CHAPTER - 1

INTRODUCTION
CONTENTS


2. Objectives of *Plantago Ovata* Forsk, and *Trachyspermum ammi* (L) Sprague.

3. Some Important Highlights in the field of Tissue Culture Research.

4. Meristem Culture

5. Organogenesis

6. Somatic Embryogenesis

7. Source of Variability in Cell Cultures for Plant Improvement

8. Somaclonal Variation

9. Protoplasts Culture and Fusion

10. Secondary Metabolites
Plant tissue culture techniques have become a powerful tool for studying the basic and applied problems in plant biology. The potential impact of these novel and powerful biotechnology on genetic improvement of crop plants is significant. However, a tissue culture system is also very often a "model" system, which allows one to investigate physiological, biochemical, genetic and structural problems related to plants and the technique is being used also as an adjunct to more traditional means in plant modification.

Medicinal plants are important components of traditional medicine in many countries. The investigation and utilization of the world's medicinal plant resources are significant and promising tasks. Development of industry and agriculture, increased domestic as well as export demand, and environmental degradation have led to over exploitation of medicinal plants. This situation has resulted in decreased production or unstable quality of many medicinal substances.

The propagation of medicinal plants in vitro and breeding of new varieties with enhanced yield, high efficacy, and good vigour are urgent. There has been much research on tissue culture of herbaceous and woody medicinal plants, and it is clear that regeneration through tissue culture techniques will be crucial for protection and conservation of resources and breeding of new varieties in medicinal and other economically useful plants.
Many investigators have focused on the cultivation of aromatic plants and increase in the quantity and quality of their products. The economic value of aromatic plants is much higher than that for other cultivars.

Plant tissue culture technique thus present new strategies for the development and improvement of agricultural crops as well as drug yielding and economically important plants through selective breeding, genetic modification, mutant selection and micropropagation of elite plants. Recent developments in recombinant DNA technology along with the isolation and culture of protoplasts have further made it possible to produce genetically engineered plants and have opened up unprecedented opportunities for the manipulations of genetic composition of the plants. Hopefully, in the years to come this may produce improved food and forest crop varieties and result in increased food and biomass production.

With above background, the present investigation is undertaken on two medicinal and economically important plants:

1. **Plantago ovata** Forsk, and
2. **Trachyspermum ammi** (L) Sprague

1. **Plantago ovata** Forsk (Isabgol)

   Family: Plantaginaceae

   Isabgol (**Plantago ovata**) known as Bengali-Isabgol, Ispaghul, Eshopgol; Hindi - Isabghul, Isapaghul, Issufgul; Bombay -
Isapghul; Tamil - Ishappukal Vibrai; Telugu - Isapagala vittulus; Persian - Ispaghul, Isparzah; Gujarati - Ghoda Jiru, is an exclusive seasonal cash crop of India, having monopoly in the world market with foreign exchange earning of over Rs.50 crores annually. 70% of the annual production is exported to U.S.A. and U.K.; 20% exported to European and other countries, while 10% is used locally in India. The seeds and seed husk are extensively used in Ayurvedic, Unani and Allopathic systems of medicine due to its safe laxative property, especially in constipation, diarrhoea, dysentry and similar disorder of digestive system including termination of pregnancy (Bhagat and Hardas, 1980).

Commercial cultivation of the said crop is confined to a few districts in North Gujarat (Mehsana, Banaskantha, and Sabarkantha), Kutch, Saurashtra, with few localities in Rajasthan and Madhya Pradesh. To some extent it is also grown in West Bengal. Presently about 4 lac acres of land is under cultivation of the crop in India. Against existing demand of 750 lac kg, only 525 lac kg seeds are produced annually in India. Thus, the yield of the crop is inadequate to meet present demands. Since there is lack of genetic variability in the present germplasm, presumably due to plant introduction from a single source, it is not possible to boost the yield and improve seed quality. Absence of genetic variability renders plants' improvement by breeding programmes very difficult.
Creation of genetic variability is desirable to overcome certain agronomical problems for crop improvement. Because traditional breeding methods have not proved useful, tissue and cell culture approaches are envisaged for generation of variability through somaclonal variation (Larkin and Scowcroft, 1981). However, regeneration of plants from cultured cells and tissues is a prerequisite in the application of tissue culture techniques for plant propagation and improvement (Rao, 1987). For Isabgol crop improvement three major agronomic problems need to be overcome:

A. Late maturity (>4 months)
B. Seed shattering, and
C. Susceptibility to downy mildew.

The objective, therefore, has been to work out methods for induction of callus and streamline procedure for regeneration of plants through organogenesis/embryogenesis. Hopefully, the latter would ultimately lead to efforts for plant improvement.

2. *Trachyspermum ammi* (L) Sprague

Family : Apiaceae (Umbelliferae)

English Name : Ammi

Hindi Name : Ajowain

Syn. *Carum Copticum*

Ajowain, as it is called, is an aromatic herb cultivated
throughout India, but mostly in districts of North Gujarat, and also in and around Indore and Hyderabad. The fruits are being used as spices and are also carminative, stimulant, antispasmodic and tonic. They are given in colic flatulence, dyspepsia, indigestion, diarrhoea, cholera, tympanites, hysteria etc. The fruits have an aromatic smell and pungent taste. Thy yield 2 to 4% of a colourless to brownish essential oil in which thymol is present to the extent of 35 to 60%. The annual production of Ajowain oil from the seeds of _T. ammi_ is over 35 metric tons which is not sufficient to meet demands. The oil is used in perfumary industries, as insecticide, and in indigenous medicine. The present market price of the oil range between Rs.250/- and Rs.300/- per kg. One kg of thymol costs about Rs.400/- and is chemically synthesized.

The Ajowain oil being an important source of thymol, the large quantities of the fruit were exported to Europe, particularly to Germany and U.S.A. before World War I for its distillation and the manufacture of thymol. During World War II and after large scale distillation of the fruit and manufacture of thymol were also organised in India. If the yield of oil could be substantially improved and the yield of fruit per acre increased, there is every chance of reviving the industry (Chopra et al., 1958). The main processors of Ajowain oil are: Devi Industries in Maduri (Tamil Nadu), Siva Industries in New Delhi, and Ashok Thymol in Indore (M.P).
Plant cell and tissue culture studies on *Trachyspermum ammi* (L) is not reported till to date. Present studies were, therefore, undertaken on the plant both for micropropagation through meristem culture and/or somatic embryogenesis.

Some important highlights in the field of Tissues Culture Research are illustrated in Table 1 and provide general background for further research in various areas of Plant Tissue Culture. Clearly, from being a method for pure academic pursuit, the techniques of plant cell and tissue culture have now become a powerful tool for biotechnological applications in agriculture, forestry and industries.

The regeneration of plants from cultured cells and tissues is a key step in the application of tissue culture methodology for plant propagation and improvement. The development of efficient protocols for reproducible high frequency plant regeneration from cultured tissue has, therefore, assumed great importance (Rao, 1987).
<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Achievements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1902</td>
<td>G. Haberlandt</td>
<td>Concept of cell culture and Totipotency</td>
</tr>
<tr>
<td>1922</td>
<td>Kotte</td>
<td>Root Culture</td>
</tr>
<tr>
<td>1934,</td>
<td>White</td>
<td>Tomato root culture</td>
</tr>
<tr>
<td>1939</td>
<td>Gautheret</td>
<td>Long term callus</td>
</tr>
<tr>
<td>1939a</td>
<td>Nobecourt</td>
<td>Cultures (Carrot, hybrid tobacco tumour)</td>
</tr>
<tr>
<td>1939b</td>
<td>White</td>
<td></td>
</tr>
<tr>
<td>1953</td>
<td>Muir</td>
<td>&quot;nurse&quot; culture</td>
</tr>
<tr>
<td>1954</td>
<td>Muir et al</td>
<td>Cell division in single isolated cells</td>
</tr>
<tr>
<td>1957</td>
<td>Torry</td>
<td>Hanging drop method</td>
</tr>
<tr>
<td>1957</td>
<td>Skoog and Miller</td>
<td>Regulation of shoot and root initiation in callus cultures by varying auxin and cytokinin ratio</td>
</tr>
<tr>
<td>1958</td>
<td>Steward F.C.</td>
<td>Totipotency</td>
</tr>
<tr>
<td>1959</td>
<td>Reinert</td>
<td>Totipotency</td>
</tr>
<tr>
<td>Year</td>
<td>Author</td>
<td>Achievement</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1960</td>
<td>Jones et al</td>
<td>Microchamber technique</td>
</tr>
<tr>
<td>1960</td>
<td>Bergmann</td>
<td>The agar-plating method</td>
</tr>
<tr>
<td>1960</td>
<td>Cocking</td>
<td>Protoplast isolation and culture</td>
</tr>
<tr>
<td>1960</td>
<td>Morel</td>
<td>Shoot-tip (meristem) culture producing virus-free Cymbidium</td>
</tr>
<tr>
<td>1962</td>
<td>Murashige and Skoog</td>
<td>Culture medium</td>
</tr>
<tr>
<td>1964</td>
<td>Morel</td>
<td>Shoot-tip (meristem) culture technique of clonal propagation in Orchids: Orchid Industry</td>
</tr>
<tr>
<td>1966</td>
<td>Guha and Maheshwari</td>
<td>Anther culture (Datura)</td>
</tr>
<tr>
<td>1967</td>
<td>Bourgin and Nitsch</td>
<td>Anther culture (Tobacco)</td>
</tr>
<tr>
<td>1972</td>
<td>Carlson et al</td>
<td>Protoplasts fusion from Nicotiana langsdorffii, Hybrid not demonstrated</td>
</tr>
<tr>
<td>1977</td>
<td>Misawa</td>
<td>Secondary products, progress at industrial level</td>
</tr>
<tr>
<td>1978</td>
<td>Melchers et al</td>
<td>Protoplasts fusion from potato and tomato, Hybrid obtained</td>
</tr>
<tr>
<td>Year</td>
<td>Author/Technique</td>
<td>Achievement</td>
</tr>
<tr>
<td>------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>1980</td>
<td>Durzan</td>
<td>Artificial seeds by coating/encapsulation</td>
</tr>
<tr>
<td>1980</td>
<td>Misawa</td>
<td>Secondary products, progress at industrial level</td>
</tr>
<tr>
<td>1981</td>
<td>Larkin and Scowcroft</td>
<td>Somaclonal variations</td>
</tr>
<tr>
<td>1983</td>
<td>Barton and Brill, Dodds and Bengochea</td>
<td>Foreign gene transfer technique</td>
</tr>
<tr>
<td>1984</td>
<td>Zhan</td>
<td>Artificial seed engineering technique for carrot</td>
</tr>
<tr>
<td></td>
<td>Sharp et al.</td>
<td></td>
</tr>
</tbody>
</table>
Plant regeneration via tissue cultures occurs mainly through the following methods:

(i) Meristem culture,

(ii) Organogenesis, and

(iii) Somatic embryogenesis.

(i) Meristem Culture:

Shoot-apex technique, first used by Morel (1960) for micropropagation of *Cymbidium*, led to the establishment of orchid industry (Morel, 1964). The scope of meristem culture soon widened for mass multiplication of elite plants, from ornamentals to economically important herbs, shrubs, and finally fruit and forest trees. Production of virus-free plants could also be achieved by the same technique.

(ii) Organogenesis:

Organogenesis can be obtained either through direct differentiation of shoot buds from explants or through callus formation in explants and subsequent formation of shoots and roots. Organ formation in *vitro* was reported as early as 1939.
when White (1939b) observed shoot found root formation in callus cultures of carrot. Following these many attempts were made with tissues of diverse plant species which led to the elegant work of Skoog and Miller (1957), who demonstrated that the balance between auxin and cytokinin determine the nature of organogenesis. Since then there have appeared numerous reports of chemical regulation of organogenesis from cultured tissues derived from different plant parts from a wide range of plant species (Butenko et al., 1968; Vasil and Vasil, 1980; Evans et al., 1981) and the list is ever expanding.

Organogenesis is controlled by three main factors: the inoculum, the medium, and the environmental conditions (Thorpe, 1980, 1982). The physiological age of the explant is a critical factor. Raju and Mann (1971) have demonstrated that in Echeveria young leaf explants initiated only roots, older leaves regenerated shoot buds and leaves of medium age produced shoots and roots.

The composition of the nutrient medium is an important parameter to be optimized to achieve plant regeneration. The major constituents of the medium comprise of inorganic and organic nutrients, carbon source, vitamins and plant growth regulators. In certain instances natural growth adjuvant such as deprotenized coconut milk, fruit pulp and juice, malt extract and yeast extract, etc are also incorporated. Auxins (IAA,
NAA, 2,4-D) and cytokinins (Kh, BAP, Z, Zip) are more often used in cultures, and their concentration and ratio often determine the nature of growth and organogenesis/embryogenesis.

Several media have been developed by various investigators and are being used in studies of tissue culture (Mehta and Bhatt, 1990), the striking difference being essentially in quantity and form of nitrogen. A comparative account of the components of various nutrient media is given by Bhojwani and Razdan (1983) and Ozias-Akins and Vasil (1985).

Likewise, seasonal variations (Fellenberg, 1963) dissolved oxygen concentration in culture (Kessel and Carr, 1972), quality and intensity of light (Murashige, 1977) and other factors also influence organogenesis in vitro. The optimum light intensity for shoot differentiation in herbaceous plants is around 1000 lux, low intensities around 300 lux, and high intensities from 3000 - 10,000 lux (Murashige, 1977). On the other hand, in Ruta graveolens in darkness cultures produced roots of various length; while under continuous illumination cultures produced full green shoots (Abou Mandour, 1982).

The cultures grow well under a temperature regime of 25 ±2°C. However, cultures of Allium and Narcissus do better at 18°C (Hussey, 1986) and those of Monstera deliciosa require a temperature at 30°C (Fonnesbech and Fonnesbech, 1980).
In organogenesis, root and shoot development are often mutually exclusive, and a sequence of media changes is necessary to generate an entire plant.

(iii) Somatic Embryogenesis:
Somatic embryogenesis is the most fascinating phenomenon which results in the formation of a complete plantlet from a single cell and is by far the most convincing demonstration of the totipotency of plant cells (Rao, 1987). It is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. This phenomenon first observed in suspension cultures of carrot (Daucus carota) by Steward et al (1958) and in carrot callus grown on an agar medium by Reinert (1959), occurs either indirectly from callus/cell suspensions or directly from an organized structure i.e. organ explants. The origin and development of adventitious embryoids in cultures have been described in a number of reviews, e.g. Street (1975); Vasil and Vasil (1980); Evans et al (1981); Ammirato (1983); Raghavan (1983); Lutz et al (1985); Williams and Maheswaran (1986); Rangaswamy (1986) and Durzan (1988).

Somatic embryo has been defined as non-zygotic embryo arising from a single cell with no vascular connections with maternal tissues (Street and Withers, 1974; Haccius, 1978). However,
Raghavan (1976) and Tisserat et al. (1979) demonstrated that in many cases apparently normal bipolar embryoids are formed from aggregates of cells. Thus, an embryo is *ab initio* a bipolarized entity (Rangaswamy, 1986), bounded by cuticle, since physical or physiological isolation of the cell/cell group is considered prerequisite for them to embark upon the embryogenic pathway (Handro et al., 1973; Street, 1979; Vasil and Vasil, 1982; Kononowicz et al., 1984).

Somatic embryogenesis provides many advantages e.g. large number of embryoide (60,000 per liter of medium); the presence of both root and shoot mexitems in the same unit; easy scale-up, transfers and other manipulations. Embryos are natural organs of perennation, many of which typically become dormant. If dormancy could be induced in somatic embryos, the possibility arises that they could be incorporated into artificial seeds either by coating or encapsulation (Durzan, 1980). In order to release improved varieties for production rapidly and effectively, an artificial seed engineering technique has been established for carrot (Zhan, 1984). Further, tissue culture derived "artificial seeds" offer scope for mechanisation and automation for planting (Durzan, 1980; Sharp et al., 1984; This technique is also suitable for seed storage and shipment.

Population of somatic embryos also provide an important
resource for the analysis of molecular and biochemical events that occur during induction and maturation. Work has been proceeding along these lines, for example, the formation of developmental (temperature sensitive) mutants in carrot cultures (Breton and Sung, 1982; Terzi et al., 1982) and isolation of embryo specific proteins (Sung and Okimoto, 1981, 1983) and even stage specific storage proteins (Crouch, 1982).

**Source of Variability in Cell Cultures for Plant Improvement:**
The assembly of genetic variability is vital to any plant breeding enterprise. Without a continued input of "new genes" plant improvement cannot be made. The plant cells in culture generate genetic variability which is proving to be of significance for plant improvement (Larkin and Scowcroft, 1981). The existence of variability among plants regenerated from cell cultures is acknowledged by many authors (Skirvin, 1978; Brottell and Ingram, 1979; Thomas et al., 1979; Shepard et al., 1980; Barbier and Dulieu, 1980; Larkin and Scowcroft, 1983; Orton, 1984; Scowcroft and Larkin, 1985; Vasil, 1986; Lorz et al., 1988; Reddy, 1989).

Since the objective of the present investigation is to pave way for improvement of the two crops species, *Plantago* and Ajowain, some approaches made to induce genetic variations are worth considering.

**Somaclonal Variation:**
Plants regenerated from established cultures (callus, suspension) are
frequently found to be variable. The callus and suspension cultures exhibit high chromosomal instability resulting into polyploids and aneuploids. This is attributed to heterogeneous population of cells in differentiated tissues. Chromosomal mosaics in callus and cell suspension cultures have been well documented (Sunderland, 1977; Ammirato, 1978). Long term cultures of callus often result in changes in the Karyotype of the plant and the regenerated plants show variation for various characters.

Similarly, callus mediated somatic embryogenesis is ideal for recovering useful variants (Lutz et al., 1985; Durzan and Gupta, 1987; Tulecke, 1987).

Somaclonal variations are already having some impact on the improvement of sugarcane and potato cultivars, and in the breeding of new floricultural varieties. Increasing role of somaclonal variation as an adjunct to conventional plant improvement is envisaged. Recent research has demonstrated the role of such requirement realised in previously recalcitrant crop plants such as wheat (Shimada and Yamada, 1979), rice (Oono, 1978b), and important legumes (Bingham et al., 1975; Beach and Smith, 1979; Phillips and Collins, 1979; Kao and Michayluk, 1980; dos Santos et al., 1980; Saka et al., 1980 and Bhojwani and Mukhopadhyay, 1986).

The greatest application of somaclonal variation for plant improvement is in selection for desirable mutations at the cellular
level. Cellular selection in conceivable for the recovery of variants resistant to antimetabolite such as amino acid analogues, antibiotic drugs, pathotoxins, herbicides and physiological stress (Maliga, 1978; Thomas et al., 1979; Brettell and Ingram, 1979; Nabors et al., 1980; Selvapandiyan et al. (1988) and Unnikrishnan et al. (1991). Many agronomically important attributes are known: resistance to host-specific toxins such as those found in Drechslera, Pseudomonas and Alternaria pathogens, tolerance to salinity, metal toxicity, temperature stress and waterlogging.

Protoplasts Culture and Fusion:
One of the most exciting development in plant tissue culture during recent years is the isolation, culture and fusion of protoplasts. Protoplast constitute an ideal "free cell" developmental system as they are separate entities capable of reforming cell walls and regenerating whole plants.

Protoplast fusion of two different plant results in the production of hybrids and cybrids which may be unknown in nature. Genetically novel plants could be engineered by transplantation of plastids, mitochondria and chromosome or by DNA mediated transformation. The genetic information that resides in the mitochondria and chloroplasts codes for a number of significant agronomic characteristics such as resistance to herbicides, male sterility, etc.

In recent years due to considerable progress made in fusion
technology, the interest of the technique has moved from the creation of novel hybrid plants to chromosome transfer and gene introgression. The Ti plasmid DNAs carried by the inciting bacteria of the crown gall disease have served in the recent past and will doubtless continue to serve as a vector for the introduction of new and desirable genetic information for genetic engineering studies designed for crop improvement. This applied aspect of the problem has led to the development of new industries. The agricultural potential of this new technology is so great that it has already begun to lead to the development of a multibillion dollar industry worldwide by the end of the present century.

Recent development in recombinant DNA and plant tissue culture technology have thus brought us one step closer to producing genetically engineered plants such as those that are insecticide and frost resistant or require less fertilizer for growth. Once a new variety of plant is developed in the laboratory, micropropagation provides the method for uniformly propagating it, bringing it to the field for testing (Gills and Morgan, 1987) and releasing it for increased productivity.

Secondary Metabolites:
Higher plants produce a variety of secondary metabolites, some of which are a source of important pharmaceuticals. Over 4/5th of about 30,000 known natural products are of plant origin (Crocomo et al., 1981). However, due to the uncontrolled exploitation
of natural habitat and fluctuations in the supply of raw materials, the production of secondary compounds by plant cell cultures in vitro may provide a viable alternative (Berlin, 1986). It is also possible to increase the secondary metabolite production in cell cultures by various methods.

In recent years, some encouraging findings have been reported from in vitro studies for a variety of medicinally promising substances (Mehta, 1984). This is achieved largely by manipulating the hormonal and nutritional factors (Tabata, M. and Fiyita, 1985) as well as isolation of variant cells with special biochemical characteristics from the normal pool of genetic variations (Heinstein, 1985). Recent novel methods of immobilising plant cells (Mehta, 1989; Subramani et al, 1989) and the use of fungal elicitors (Ravishankar et al, 1989; Jacob John et al, 1990) as a means of producing enhanced yields of secondary metabolites are greatly promising (Lindsey and Yeoman, 1983; Heble, 1989; Jain et al, 1989). About a dozen plant products are now commercially produced by employing cell technology methods (Khanna, 1991). In the present studies preliminary attempt is made for detection of thymol in seeds, undifferentiated callus and regenerated plantlets of Trachyspermum ammi (L).