esters were as Digitoxigenin stearate, digitoxigenin palmitate and digitoxigenin myristate.

Two new glycosides were

3 epidigitoxigenin beta-D-gentiobioside and digitoxigenin beta-D-sophoroside.

Seven previously reported compounds were also isolated.

**Glucosides from *Lobelia sessilifolia*\(^ {51}\)**

Two new glucosides (−)− epiafzelechin 7-O- beta glucopyranoside and protocatechuic acid 3- O-beta-D-glucopyranoside were isolated from hairy roots of *Lobelia sessilifolia* transformed by *A. rhizogenes* after incubation with (−)-epicatechin or protocatechuic acid.

**Two new anthraquinones from *Sesamum indicum*\(^ {45}\)**

Two new anthraquinone 2(4-methyl-1,3-pentadienyl) anthraquinone and 2 (4-methyl-3-pentadienyl) anthraquinone were isolated from *A. rhizogenes* ATCC 15834 transformed cultures of *S. indicum*.
New astragaloside from *Astragalus membranaceus*\(^{52}\)

In Chinese system of medicine *A. membranaceus* is used as antiperspirant, a diuretic and tonic. A new astragaloside named agroastragaloside I, and four known astragalosides (acetyl astragaloside I, II, III and IV) were isolated from hairy root cultures of *A. membranaceus*.

Unique quinine from hairy root cultures of *Lithospermum erythrorhizon*\(^{53}\)

Unique colorless quinine, named rhizonone, showing strong antifungal activity was isolated from hairy root cultures of *L. erythrorhizon* in MS medium. The structure elucidated on the basis of spectroscopic data was 5a- methyl-5a, 10b, 10c-tetrahydro-2H-anthra [9,1 - bc] furan-7, 10 dione. This unique quinine was not previously known among the compound isolated from this plant species.

Three new flavonoids from *Glycyrrhiza pallidiflora*\(^{54}\)

Three new flavonoids named licoagrosides D, E and F together with 4 known flavonoids medicarpin 3- O- glucoside, Calycosin 7 – O- glucoside, formononentin 7- O-( 6" malonylglucoside) and 2' hydroxyformononetin 7- O-glucoside were isolated from *G. pallidiflora*.

New alkaloids from *Rauwolfia serpentine*\(^{55,56}\)

Three new monoterpenoid indole alkaloids, 19 (S), 20(R)-dihydroperaksine (1), 19 (S), 20 (R)- dihydroperaksine-17-aI (2) and 10 hydroxy 19 (S), 20 (R) - dihydroperaksine (3) were isolated from hairy root cultures of *R. serpentine* along with 16 known alkaloids. A new monoterpenoid indole alkaloid 10- hydroxy- N (alpha)- demethyl-19, 20-dehydroraumacline of raumacline group was isolated as E- and Z- mixture isomer from hairy root culture of *R. serpentine Benth. Ex Kurz*

Table -9, shows some important new compounds isolated from some hairy root cultures.
### Table -9: New compounds isolated from hairy root culture

<table>
<thead>
<tr>
<th>Plant</th>
<th>Family</th>
<th>Compound type</th>
<th>Compound</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhiza glabra</td>
<td>Fabaceae</td>
<td>Flavonoids</td>
<td>3-prenyl 2',4,4'-trihydroxychalcone (6αR,11αR)-4-prenyl-3-hydroxy-9-methoxypterocarpan</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>licoagrodione</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prenylated biaurone (licoagrone)</td>
<td>58</td>
</tr>
<tr>
<td>Catharanthus trichophyllis</td>
<td>Apocynaceae</td>
<td>Epiallo-yohimbine derivatives</td>
<td>Anthraserpine, Dimethoxyanthraserpine</td>
<td>47</td>
</tr>
<tr>
<td>Swertia japonica</td>
<td>Gentinaceae</td>
<td>Phenyl glucosides</td>
<td>5-(3'-glucosyl)benzoyl oxygentisic acid 2,6-dimethoxy-4-hydroxyphenol1-glucoside</td>
<td>48</td>
</tr>
<tr>
<td>Lobelia inflate</td>
<td>Campanulaceae</td>
<td>Polyacetylene compounds</td>
<td>Lobetyolin, Lobetyl</td>
<td>49</td>
</tr>
<tr>
<td>Panax pseudoginseng</td>
<td>Araliaceae</td>
<td>3 esters 2 glycosides</td>
<td>Digitoxigenin stearate, Digitoxigenin palmitate Digitoxigenin myristate 3-epidigitoxigenin beta-D-gentiobioside digitoxigenin beta-D-sophoroside</td>
<td>50</td>
</tr>
<tr>
<td>Lobelia sessilifolia</td>
<td>Campanulaceae</td>
<td>Glucosides</td>
<td>(-)-epiaflzelechin 7-O-beta-D-glucopyranoside protocatechinacid 3-O-</td>
<td>51</td>
</tr>
<tr>
<td>Plant</td>
<td>Family</td>
<td>Compound/Alkaloid</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------</td>
<td>--------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><em>Sesamum indicum</em></td>
<td>Pedaliaceae</td>
<td>Beta-D-glucopyranoside</td>
<td>2(4-methyl-1,3-pentadienyl) anthraquinone 2(4-methyl-3-pentenyl)anthraquinone</td>
<td></td>
</tr>
<tr>
<td><em>Astragalus membranaceus</em></td>
<td>Fabaceae</td>
<td>Astragaloside</td>
<td>Astragaloside 1</td>
<td></td>
</tr>
<tr>
<td><em>Lithospermum erythrorhizon</em></td>
<td>Boraginaceae</td>
<td>Quinine</td>
<td>Rhizonone (very strong antifungal)</td>
<td></td>
</tr>
<tr>
<td><em>Glycyrrhiza pallidiflora</em></td>
<td>Fabaceae</td>
<td>Flavonoids</td>
<td>Licoagaroside D,E &amp; F</td>
<td></td>
</tr>
<tr>
<td><em>Rauvolfia serpentine</em></td>
<td>Apocynaceae</td>
<td>Monoterpenes</td>
<td>19(S),20(R)-dihydroxperaksine, 19(S),20(R)dihydroxyperaksine-17-al, 10-hydroxyl-19(S), 20(R) dihydroperaksine,</td>
<td></td>
</tr>
<tr>
<td><em>R. serpentine</em></td>
<td>Apocyanaceae</td>
<td>Monoterpenoid indol alkaloid</td>
<td>10-hydroxyl-N (alpha) demethyl19,20-dehydroraumacline.</td>
<td></td>
</tr>
</tbody>
</table>

### Some Significant Studies on Hairy Root Cultures

**Labdane alkaloids from *Coelous forskohlii***

Three main alkaloids isolated from the hairy roots of *C. forskohlii* were 1, 9-dideoxyforskolin, forskolin and coleol. The transformation was brought about by using *A. rhizogenes* pRi 15834.

It was studied and observed that MS medium favored forskholin and coleol formation and B5 medium enhanced 1,9-dideoxyforskolin formation.
Hairy root cultures of *Digitalis Lanata* \(^6\)

The leaves of axenically grown shoots of *D. lanata* were infected with various strains of microorganisms. Hairy roots were successful with only 3 strains all agropine type bacteria. The plants were regenerated from hairy roots via somatic embryos and shoots. Hairy roots and transgenic plants were examined for cardenolide contents.

No cardenolides were detected in hairy roots. Anthraquinones and flavonoids were shown to occur the contents of these compounds were higher in hairy roots than in untransformed roots. Shoots and regenerated plants showed cardenolide content similar to untransformed shoots and plants.

Roots of untransformed and transformed plant contained cardenolides that were probably formed in shoots and transported into the roots.

Production of cycloartane saponins \(^29\)

The sterile grown seedlings of *A. mongolicus* were transformed using different strains of *A. rhizogenes* (LBA 9402, ATCC 15834 R 1601 and TR 105). Different strains influenced the growth rate, saponin production and ratio of astragaloside.

Transformed roots grew best in MS medium free from ammonium nitrate and without plant growth regulators.

Saponin production was increased when sitosterol was added in culture media.

Alkaloid production in *Atropa belladonna* \(^51\)

After inoculation of stems of sterile plants of *A. belladona* with *A. rhizogenes* the roots were formed root tips, were removed and transferred to a medium without carbenecillin. The fresh weight of root increased 60 folds in a month. After one month in liquid medium the atropine and scopolamine content was found comparable to that of normal plant. The atropine and scopolamine were produced continuously in hairy roots. This clearly indicated that tropane alkaloid synthesis is closely related to root differentiation and hairy roots can provide better alternative for large scale production of these alkaloids.
Introduction

Steviol glucosides from *Stevia rebaudiana*  
The leaves of *S. rebaudiana* contain two glycosides stevioside and rebaudiana which are used as natural sweeteners, but the site of synthesis of these glycosides was not clear. Steviolbioside, the precursor of stevioside was produced in hairy root cultures developed specially to study the site of synthesis of stevioside. However stevioside was not found in roots. High concentration of sucrose was seen to increase steviolbioside level in roots. Callusing of hairy root was seen on addition of 30 μM kinetin into the media.

Scopolamine production by root cultures of *Duboisia myoporoides* 
The scopolamine content was seen to decrease in hairy root culture established by infecting cultured shoots with *A. rhizogenes* strain HRI. However the hyoscyamine content increased as compared to non transformed roots.

Tropine alkaloids from *Scopolia japonica* 
Hairy root clones of *S. japonica* were established by selecting adventitious roots formed on root segments inoculated with *A. rhizogenes* 15834. Two clones S1 and S22 were selected for high content of tropine alkaloids. For S1 the optimum growth medium was Heller’s supplemented with 0.078mM FeNaEDTA instead of FeCl₃, after 4 weeks in darkness root tissue increased 40 folds. Maximum scopolamine content was seen in Heller medium supplemented with 1 % casamino acid instead of NaNO₃. For S22 Schenk- Hildebrandt medium with pH 7.0 instead of 5.8 Showed 102 fold increase in weight.

Diterpenoid production in *Salvia miltiorrhiza* 
*S. miltiorrhiza* is used to treat haematological abnormalities and cardiovascular disease in China. The sterile grown plants were infected with various strains of *A. bacterium*. Diterpene production was highest in absence of ammonium nitrate.

High biotransformation potential of ginseng hairy root cultures 
*Panax pseudoginseng* established by transformation with Ri plasmid of *A. rhizogenes* had a higher potential to biotransformation.
(RS)-2-phenylpropionic acid to (RS)-2-phenylpropionyl beta-D-glucopyranoside (71 % conversion ratio), (2RS)-O-(2-phenylpropionyl)-D glucose (8%), (2S)-2-phenylpropionyl 6-O-beta-D-xylopyranosyl-beta-D-glucopyranoside (10 %) and (2R)-2-phenylpropionic acid (5 %).

Valepotriates from Centranthus rubber

The hairy root cultures of C. ruber were established by infection of stems from in vitro grown seedlings with A. rhizogenes strain R1601. Transformed roots were grown in twelve liquid hormone free media and valtrate, iso valtrate, 7-desisovaleroyl-7-acetylvaltrate, 7-homovaltrate, didrovaltrate and isovaleroxyhydroxydiodrovaltrate were quantified by HPLC. The highest total valepotriate content were found in half strength B5 medium which was same as that found in roots of non transformed control plants grown in field. Hairy root cultures in half B5 liquid medium supplemented with 3 % sucrose for 45 days produced over 31 mg / g dry weight valepotriate.

Catharanthine and ajmalicine synthesis in Catharanthus roseus hairy root cultures

The changes in the culture medium and the addition of biotic elicitors (from Aspergillus spp., Trichoderma viride, T. reseeii and Rhodotorula mariana) and hydrolytic enzymes were tested for their ability to increase alkaloidal yield. The medium component containing 4.5 % sucrose showed a 45% increase in the yield of ajmalacine as compared to those cultured in 4 % sucrose containing media. Bio elicitors showed no response to alkaloidal content. However addition of macro enzyme methyl jasmonate increased the yield of both alkaloids.

Coumarin production from Cichorium intybus

The effect of putrescine on the growth and production of two coumarins esculin and esculetin in the hairy roots of chicory was examined. Putrescine treatment at 1.5 mM produced 1.9 folds increase in the growth of hairy roots as well as production of two alkaloids.
Introduction

Genetic Engineering for the production of alkaloids in *Cinchona officinalis 'Ledgeriana'*. The hairy root cultures of *C. officinalis* were initiated containing constitutive expression construct of cDNAs encoding the enzymes tryptophan decarboxylase (TDC) and striccosidine synthase (STR) from *Catharanthus roseus*, two key enzymes in terpenoid indole and quinoline alkaloid synthesis. The successful integration of these two genes along with *gus* gene as a marker was demonstrated using southern blotting PCR. Quinine and quinidine levels were found to rise up to 500 and 1000 μg/l dry weight respectively.

Improvement of artemisin accumulation in hairy root cultures of *Artemisia annua*. The effect of fungal elicitor, derived from mycelial extract of *Penicillium chrysogenum* 3446 on artemisin production in hairy root cultures of *A. annua* was studied. It was seen artemisinin production in 21 days in hairy root cultures of *A. annua* treated with 0.3 mg total sugar/ml medium elicitor for 3 days reached 549.1 mg/L.

Enhancement of scopolamine production in *Hyoscyamus muticus*. 3535 S- h6h transgene that codes for the enzyme hyoscyamine-6 beta-hydrolase was introduced into the *Hyoscyamus muticus* strain Cairo. This plant was chosen for its ability to produce high amounts of tropane alkaloids. Great variation was observed in tropane alkaloid production among 43 positive transformants. The best clone produced 17 mg scopolamine/L which was over 100 times more than control clones. The expression of h6h was proportional to scopolamine production and was the main reason behind the variation in scopolamine/hyoscyamine ratio in hairy root clones.

Essential oils from *Achillea millefolium*. The essential oil isolated from the roots of two Portuguese *A. millefolium* populations (BGL and CGA) and from two hairy root cultures (A4 and LBA) derived by transforming BGL with *A. rhizogenes* were analyzed by GC and GC–MS. Compared on the DW basis the yield from the hairy root cultures of essential oil was comparable or higher than that from the plant roots.
Ginsenoside production by Hairy root cultures of *Panax ginseng*. Hairy roots of *P. ginseng* were obtained by infecting roots stem and leaves of the plant with *P. ginseng* on half strength MS medium supplemented with 300 mg of cefotaxim sodium / litre. The selected root lines were propagated in 5 L bioreactor supplemented with 2.0 mg NAA and 30 mg sucrose / L. To increase the ginsenoside content Jasmonic acid in various concentrations was added after 30 days of culture and root were cultured for 7 more days until harvest. Total ginsenoside increased with increasing concentration of jasmonic acid, but higher concentrations inhibited the growth of roots. Ginsenoside production was greatest at 2.0 mg jasmonic acid / L.

Volatile compounds from hairy roots of *Cichorium intybus*. Hairy root cultures of *Cichorium intybus* produced volatile compounds under the influence of fungal elicitors. It was observed that intensity of volatile oil reached the maximum on 21st day with 10 ml / 1 media filtrate of *Phytophthora parasitica* var nicotianana. The change in the morphology with cell wall and shear in hairy roots was also seen. The component of volatile oil was identified as propyl isovalerate, udecanal nonanol, isoamyl nonanoate and 2- decene- 1-ol.

Secreation of alkanin by hairy root cultures of *Arnebia hispidissima*. Transformed hairy root cultures of *A. hispidissima* cultured on half strength MS solid medium produced red pigments. However hairy root cultures in liquid medium produced a larger amount of these pigments, which were released in to the liquid medium. The addition of adsorbents like charcoal to the liquid culture medium stimulated the alkanin production by three folds. Liquid paraffin and sesame oil were suitable solvent for alkanin. The addition of 40ml / flask of liquid paraffin with varying concentrations of sucrose and boric acid elevated the level of alkanin 3 folds as compared to normal hairy roots.

Shikonin biosynthesis in hairy roots of *Lithospermum erythrorhizon*. 4- hydroxyl benzoate (4HB) is a precursor in the synthesis of shikonin. This precursor was modified in *L. erythrorhizon* hairy roots by the introduction of bacterial gene *ubiC*. 
This gene is in *E. coli* not normally present in plant and codes for an enzyme chorismate pyruvate-lyase (CPL) that converts chorismate to 4HB. The resulting hairy root cultures showed high CPL activity. The 4HB produced by CPL reaction was utilized for shikonin biosynthesis as shown by in vivo inhibition of native pathway to 4 HB with 2-aminoindan-2-phosphonic acid (AIP), an inhibitor of phenylalanine ammonia lyase. A feeding experiment with {1, 7 - 13C2} shikimate showed that in absence of AIP the artificially introduced CPL reaction contributed 20 % of overall 4HB biosynthesis in the transgenic cultures. *UbiC* transformation did not lead to statistically significant increase of shikonin formation.

**Elicitors in hairy root cultures**

The *Catharanthus roseus* hairy root cultures elicited with pectinase and jasmonic acid were studied. Pectinase showed a decrease in indol concentration as ratio of fresh weight to dry weight whereas jasmonic acid had no effect on this ratio. There was an increase of 150% in the specific yield of taberosine. The yield of taberosine the likely precursor of lochericine and horhamericine decreased with lower levels of jasmonic acid and increased with increased jasmonic acid concentration.

The *Panax ginseng* hairy root cultures were elicited with jasmonic acid and some other elicitors. Jasmonic acid in the concentrations of 1.0 – 5.0 mg/ l improved ginsenoside production.

The hairy root cultures of *Salvia miltiorrhiza* upon elicitation with yeast elicitors enhanced the production of both phenolic acids and tashinones.

The production of indigo in hairy root cultures of *Polygonum tinctorium* increased with the addition of 200mg chitosan / l and 20U pectinase.

The hairy root cultures of *Solanum tuberosum* the elicitor treated hairy root cultures showed an increase in production of sesquiterpene and lipoxygenase metabolites.

Table -10, records some significant studies on hairy root cultures.
Table 10: Some significant studies on hairy root culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Significant compound / study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleus forskohlii</td>
<td>Laminaceae</td>
<td>Labdane alkaloids</td>
<td>59</td>
</tr>
<tr>
<td>Digitalis lanata</td>
<td>Scrophulariaceae</td>
<td>No cardiacolides seen in hairy roots but an increased flavonoid, anthraquinone production</td>
<td>60</td>
</tr>
<tr>
<td>Atropa belladonna</td>
<td>Solanaceae</td>
<td>Atropine and scopolamine production was at par with normal plant</td>
<td>61</td>
</tr>
<tr>
<td>Duboisia myopoides</td>
<td>Solanaceae</td>
<td>Increased hyoscymine and reduced scopolamine production</td>
<td>37</td>
</tr>
<tr>
<td>Ruta graveolens</td>
<td>Rutaceae</td>
<td>S-adenosyl-l-methionine: 1, 3 dihydroxy N-methyl acridone a new enzyme from hairy root cultures.</td>
<td>81</td>
</tr>
<tr>
<td>Geranium thumberghi</td>
<td>Geraniaceae</td>
<td>9 tannins of comparable amount isolated</td>
<td>82</td>
</tr>
<tr>
<td>Panax pseudoginseng</td>
<td>Araliaceae</td>
<td>Hairy roots have higher potency for biotransformation of (RS)-2-phenylpropionic acid to beta-D-glucopyranoside</td>
<td>64</td>
</tr>
<tr>
<td>Centaranthus rubber</td>
<td>Valerianaceae</td>
<td>Valepotriates contents comparable to plant</td>
<td>65</td>
</tr>
<tr>
<td>Achillea millefolium</td>
<td>Asteraceae</td>
<td>Essential oil yield on dry weight was comparable.</td>
<td>71</td>
</tr>
<tr>
<td>Solanum laciniatum</td>
<td>Solanaceae</td>
<td>Solacidine content similar to normal plant</td>
<td>83</td>
</tr>
</tbody>
</table>
Some Significant *Agrobacterium tumefaciens* transformed cultures

**Transgenic cabbage resistant to insects**

Transgenic head cabbage resistant to diamond moth back was developed through *A. tumefaciens* mediated transformation with *Bacillus thuringensis* (Bt) cry genes using a modified procedure. Cabbage plant transformed with a synthetic Bt gene and cry1Ab3 gene were resistant to diamond back moth.

**Sugarcanes resistant to osmotic stress**

Tetrahalose synthase [maltose alpha-D-glucosyl transferase] gene (TSase) from *Grifola frondosa* was introduced into sugarcane by *A. tumefaciens* mediated transformation of callus after incubation produced highest frequency of phosphinothricine resistant plant reaching 4.5% on an average.

**Regeneration of herbicide resistant onion**

Transgenic onion plants tolerant to herbicide containing active ingredients glycophosate and phosphinothricin were recovered from immature embryos of open pollinated and hybrid parent onion lines at a maximum transformation frequency of 0.9%. Transformants of different onion cultivators thrived when sprayed with different herbicides.

**Berberine production in *Thalictrum flavum***

Thalictrum species contain quaternary protoberberine alkaloid berberine and quaternary aporphine alkaloid magnafluorine. Berberine stimulates GIT, depresses auricle and ventricles and dilates heart, whereas magnafluorine induces hypotension and exhibits ganglionic blocking effects. Transgenic *T. flavum* callus tissues were produced by infecting with *A. tumefaciens* oncogenic strains A281 and C58. The transformation process did not alter the berberine content in transgenic roots or cell culture.
Shikonin from crown gall

Crown gall tissue lines of *lithospermum erythrorhizon* were established by transformation with *A. tumefaciens* C58. The content of shikonin in crown gall is 1.8% (dry tissue). The crown gall tissue culture was a new method to produce shikonin.

Production of hG-CSF

The recombinant human granulocyte colony stimulating factor (hG-CSF) production was done through transgenic tobacco cell suspension culture. The hG-CSF gene was cloned with its own signal peptide from TPA stimulated cell line. The gene was subcloned into the plant expression vector, pMY27 and transformation of tobacco was conducted by *A. tumefaciens* harboring hG-CSF gene. Cell suspension culture of leaf derived calluses of transgenic tobacco were established. The biological activity of produced hG-CSF was confirmed. The maximum concentration of hG-CSF produced and secreted by tobacco suspension about 105 µg/L. Table -11, records *A. tumefaciens* transformed plants.

Table - I1: Shows some of transformed plants by *A. tumefaciens*

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiana plumbaginifolia</em></td>
<td>90</td>
</tr>
<tr>
<td>Petunia</td>
<td>91</td>
</tr>
<tr>
<td>Tobacco</td>
<td>92</td>
</tr>
<tr>
<td>Tomato</td>
<td>93</td>
</tr>
<tr>
<td>Potato</td>
<td>94</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>95</td>
</tr>
<tr>
<td><em>Medicago varia</em></td>
<td>96</td>
</tr>
<tr>
<td>Cotton</td>
<td>97</td>
</tr>
</tbody>
</table>
Review on *Plumbago zeylanicum* linn.

**Family** – Plumbaginaceae  
**Genus** – Plumbago

**Common names of plant**

- English name: Whiteleadwort  
- Sanskrit name: Chitraka  
- Arabic: Shitraj  
- Bengali: Chitrak  
- Bombay: Chitra  
- Deccan: Chitaro  
- Gujarati: Bhigbartnde shitarak  
- Persian: Chitu  
- Nepal: Pai hua T’eng  
- Chinese: Chitalarki  
- Urdu.

**History**

In the Nighantus, there are passages that describe the herbs burning and acrid properties. Several European writers of Indian drugs have mentioned this plant.

**Habit**

*Plumbago zeylanicum* is one of the most important medicinal plants grown throughout India. It is a perennial, sub-scandent shrub, the leaves are ovate and glabrous, the flowers are white elongated spikes, found wild in India, West Bengal, Malay Peninsula, Ceylon and throughout Arabia and Western Africa. It is also cultivated as an ornamental plant.

This species is more widespread and common than *P. indica*. It contains 2n = 24 chromosomes in contrast to 2n = 1 in *P. indica* and other species.

**Morphology**

A perennial herb, sometimes, in shady places, subscandent. (Figure -1, Shows plant of *P. zeylanicum*)

**Stems**: 0.6-1.5 m. long somewhat woody,

**Leaves**: Are thin 3.8- 7.5 by 2.2 – 3.8 cm, ovate, subacute, entire, glabrous, somewhat glaucous beneath, reticulate veined, shortly and abruptly attenuated into a short petiole,  
**Petiole**: Narrow, amplexicaul at the base and often dilated into stipule-like auricles.
Figure 1: Plant of *Plumbago zeylanicum* Linn

Figure 2: Flowering shoot of *P. zeylanicum*
Flowers: Are elongated spikes, rachis glandular, striate, bracteoles ovate, acuminate, shorter than the calyx, glandular or not. (Figure -2, Shows flowering shoot of *P. zeylanicum*).  
Calyx : 1- 1.3 cm. long narrowly tubular, persistent, densely covered with stalked glands; teeths small with membranous margins.  
Corolla: white, slender, tube 2-2.5 cm. Lobes are long 8mm. Filaments as long as the corolla tube. Anthers exerted just beyond the throat. Capsule oblong. Pointed pericarp thin below, thick and hardened above.  
Roots: as sold in market are cylindrical pieces of varying length and less than 1.25 cm in thickness. The external color of root is light yellow when fresh and reddish brown when dried. Internal color is brown and striated. The surface is thick, smooth and irregularly fissured brittle bark marked here and there with small projections representing scars of rootlets. Roots have a short fracture, an acrid and biting taste and disagreeable odor.  

Anatomy  
In transverse section roots show a very small cork represented by 8- 10 layer of cells and very wide cortex. The size of the wood is medium. The phelloderm is a wide zone 1/2 to 2/5 of total root diameter. The cells are thick walled and polyhedral. The phelloderm cells contain starch. The cortical cells are polygonal, parenchymatous and also contain starch. The important feature of cortex is presence of many non lignified fibers scattered throughout the cortex in groups or chains. The wood is characterized by presence of numerous vessels of different sizes and is mainly composed of tracheids. The xylem rays are thin to moderately broad many in numbers. The walls of vessels and tracheids are pitted and the vessels generally have perforations.  
The powdered roots are olive green but appear green when mounted in nitrocellulose in fluorescent light.  
Plumbagin is distributed mostly in secondary cortex and medullary rays cells in roots as an amorphous yellow substance. The root powder also shows simple and compound starch grain.  
The following are microscopic and macroscopic characteristic of roots of *P. zeylanicum*.  

46
Macroscopical characters:

Color: Light yellow when fresh, reddish brown on drying
Texture: Uniform and smooth
Taste: Acrid and biting
Cortex: Cream to light yellow with numerous small scattered spots, central part is woody with lighter tinge.

Microscopic characters:

Phelloderm: Wide zone, cell thick walled and polyhedral
Phelloderm fibres: Lignified and simple pitted
Wood: ½ to 2/5 diameter of roots
Xylem fibres: Elongated and simple pits
Fibre tracheids: Absent
Medullary rays: Rays cells are simple pitted
Starch: Absent

The following are characters of powdered root:

Taste - Acrid, Odor - irritant
Microscopy - Both simple and compound starch grains are present. Simple grains are round and concentric. Compound grains are in group of 12-18. Diameter of starch grain is 2-16μ. Starch index - 42% specific number 8. Non lignified fibres present are linear with pointed ends. 100 – 200 μ, breadth 12-16 μ.

Cultivation

*Plumbago zeylanicum* is one of the most important plants grown throughout India. An experiment conducted during 1998-99 and 1999-2000 for optimizing planting dates of *P. Zeylanicum* for the maximum growth and root productivity. The results revealed that planting of *P. zeylanicum* in the months in the month of July is the best time because of better performance in respect of growth and yield parameters. Observation recorded at 8, 10 and 12 months after planting of *P. zeylanicum* produced maximum root yield of
880, 1187.20 and 1376.80 Kg/ha respectively than that of September and November planting. The plant height, number of branches, root fresh and dry weight was highest in July planting than that of other months planting. Leafing was enhanced by spraying of GA and number of days of flowering is decreased to 60 days.\(^{103}\)

**Chemical constituent**

*Plumbago zeylanicum* contain plumbagin as principal constituent. Plumbagin is a naphthoquinone closely related to juglone group. It was isolated from *P. europea* and assigned the formula \(C_{11}H_{8}O_3\) \(^{104}\)(2-methyl-5 hydroxyl, 4 naphthaquinone). The synthesis of plumbagin in the laboratory confirmed the position of methyl group to be 2. \(^{105}\) Thereafter it was investigated thoroughly by using MS \(^{106}\), IR \(^{107}\) and NMR techniques. The melting point of plumbagin is: 78 - 79⁰.

MS \((m/e)\): 189(28), 188 (100), 174(6), 173 (22), 160 (18), 145 (6), 132 (13), 131(18), 121(14), 120(18), 92 (14), 77(8), 64(6), 63(10), 51(6), 39(8).

UV \(\lambda_{max}\) (EtOH) 212, 266, 410, 423 with respective \(\log \epsilon\) 4.35, 3.92, 3.39 and 3.40.

IR \(v_{max}\) \((1\% \text{ in KBr})\): 1640, 1660 strong \((C = O)\), 1605 Strong \((C = C)\) and 3440 broad, weak \((\text{hydrogen bonded OH})\) / cm.

**Solubility:** High solubility in alcohol, acetone and other organic solvents.

**Storage:** Tends to deteriorate on storage under natural conditions.

**Plumbagin as marker in taxonomic classification** \(^{107}\)

55 species, representing ten of the 11 family of genera plumbaginaceae have been surveyed for flavonoids and other phenolic constituents in roots, leaves and flowers. The most important chemical marker which occur in roots of ten taxa examined of tribe Plumbaginaceae and which is uniformly absent from plant of tribe Staticeae.
Plumbagin is major constituent of roots and leaf of *P. zeylanicum*. The flowers petals contain mainly azaleatin 3 - Rhamnoside.

The biogenesis of plumbagin studied as early as 1971 revealed to occur from acetate. \[\text{CH}_3 \text{CO-CoA} + \text{CH}_3 \text{CO-CoA} \rightarrow \text{CH}_3 \text{CO-CoA} \]

Plumbagin is present in various parts of *P. zeylanicum* plant as

- Leaves: 1 mg / 100 g (Fresh weight)
- Green stems: 37.5 / 100 g (Fresh weight)
- Roots: 650 / 100 g (Fresh weight).

Plumbagin (I), 3- chloroplumbagin (II) and new substance 3, 3 biplumbagin (III) was isolated from the roots of *P. zeylanicum*. \(^{111}\)

New binaphthaquinone Chitranone(IV) along with Zeylinone (V), isozyelinone (VI), eliptinone and droserone were isolated from the roots later. \(^{112}\)

Catechol tannin, glucose and steroidal glycosides from the roots of *P. zeylanicum* were also reported along with main constituent plumbagin. \(^{113}\)

1,2 (3)- tetra hydro- 3, 3' biphumbagin (VII) was isolated from plant growing in Sri Lanka. \(^{114}\)

The flowers of plant contain azulein and 3- rhamnoside of delphinidin. \(^{115}\)
Review of literature

Plumbagin

3 chloroplumbagin

3-3'-biplumbagin

Chitranone

Zeylanone

Isozeylanone
1, 2 (3) - tetrahydro3, 3' biplumbagin.

VII
Pharmacology

This is one of the important species in Ayurveda. The root is used as abortifacient, appetizer, used in sore diseases, diarrhea, dyspepsia. A paste of root is used as external applicator in leprosy and other ailments. It is also used in influenza and black water fever. The root bark is used as tincture in antiperiodic. The milky juice of plant is used in scabies and ulcer.99

Plumbago contains about 1.25% of an orange colored pigment plumbagin. Plumbagin is an irritant, powder of plumbagin is germicide. Plumbagin stimulates muscle tissues in smaller and paralyses in larger doses. It includes contraction of muscle of heart and intestine. Plumbagin stimulates secretion of sweat and urine. Plumbagin is also nervine stimulant and anthelminthic. Plumbagin has antimicrobial activity against staphylococci, certain pathogenic fungi and parasitic protozoa. Antispasmodic activity of plumbagin is also well recorded. Plumbago is insecticidal particularly against Spondoptera exempta and Spondoptera littoralis. Plumbagin is also present in the species of Drosera (carnivorous species of Droseraceae).

In Nigeria, The leaves are used in soup as remedy against intestinal worms. In Ghana root is administered an enema. In Ivory coast the root is used to treat leprosy.

Plumbagin has been in use since old times for various ailments, its reference could be traced to the times of Charka and Sushitra.

The root extract of plumbago has been incorporated in large number of indigenous drug formulation. As many as 32 formulation contained root extract of P. zeylanicum in 1918-1920 this figure must have improved at least ten times to this figure by now.116

Plumbagin was isolated about 170 years back and is often considered synonymous in its uses with root extract of the plant.

A large number of Indian traditional medicines like Asokarishtam, Aswagandharishtam, Arogyavardhani, Dasamula talia, Karunkoduveri Majoon -E - Flasfa, Shilladhatri vatti and siddharmulam contain plumbago extracts.

The root extract of plumbago are incorporated in number of ayurvedic preparations like Livospin, Livrotrit, Livomyn, Livokin and Livin sold as hepatoprotective agents.117
The plant is described as "Deepen -Pachan- dravya" by Charka this itself tells the importance of the plant.

The extract of plumbago have multifarious pharmacological activity as abortifacient\textsuperscript{118}, antidiabetic\textsuperscript{119}, anti inflammatory\textsuperscript{120}, anti cancer\textsuperscript{121,122}, antifertility\textsuperscript{123,124,125}, anti cancer\textsuperscript{122} and anti parkinsons effect\textsuperscript{120}, antimicrobial\textsuperscript{126,127}, antirheumatism,\textsuperscript{128} anti snake bite\textsuperscript{129}, gastro intestinal flora normalizer\textsuperscript{130}, post partum hemorrhage antiseptic\textsuperscript{131} and veterinary medicines\textsuperscript{132,133}. It is used as a stimulant, adjunct to other preparations. It enters in numerous compound preparations. Small doses of it are a powerful stimulant of mucus membrane of digestive organs.

The metabolic effects extract of crude powdered drug and ethanolic extract of plumbago have been reported to increase blood sugar level and reduce nucleic acids and liver acid phosphotase\textsuperscript{134}. The methanolic extract of \textit{Plumbago zeylanicum} when administered IP to Proton albino rats 500 mg/ Kg showed 8.4 % decrease in overall following four serum parameters.

<table>
<thead>
<tr>
<th>SGOT</th>
<th>SGPT</th>
<th>SALP</th>
<th>SBHM</th>
<th>Overall Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>129.3 ± 11.43</td>
<td>146.9 ± 23.95</td>
<td>171.2 ± 11.19</td>
<td>4.9 ± 0.63</td>
<td>8.4 %</td>
</tr>
</tbody>
</table>

Rats treated with ethanolic extracts of roots of \textit{P. zeylanicum} showed a decrease in activities of following key role enzymes\textsuperscript{135}. Hexokinase, phosphofructokinase, private kinase and lactate dehydrogenase. The reduction in the activities of these key enzymes of glycolysis and its end product suggest a reduction in flux across glycolytic pathway. This in turn results in impaired delivery and utilization of glucose to peripheral tissues thus giving hypoglycaemic effect in extract treated rats.

\textbf{Metabolism of plumbagin}

The metabolism of plumbagin as studied by Chandrasekaran and Nagarajan\textsuperscript{136} observed the rapid metabolism in urine within 24 hours of its intramuscular administration.
Toxicology

No adverse effect is reported on use of this plant as a drug.

Tissue Culture Work on *Plumbago spp.*

*Plumbago spp.* contains plumbagin as the main constituent with strong anti-cancer, anti-fungal, antibacterial, and antimicrobial activity. In recent years, an approach has been made to increase the yield of plumbagin through some tissue culture works.

In one of the reports, chemical analysis of three different cell lines derived from stem explants of *P. zeylanicum* were maintained. These lines were designated as (Pz1) pale yellow, (Pz2) dark brown, and (Pz3) intense red on the basis of pigmentation in cell strains differing in their pigmentation in the second passage.

The plumbagin estimation was done by spectrophotometry at 415 nm, showing that Pz3 contained the highest content of plumbagin.

Multiple shoot formation in *P. rosea* has also been reported. The callus induction was done on MS medium supplemented with 2,4-D (2.5 ppm) and kinetin (1.5 ppm). Addition of 6 BA (2 ppm) and NAA (1 ppm) brought about shooting. Rooting was seen on transferring these shoots in medium supplemented with 6 BA (1 ppm). Regenerated plants thus formed were transferred to pots with 60% survival rate.

Rapid plant regeneration in the callus cultures derived from leaf and stem explants of *P. zeylanicum* has also been reported.

MS medium supplemented with 4.44 μM 6BA, 1.42 μM IAA, and 3% sucrose was used for induction of callus from stem and root explants. The shoot bud regeneration was positively correlated to concentration of growth regulators. The leaf explants were more efficient in shoot regeneration than stem explants in medium supplemented with 1.42 μM IAA and 4.44 μM 6BA. The regenerated plants were transferred to soil which grew with a survival rate of 90%.

A rapid and highly efficient method of micropropagation from vegetative shoots was also established for *P. zeylanicum*. Multiple roots were proliferated from nodal explants cultured on MS medium supplemented with 6 BA (0.25-1.0 mg/l). Excised microshoots cultured hormone-free medium rooted within 4-5 days. The 100% survival was seen by transferring the rooted shoots in the potting soil.
An efficient protocol\textsuperscript{141} was developed for in vitro clonal propagation of \textit{P. Zeylanicum} through nodal culture. Multiple shoots were induced from nodal explants of \textit{P. zeylanicum} on MS medium supplemented with 6 BA a (0.5- 0.1 mg / l) + IAA (0.01 mg / l). Rooting was readily achieved on transferring the shoots on Half strength MS medium supplemented with IBA (0.25 mg/ l) and 2 % sucrose. Micropropogated plants were successfully established in soil.

The root cultures of \textit{P. rosea} linn. were established from young leaf explant on Gamborg’s B5 (B5) medium\textsuperscript{142} supplemented with a Naphthalene acetic acid (NAA) + Kinetin in the concentration range of 0.5-2.0 mg/l and 0.1- 0.5 mg/l respectively. The production of plumbagin determined by TLC desitometry was higher (0.016 ± 0.003% DW) in cultured root obtained from B5 medium supplemented with NAA (1.0 mg/ l) +Kinetin (0.1 mg/ l). The variation in nitrogen [(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}] and sucrose concentration in B5 media also showed increase in plumbagin synthesis to 0.020 ± 0.0015 % and 0.023 ± 0.0017 respectively.

\textbf{Transformed cultures}

A protocol for rapid in vitro multiplication of \textit{P. zeylanicum} L. through axillary bud proliferation was developed as to conduct genetic transformation study.\textsuperscript{143} A maximum of 35 ± 0.5 shoots were produced from a single nodal segment of 4 year old field grown plant after 4 weeks of transfer to MS medium supplemented with BAP (8.87 mmol / l) + IBA (0.49 mmol / l). On transferring the individual regenerant to ½ strength MS medium with 0.49 mmol / l IBA optimum root induction was seen.

The hairy roots were induced by using A4 strain of \textit{Agrobacterium rhizogenes}. The optimum growth of hairy roots was seen in MS medium supplemented with ½ MS medium supplemented with 4% sucrose. Growth kinetic study revealed 21 fold increase in biomass in 6 weeks of culture.

The fresh hairy roots produced 2.5 times higher amount of plumbagin than the fresh untransformed roots of control and dried hairy roots of the same age.
Justification of the present research work

Plumbago zeylanicum is an important medicinal plant used in Indian system of medicine. It is important drug because of presence of plumbagin.

In the recent years attempt has been made to increase the yield of this pigment by tissue culture technique.

The cell lines were raised from stem of P. zeylanicum. Rapid plant regeneration in the callus cultures derived from leaf and stem explants of P. zeylanicum has also been reported. The hairy roots were also developed to improve plumbagin yield. Thus it may be noted that Ti and Ri mediated cell lines also sometimes improve the yield of active constituents in medicinal plants and here the present investigation is directed towards production of plumbagin in different cultures and a systematic study was carried out with a view to improve the content of plumbagin in normal culture (static and suspension) and Ti and Ri mediated transformed cultures by A. tumefaciens and A. rhizogenes. The antimicrobial activity of the biomass developed in the present investigation was also tested.

P. zeylanicum is considered to be a potent hepatoprotective drug in Indian system of medicine. A number of liver protective herbal drugs contain the extract of P. zeylanicum as one of the important ingredient of these herbal formulations. It was thought worthwhile to undertake the hepatoprotective studies of normal as well as genetically transformed cultures.