CHAPTER - 4

STUDIES WITH TRACHYSPERMUM AMMI SPRAGUE
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A. MORPHOGENESIS IN APIACERE:

Like Solanaceae, the family Apiaceae has been extensively examined for morphogenetic responses. Starting from pioneering experiments of F.C. Steward (1958) and Reinert (1959) on Daucus carota, most of the reports on regeneration in the Apiaceae family is through embryogenesis, rather than via organogenesis. Similarly, except for carrot there are hardly any reports on the anther and protoplast culture with the species of Apiaceae (Jha et al., 1981). A tentative list of plants in which propagation is achieved through somatic embryogenesis is presented in Table 16. The details of media along with hormonal and other supplements are given in Table 17.

The above summary of work done clearly shows that using a variety of media formulations and hormonal and other supplements regeneration is obtained chiefly through somatic embryogenesis from diverse explant sources.

The present chapter describes experiments conducted with Trachyspermum ammi (L) Sprague.
Table - 16

Members of Apiaceae in which somatic embryogenesis has been achieved in vitro.

<table>
<thead>
<tr>
<th>Plant Explant</th>
<th>source</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Ammi majus Ovary</td>
<td>Sehgal (1972)</td>
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</tr>
<tr>
<td>Hypocotyl</td>
<td>Grewal et al (1976)</td>
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</tr>
<tr>
<td>Koul et al (1985)</td>
<td></td>
<td></td>
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<tr>
<td>Anethum graveolens Hypocotyl</td>
<td>Johri and Sehgal (1966)</td>
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<tr>
<td>cotyledon Embryo</td>
<td>Steward et al (1970)</td>
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<tr>
<td>Petiole, root, leaf, fruit</td>
<td>Ratnamba and Chopra (1974)</td>
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</tr>
<tr>
<td>Inflorescence</td>
<td>Sehgal (1978)</td>
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</tr>
<tr>
<td>Hypocotyl</td>
<td>Chopra and Khanna (1982)</td>
<td></td>
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<tr>
<td>Apium graveolens (Celery) Leaf, fruit</td>
<td>Reinert et al (1966)</td>
<td></td>
</tr>
<tr>
<td>Petiole</td>
<td>Williams and Collin (1976)</td>
<td></td>
</tr>
<tr>
<td>Chen (1976)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td>Petiole</td>
<td>Orton (1984)</td>
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<tr>
<td>Bunium persicum</td>
<td>Immature seed (mericarp)</td>
<td>Wakhu et al (1990)</td>
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<td>Carum carvi (caraway)</td>
<td>Petiole</td>
<td>Ammirato (1974)</td>
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<td></td>
<td>Anther</td>
<td>Egorova and Reznikova (1982)</td>
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<td>Root</td>
<td>Halperin and Watherell (1964)</td>
</tr>
<tr>
<td></td>
<td>Hypocotyl, leaf, petiole, penduncle, phloem, root, root tip, stem</td>
<td>Tisserat et al (1979)</td>
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<tr>
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<td>-----------------------------</td>
</tr>
<tr>
<td>Dadiscus coorulea</td>
<td>Stem</td>
<td>Ball and Joshi (1966)</td>
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<tr>
<td>Foeniculum vulgare (Fennel)</td>
<td>Stem</td>
<td>Maheshwari and Gupta (1965)</td>
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<tr>
<td>Petronolium hortense (Parsley)</td>
<td>Petiole</td>
<td>Vasil and Hilderbrandt (1966a)</td>
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<td>Pimpinella anisum (anise)</td>
<td>Fruit</td>
<td>Huber et al (1978)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ammirato and Steward (1971)</td>
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Table - 17

Media and supplements for Somatic Embryogenesis in Apiaceae family.

<table>
<thead>
<tr>
<th>Plant sps</th>
<th>Medium</th>
<th>Hormonal/Organic supplements</th>
<th>Morphogenesis</th>
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<tbody>
<tr>
<td>Ammi majus</td>
<td>MS</td>
<td>IAA</td>
<td>Polyembryony</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Organogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(shoots)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Embryogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAA + Kn</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Ammi visnaga</td>
<td>MS</td>
<td>2,4-D + Kn</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Anethum graveolens</td>
<td>WM</td>
<td>CM + NAA or IAA + CH + Yeast extract</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thalloid seedlings, (neomorphs)</td>
</tr>
<tr>
<td>Apium gravelones</td>
<td>MS</td>
<td>2,4-D + Kn (-2,4-D)</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>(Celery)</td>
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<td>Organogenesis</td>
</tr>
<tr>
<td>Bonium persicum</td>
<td>MS</td>
<td>2,4-D + Kn</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Carum carvi</td>
<td>WM</td>
<td>CH + NAA, hormone-free + ABA</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>(Caraway)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conium maculatum</td>
<td>MS</td>
<td>CM + NAA</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Coriandrum sativum</td>
<td>WM</td>
<td>CH + NAA + CM or CH + CM</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Plant sps</td>
<td>Medium</td>
<td>Hormmonal/Organic supplements</td>
<td>Morphogenesis</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------</td>
<td>-------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Cuminum cymium</td>
<td>B-5</td>
<td>2,4-D/NAA + BAP or NAA + Kn</td>
<td>Shoots</td>
</tr>
<tr>
<td>Daucus carota (Carrot)</td>
<td>WM</td>
<td>NAA + CH</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>2,4-D + Z</td>
<td></td>
</tr>
<tr>
<td>Dadiscus coorlea</td>
<td>Agar salts</td>
<td>2',4-D + CW</td>
<td>Embryogenesis</td>
</tr>
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<td></td>
<td>complex</td>
<td></td>
</tr>
<tr>
<td>Foeniculum vulgare (Fennel)</td>
<td>NM</td>
<td>CM + 2,4-D + Kn alone or in combination</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td></td>
<td>LS</td>
<td>2,4-D + Kn</td>
<td>Embryogenesis</td>
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<tr>
<td>Petronolium hortense (Parsley)</td>
<td>MS</td>
<td>Adenine Sulphate</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Pimpinella anisum (anise)</td>
<td>WM</td>
<td>CM + NAA or CM</td>
<td>Embryogenesis</td>
</tr>
<tr>
<td>Sium suave (water parsnip)</td>
<td>WM</td>
<td>CM + NAA + CH, (-NAA)</td>
<td>Embryogenesis</td>
</tr>
</tbody>
</table>
B. TRACHYSPERMUM AMMI (L) SPRAGUE:

1. Important Uses:

Trachyspermum ammi (L) is a highly important economic and medicinal plant. The fruits and roots are the economically and medicinally useful parts. The main uses are as under:

Fruits:
1. Fruits are widely used as a spice.
2. Fruits are stimulant, antispasmodic, tonic and carminative, administered in flatulence, dyspepsia and diarrhoea, and also recommended in cholera.
3. Ajowan is used for relaxed sore throat and bronchitis and is a common ingredient or cough mixtures.
4. A paste of crushed fruits is applied to the abdomen externally for relief from colic.
5. It shows antibiotic activity and is used in lotions and ointments applied for checking chronic discharge.
6. Essential oil, yielded by fruits, is called Ajowan oil. Principal constituent of the oils is thymol which can be easily crystallized from the oil and is known as Flowers of Ajowan.
7. Ajowan oil is employed as an aromatic, carminative and antiseptic; also expectorant in emphysema, bronchial pneumonia and some other respiratory ailments.

8. Aqueous solution left after separation of essential oil is called Omum water, used as carminative in flatulence and gripe.

9. Extracted fruits may be used as cattle feed.

10. Fruits also yield a fatty oil, used externally on rheumatic swellings, also used in soaps and in the preparation of epoxy derivatives, used as plasticizers in vinyl industry.

11. Oil cake used as a cattle feed and fertilizer.

12. Roots are carminative, diuretic, used in febril conditions and stomach disorders.

2. Explant source:

In order to identify the plant part ideal for use as explant, various parts such as roots, hypocotyl, leaves and shoot tip of aseptically raised seedling were cultured on Murashige and Skoog's medium. Various hormonal supplements made included IAA, NAA, 2,4-D, Kn and BAP, in the range $1 \times 10^{-6}$ - $1 \times 10^{-5}$M, individually and in combinations. Of the various explants tried, the shoot tip and hypocotyl responded the best; segments of leaves and roots invoked poor response.
3. Induction of callus cultures from hypocotyl and shoot tip

Aseptic seedlings were raised from seeds on MS basal medium. Hypocotyl and shoot tips were excised from 15 days old seedlings and callus cultures were initiated on MS medium with different phytohormones (IAA, NAA, 2,4-D, BAP and Kn) singly and in combinations. After 10 days callus was induced. The culture response to concentration range tested is presented in Table 18.

Best callus growth was obtained in MS medium supplemented with 2,4-D and Kn as well as with NAA and Kn. The response of hypocotyl explants in terms of percentage was slightly lower than the shoot tip. However, the nature and growth of the callus were found similar in both the explants. Callus cultures were maintained on respective media by regular transfers every 3-4 wks.

Of the three auxins tried singly, 2,4-D induced only callusing. Best results in terms of response and callus growth was obtained at 2,4-D conc of 1x10^{-5}M. Reduced conc (1x10^{-6} and 5x10^{-6}M) exhibited moderate response and formed pale yellow semifriable callus. NAA at 1x10^{-5}M conc in the medium demonstrated maximum response and induced pale yellow semifriable callus with
few roots. The response in terms of percentage and callus growth was observed moderate at low cone of NAA ($1 \times 10^{-6}$ and $5 \times 10^{-6}$M) in the medium, with the induction of pale yellow semifriable callus. IAA as a supplement invoked poor response and initiated brown, slow growing callus at all the three cone it was supplemented ($1 \times 10^{-5}$, $5 \times 10^{-6}$ and $1 \times 10^{-6}$M).

The medium supplemented with BAP ($1 \times 10^{-5}$M) induced green, nodular, slow growing callus with few roots. Lower conc of BAP ($1 \times 10^{-6}$ and $5 \times 10^{-6}$M) induced pale green, semifriable callus. Addition of Kn at $1 \times 10^{-5}$M in the medium produced green nodular and slow growing callus with few roots; while at low Kn conc ($1 \times 10^{-6}$M), pale green compact callus was formed. The best response (85%) and fastest growth was achieved when the Kn conc was adjusted at $5 \times 10^{-6}$M for both the explants.

In another set of experiments, Kn was applied in combination with different auxins to evaluate whether the effect was additive or antagonistic. From among the combinations tried, Kn ($5 \times 10^{-6}$M) in combination with NAA ($1 \times 10^{-5}$M) invoked 100 percent response and induced good pale yellowish green semifriable callus. The application of Kn ($5 \times 10^{-6}$M) with low levels of NAA ($1 \times 10^{-6}$ and $5 \times 10^{-6}$M) showed moderate response and induced light semifriable callus with slow growth.
2,4-D at $1 \times 10^{-6}$M and $5 \times 10^{-6}$M with Kn ($6 \times 10^{-6}$M) conc exhibited good response by promoting pale yellowish green semifriable callus. However, the best callus growth was achieved when the 2,4-D conc was tried at $1 \times 10^{-5}$M with Kn ($5 \times 10^{-6}$M). Pale yellowish green compact to semifriable callus was formed in 100 percent of the cultures. IAA + Kn induced slow growing brown callus.

Supplementing the medium with BAP ($5 \times 10^{-6}$M) in combination with different auxins at different conc proved less effective than Kn. The best results in terms of percent response and callus growth were obtained in the medium with BAP ($5 \times 10^{-6}$M) and 2,4-D/NAA ($1 \times 10^{-5}$M). BAP ($5 \times 10^{-6}$M) with low levels of NAA and 2,4-D ($1 \times 10^{-6}$ and $5 \times 10^{-6}$M) induced light green, compact and slow growing callus. BAP ($5 \times 10^{-6}$M) with all the conc of IAA in the medium invoked poor response and induced slow growing brown callus.
Table - 18

Effects of various phytohormones on callus induction on shoot tip and hypocotyl of *Trachyspermum ammi* L.

<table>
<thead>
<tr>
<th>MS + Sucrose + Phytohormones (μM)</th>
<th>Response(%)</th>
<th>Morphogenetic Response</th>
<th>Callus growth</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Shoot (3%)</td>
<td>Hypo-cotyl</td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>3</td>
<td>4</td>
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</table>

**Auxins**

<table>
<thead>
<tr>
<th></th>
<th>IAA</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>1x10^{-6}M</td>
<td>30 25</td>
<td>Brown callus</td>
<td>+</td>
</tr>
<tr>
<td>5x10^{-6}M</td>
<td>20 15</td>
<td>Brown callus</td>
<td>*</td>
</tr>
<tr>
<td>1x10^{-5}M</td>
<td>25 20</td>
<td>Brown callus</td>
<td>*</td>
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<table>
<thead>
<tr>
<th></th>
<th>NAA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10^{-6}M</td>
<td>40 35</td>
<td>Pale yellow, semifriable callus</td>
<td>*</td>
</tr>
<tr>
<td>1x10^{-5}M</td>
<td>75 70</td>
<td>Pale yellow, semifriable callus with few roots</td>
<td>**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>2,4-D</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10^{-6}M</td>
<td>60 55</td>
<td>Pale yellow, semifriable callus</td>
<td>*</td>
</tr>
<tr>
<td>5x10^{-6}M</td>
<td>70 65</td>
<td>Pale yellow, semifriable callus</td>
<td>*</td>
</tr>
<tr>
<td>1x10^{-5}M</td>
<td>80 75</td>
<td>Pale yellow, compact callus</td>
<td>$\emptyset$</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>BAP</td>
<td>1x10^{-6}</td>
<td>60</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>BAP</td>
<td>5x10^{-6}</td>
<td>65</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>BAP</td>
<td>1x10^{-5}</td>
<td>60</td>
</tr>
<tr>
<td>Kn</td>
<td>1x10^{-6}</td>
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<tr>
<td>Kn</td>
<td>1x10^{-5}</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>Auxin + Cytokinin</td>
<td>IAA + BAP</td>
<td>1x10^{-6} + 5x10^{-6}</td>
<td>45</td>
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<td>Auxin + Cytokinin</td>
<td>IAA + BAP</td>
<td>5x10^{-6} + 5x10^{-6}</td>
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</tr>
<tr>
<td>Auxin + Cytokinin</td>
<td>IAA + BAP</td>
<td>1x10^{-5} + 5x10^{-6}</td>
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<td>Auxin + Cytokinin</td>
<td>NAA + BAP</td>
<td>1x10^{-6} + 5x10^{-6}</td>
<td>55</td>
</tr>
<tr>
<td>Auxin + Cytokinin</td>
<td>NAA + BAP</td>
<td>5x10^{-6} + 5x10^{-6}</td>
<td>70</td>
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<tr>
<td>Auxin + Cytokinin</td>
<td>NAA + BAP</td>
<td>1x10^{-5} + 5x10^{-6}</td>
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<tr>
<td>2,4-D + BAP</td>
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<td>---</td>
</tr>
<tr>
<td>1x10^{-6} + 5x10^{-6}</td>
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<td>55</td>
<td>Light green, semifriable callus</td>
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<td>5x10^{-6} + 5x10^{-6}</td>
<td>70</td>
<td>66</td>
<td>Light green, semifriable callus</td>
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<td>1x10^{-5} + 5x10^{-6}</td>
<td>75</td>
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<td>Pale yellowish green, semifriable callus</td>
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<table>
<thead>
<tr>
<th>IAA + Kn</th>
<th>1</th>
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<td>Brown, compact callus</td>
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<tr>
<td>5x10^{-6} + 5x10^{-6}</td>
<td>55</td>
<td>45</td>
<td>Brown, compact callus</td>
<td>+</td>
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<tr>
<td>1x10^{-5} + 5x10^{-6}</td>
<td>60</td>
<td>50</td>
<td>Brown, compact callus</td>
<td>+</td>
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<table>
<thead>
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<tr>
<td>1x10^{-6} + 5x10^{-6}</td>
<td>60</td>
<td>55</td>
<td>Light green, semifriable callus</td>
<td>*</td>
<td></td>
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<tr>
<td>5x10^{-6} + 5x10^{-6}</td>
<td>75</td>
<td>70</td>
<td>Pale yellowish green, semifriable callus</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>1x10^{-5} + 5x10^{-6}</td>
<td>100</td>
<td>90</td>
<td>Pale yellowish green, semifriable callus</td>
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<table>
<thead>
<tr>
<th>2,4-D + Kn</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>1x10^{-6} + 5x10^{-6}</td>
<td>70</td>
<td>65</td>
<td>Light green, semifriable callus</td>
<td>*</td>
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<tr>
<td>5x10^{-6} + 5x10^{-6}</td>
<td>80</td>
<td>75</td>
<td>Pale yellowish green, semifriable callus</td>
<td>*</td>
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</tr>
<tr>
<td>1x10^{-5} + 5x10^{-6}</td>
<td>100</td>
<td>90</td>
<td>Pale yellowish green, semifriable callus</td>
<td>#</td>
<td></td>
</tr>
</tbody>
</table>

+ Poor
* Moderate
Ø Good
# Best
C. MORPHOGENESIS:

1. Plant regeneration through apical and axillary meristems of seedling

Terminal parts of aseptically raised seedlings (15 days old) measuring about 2 cms in length with two nodes were excised and cultured on MS medium containing 3% (w/v) sucrose and supplemented with Kn and NAA/2,4-D.

On the 8th day of the incubation, shoot buds were induced from the apical and/or axillary meristems of explants (2 cms); placed vertically on the medium in all the hormonal combinations tried. However, the degree of differentiation varied according to the treatment (Table 19). No shoot bud was formed from the explants (2 cm) when inoculated horizontally on the medium in any of the hormonal combinations tried. On the other hand, only callus was induced from the 1 cm long explants in all the media combinations, after 1 wk of incubation, irrespective of their position.

Shoot buds (1-2) were induced in 90% of the cultures from apical meristems of the explants in the medium containing high NAA (1x10^{-5}M) and low Kn (1x10^{-6}M). The regenerated shoots developed further, initiated roots and formed completed, but feable plantlets on the same
Plate 10

Fig. 44 Regenerated plantlet from shoot apex (tip) of T. ammi in 4 wks old cultures

Medium: MS + NAA (1x10^{-5}M) + Kn (1x10^{-6}M)

X 1:5

Fig. 45 Regenerated shoots from axillary meristems of T. ammi in 2 wks old cultures

Medium: MS + NAA (1x10^{-5}M) + Kn (5x10^{-6}M)

X 1:6

Fig. 46 Regenerated shoots from apical meristems of T. ammi in 2 wks old cultures

Medium: MS + 2,4-D (4x10^{-6}M) Kn (2x10^{-6}M)

X 1:7

Fig. 47 Regenerated shoots from apical meristems of T. ammi in 2 wks old cultures

Medium: MS + NAA/2,4-D (1x10^{-5}M) + Kn (2.5x10^{-6}M)

X 1:7
medium by 4 wks of culture (Fig. 44). At the same time, 
a scanty, white to pale green, callus was formed from 
the cut ends of the explants near the medium surface.

Multiple (3-5) shoot buds were induced from the axillary 
meristems of the explants in 80% of the cultures, in the 
medium supplemented with NAA (1x10^{-5}M) and Kn 
(5x10^{-6}M). The regenerated shoots failed to induce roots 
even after 4 wks of incubation in the same medium. 
Little callus occurred at the cut ends of the explants near 
the medium surface (Fig. 45).

Induction of shoot buds (3-5) was also observed from the 
apical meristem of the explant in 70% of the cultures in 
the medium containing 2,4-D (4x10^{-6}M) and Kn (2x10^{-6}M) 
after 2 wks of incubation (Fig. 46). Whitish brown, 
callus was formed from the cut ends of the explants near 
the medium surface.

The medium supplemented with Kn (2.5x10^{-6}M) and 
NAA/2,4-D (1x10^{-5}M) proved less effective and cultures 
exhibited comparatively poor response in terms of shoot 
formation. 2-3 shoots initiated from the apical meristems 
of the explants in 60% of the cultures. Callus formation 
was, however, more from the cut ends of the explants 
early the medium during 2 wks of incubation (Fig. 47).
### Table 19

Effect of NAA, 2,4-D and Kn on shoot regeneration from shoot tip in *Trachyspermum ammi* L.

<table>
<thead>
<tr>
<th>MS + Sucrose 3% (w/v) + Phytotigones (µM)</th>
<th>Response Percentage (%)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA (10^{-5}M) + Kn (5x10^{-6}M)</td>
<td>80</td>
<td>@ Shoots (3-5/explant), scanty, yellow, friable callus at cut ends.</td>
</tr>
<tr>
<td>NAA (10^{-5}M) + Kn (2.5x10^{-6}M)</td>
<td>60</td>
<td>+ Shoots (2-3/explant), green friable callus at cut ends.</td>
</tr>
<tr>
<td>NAA (10^{-5}M) + Kn (10^{-6}M)</td>
<td>90</td>
<td>@ Shoots (1-2/explant), scanty, pale green callus at cut ends.</td>
</tr>
<tr>
<td>2,4-D (10^{-5}M) + Kn (2.5x10^{-6}M)</td>
<td>60</td>
<td>+ Shoots (2-3/explant), yellow callus at cut ends.</td>
</tr>
<tr>
<td>2,4-D (4x10^{-6}M) + Kn (2x10^{-6}M)</td>
<td>70</td>
<td>* Shoots (3-5/explant), whitish brown callus to cut ends.</td>
</tr>
</tbody>
</table>

* Poor
* Moderate
@ Good
Plate 11

Fig. 48 Regenerated shoots for growth and hardening in 3 wks cultures

Medium: MS + NAA (1x10^{-6} or 1x10^{-7}M) + Kn (1x10^{-6}M)

Fig. 49 Root induction on regenerated shoots of T. ammi

Medium: MS + IBA (2.5x10^{-6}M)

Fig. 50 Plantlets of T. ammi Transferred to Pot

X | · 2.

X | · 5
These shoots did not develop further even after 4 wks on the same medium.

The developing shoots growing in the medium supplemented with NAA (1x10^{-5}M) + Kn (1x10^{-6} and 5x10^{-6}M) and 2,4-D (4x10^{-6}M) + Kn (2x10^{-6}M) were excised and individual shoots transferred to a medium containing NAA (1x10^{-6} or 1x10^{-7}M) and Kn (1x10^{-6}M) for further growth and hardening. Within 2 wks, the shoots developed to maturity (Fig. 48).

Root induction on regenerated shoots:
The well developed shoots were transferred to half-strength MS medium with various conc and/or combinations of NAA and IBA for root induction on the regenerated shoots (Table 20).

IBA (2.5x10^{-6}M) in the medium triggered root induction on the shoots to form complete plantlets after 10 days of incubation (Fig. 49). On the other hand, the medium containing NAA/IBA (5x10^{-6}M) induced callusing from the cut ends of the shoots near the medium surface and failed to induce rooting on the regenerated shoots.

Transfer to pots:
Healthy plantlets when attained 14-16 cm length with
Table - 20

The effects of NAA and IBA on root induction on regenerated shoots in *T. ammi*

<table>
<thead>
<tr>
<th>MS (1/2) + Phytohormones (µM)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA (2.5x10⁻⁶M) + IBM (2.5x10⁻⁶M)</td>
<td>-</td>
</tr>
<tr>
<td>IBA (2.5x10⁻⁶M)</td>
<td>Roots</td>
</tr>
<tr>
<td>NAA (5x10⁻⁶M)</td>
<td>Callusing only, at cut ends of the shoots</td>
</tr>
<tr>
<td>IBA (5x10⁻⁶M)</td>
<td>Only callusing at cut ends of the shoots</td>
</tr>
</tbody>
</table>

- Indicates no response
4-6 leaves were transferred to sterile pots containing garden soil, vermiculite and sand (1:1:1) (Fig. 50) and kept in moist chamber. The technique adopted for *P. ovata* in transplanting the regenerated plantlets to pots is also followed for *T. ammi*.

2. Somatic Embryogenesis:

Experiments were next conducted to induce somatic embryos from callus and cell suspensions derived from different explants. Somatic embryos were formed from the callus derived from 15 days old aseptically raised seedling explants such as cotyledonary leaf and hypocotyl. Cell suspensions obtained from callus cultures also formed embryoids as described below:

(a) Induction of somatic embryos from cotyledonary leaf callus

Callus was induced from the cotyledonary leaf explants cultured on MS medium containing 3% (w/v) sucrose, and supplemented with NAA (1x10^{-5} M) and Kn (5x10^{-6} M) after one wk of incubation. The callus initiated from the cut ends of the explants but eventually spread over the entire explant surface and proliferated further to form considerable pale green semifriable callus mass within 3 wks in culture. The callus was subcultured at every
Plate 12

Fig. 51 3 wks old callus of *T. ammi*
\[ \times 1.3 \]

Fig. 52 Root induction from cotyledonary leaf callus of *T. ammi*
Medium: MS + NAA (1x10^{-5}M)
\[ \times 1.5 \]

Fig. 53 Embryogenic cotyledonary leaf callus of *T. ammi*
(2 wks old cultures).
Medium: MS + NAA (1x10^{-5}M) + Kn (1x10^{-6}M)
\[ \times 1.9 \]
4 wk intervals and maintained on the same medium (Fig. 51). It remained undifferentiated on this medium.

In order to induce somatic embryogenesis, actively growing callus was then transferred onto MS medium containing NAA (1x10⁻⁵M) with/without low Kn (1x10⁻⁶M). In absence of Kn, the callus proliferated and induced few roots from its periphery; but no somatic embryos were formed even after 4 wks of incubation (Fig. 52).

On the other hand, in presence of Kn (1x10⁻⁶M), the callus turned embryogenic and somatic embryos were induced after 10 days of incubation. Several shining globular structures were observed scattered over the callus (Fig. 53). Microscopic observations revealed that they were somatic embryos (Fig. 54). The somatic embryos developed further into plantlets.

Various stages (globular, heart-shaped, torpedo, cotyledonary) of somatic embryos could be seen in the same callus indicating their asynchronous development. The close proximity of the embryo initiation sites produced frequent fasciations and distortions of cotyledonary lobes. During its growth the typical embryoid developed a notch in the medium region from
Plate 13

Fig. 54 Various stages of somatic embryos in *T. ammi*

(a) Globular (X 60)
(b) Heart shaped (X 60)
(c) Torpedo shaped (X 20)
(d) Cotyledonary mature embryo (X 20)

Fig. 55 Stages of somatic embryo germination in *T. ammi*

Medium: MNS + NAA (1x10^{-5}M) + Kn (1x10^{-6}M)
(X 2.5)
which shoot meristems differentiated later. Embryoids were attached to the parent tissues at the root pole and could be detached easily.

Cotyledonary leaf explants cultured directly on MS medium supplemented with NAA (1x10^{-5} M) and low Kn (1x10^{-6}M) induced callus after one wk. The callus proliferated and formed pale yellowish green callus mass but failed to induce somatic embryogenesis even after 4 wks of incubation. Clearly, callus induction on high Kn medium and subsequent transfer to low Kn medium was favourable for embryoid induction.

Maturation and germination of somatic embryos occured on the same medium i.e. MS medium containing NAA (1x10^{-5}M) and Kn (1x10^{-6}M), on which the embryoids were induced. However, the germinated embryoids developed into plantlets when transferred on to only Kn (1x10^{-6} M) containing medium (Fig. 55) as shown in flow chart (Table 21).

(b) Induction of somatic embryos from hypocotyl callus:

Hypocotyl segments formed pale yellow callus within 10 days of incubation on MS medium supplemented with 2,4-D (9 μM) and Kn (2.3 μM). The callus was maintained by regular subculture on the same medium at every 4 wk intervals (Fig. 51).
In order to induce somatic embryogenesis from the above callus, the level of 2,4-D was reduced gradually, while original conc of Kn was maintained on each subculture. Direct transfer of callus to a medium devoid of 2,4-D in presence or absence of Kn failed to induce embryogenesis. Similarly, reduction of 2,4-D level to 4.5 μM from 9.0 μM yielded nor esponse even after 4 wks. Maintaining the Kn level at 2.3 μM and reduction in 2,4-D conc from 4.5 μM to 2.25 μM in the next subculture also did not induce embryoid formation. Still further reduction of 2,4-D level to 0.45 μM and finally complete withdrawl of it from the MS medium containing Kn (2.3 μM) also proved ineffective to elicite embryoid induction. Similarly, transfer of callus from 2,4-D (2.25 μM) and Kn (2.3 μM) containing medium to BA (4.4 μM) and Kn (2.3 μM) medium also did not favour embryogenesis even after 4 wks though the callus turned green and its growth enhanced.

However, on transfer of callus from 2,4-D (2.25 μM) and Kn (2.3 μM) containing medium to lower conc of Kn (1 μM) in absence of 2,4-D resulted into embryogenic callus. Somatic embryos were noticed along the periphary of the callus within 4 wks. The developmental stages of embryoids which are shown in Fig. 54, recapitulated zygotic embryogenesis. These embryoids on transfer to MS
basal medium with/without Kn (1 μM) differentiated further and formed numerous new embryoids and secondary embryoids.

The somatic embryos germinated (Fig. 55) and developed into plantlets on MS medium supplemented with Kn (1 μM) alone (Flow Chart, Table 21).

To examine additive effect if any of myo-inositol (200 mg/l) and casein hydrolysate (100, 200 and 400 mg/l) on embryo formation, they were incorporated separately and simultaneously in the medium. This resulted into initial fast growth, but subsequent browning of callus, with few root formation. Elevated cytokinin levels (4.4 μM BAP + 2.3 μM Kn) in presence of myo-inositol as well as casein hydrolysate in the 2,4-D free MS medium also showed no indication of embryogenesis. At the same time attempts to omit Kn and 2,4-D in presence of myo-inositol or casein hydrolysate also proved ineffective.

(c) Induction of somatic embryos in hypocotyl cell suspension cultures:

Callus derived from the hypocotyl segments cultured on MS medium containing 3% (w/v) sucrose, supplemented
with 2,4-D (4 μM) and Kn (2 μM) and subcultured on the same medium every 4 wk remained undifferentiated (Fig. 51).

After several subcultures, the callus was transferred on to hormone-free MS basal medium to remove any carry over effect of 2,4-D and Kn. The callus which proliferated on this medium was next transferred after 10 days to MS liquid medium containing ABA (0.1, 0.3, 0.75 and 1.0 μM) or CM (0.5, 3.5, 5.0 and 1.0% v/v) to evaluate their effect on induction of somatic embryogenesis.

Somatic embryos of globular stage were formed within one wk in the medium supplemented with ABA (0.3 μM). Further development of embryoids was however, arrested in the medium and only globular embryoids with proliferated callus clumps were observed even after 4 wks of incubation. The other ABA conc (0.1, 0.75 and 1 μM) tested in MS liquid medium failed to induce somatic embryos and supported only callus formation with few roots in 3-4 wks.

CM at 3.5% (v/v) in MS liquid medium was found favourable for the induction of somatic embryos. Within one wk, globular embryoids were formed in this
treatment. After 3-4 wks numerous embryoids at different stages (globular, heart-shaped torpedo, cotyledonary) of development were observed (Fig. 54). The developmental sequence of the somatic embryos resembled that of the zygotic embryos except that the embryoid formation was asynchronous.

Various other conc of CM (.0.5, 5.0 and 10%) tried in MS basal liquid medium invariably promoted callus growth but failed to induce any somatic embryos even after 4 wks in cultures.

Isolated embryoids and clusters of somatic embryos along with embryogenic callus clumps, when plated on MS basal agar medium readily germinated (Fig. 55). The germinated embryoids, on transfer to Kn (1 μM) containing medium, developed into plantlets.

The entire protocol is presented in the flow chart (Table 21).

(d) Somatic embryogenesis in cell suspensions from shoot explant:

Callus was derived from shoot explants cultured on MS medium supplemented with 2,4-D (2.25 μM) and Kn
(4.5 µM). The callus was subcultured on the same medium at every 3 wk intervals (Fig. 51).

After several subcultures the callus was transferred to liquid MS basal medium and kept on shaker for somatic embryogenesis. Embryoids were observed after 7 days of incubation.

The embryos were harvested to study the different stages of development under microscope. Globular, heart shaped, torpedo shaped and mature embryos with cotyledons (Fig. 54) were observed.

After 4 wks these embryoids were plated onto agar MS basal medium where root and shoot primordia developed. Embryoids, however, did not germinate when maintained in liquid MS basal medium even after 4 wks of incubation. The liquid medium was essential for only induction, but the embryoids had to be later transferred to agar medium for further development into plantlets. The plantlets were found well developed on subsequent transfer of germinated embryoids (Fig. 55) onto Kn (1 µM) containing medium (Flow Chart, Table 21).

The scutellar part of embryoids turned green and leafy. The shoot apex produced leaves within 2 wks. The first
Plate 14

Fig. 56  First and second leaves on regenerated shoots typically cotyledon like and unifoliate in *T. ammi* (2 wks old cultures).

Medium: MS + Kn (1x10^-6 M)

(X 1.6)

Fig. 57  A dense mass of entangled plantlets and mixture of shoots, roots and embryoids in 3 wks old cultures of *T. ammi*

Medium: MS + Kn (1x10^-6 M)

(X 2.0)

Fig. 58  Fully grown plantlets on MS + Kn (1x10^-6 M) - transferred to MS basal medium (4 wk old cultures of *T. ammi*)

(X 1.5)

Fig. 59  Fully grown 12-14 cm long plantlet in 4 wk old cultures of *T. ammi*

Medium: MS basal

(X 1.3)
and second leaves on shoots were typically cotyledon like and unifoliate respectively (Fig. 56). This was followed by root formation from the root pole and fully developed green plantlets were formed within 3 wks. A dense mass of entangled plantlets and mixture of shoots/roots/embryoids resulted in the culture after 4 wks of incubation (Fig. 57).

Secondary embryogenesis was also evident. Multiple embryoids of early and late globular stages, developed on the young leaves of regenerated plantlets within 3-4 wks, and 80-90 plantlets, 0.5 to 6.0 cm tall bearing 2-10 leaves each, were formed (Fig. 58). The clusters of embryogenic callus, embryoids and plantlets on transfer to fresh medium of the same composition resulted into further proliferation and development of mature plantlets with nearly 100 plantlets formed in each culture flask, after 4 wks of incubation.

(e) Transfer to pots:

Plantlets appearing normal (14-16 cm long with 2-10) leaves (Fig. 59) were transferred to sterile pots containing garden soil, vermiculite and sand (1:1:1) (Fig. 60), and kept in moist chamber. The plantlets were watered with MS (1/4) medium without sucrose at two days interval. After 15 days the plantlets, thus
Plate 15

Fig. 60  Regenerated plantlets transferred to pot.
        (X 1.5)

Fig. 61  A typical somatic embryos with multicotyledons in
        *T. ammi*
        (X 60)
established in pots, were transplanted to the soil. Of the total plantlets produced, about 30% could be successfully transplanted to soil in the field.

(f) Development of atypical somatic embryos:

The frequency of atypical somatic embryos was very low. Mainly, two anomalous types were observed. The first is, the unipolar embryoids in which polarity of root development was strong, but the shoot did not develop at all (Fig. 61). The second is multicotyledonary embryoid. Normal embryoids of T. ammi have two equal sized cotyledons like the sexual embryos. However, a few embryoids that developed in vitro had three to four cotyledons (Fig. 61).

(g) Morphogenetic potential:

The morphogenetic potential of the callus did not suffer any change with age in NAA containing medium. The cotyledonary leaf derived callus maintained on the MS medium supplemented with NAA (1x10^{-5} M) and Kn (5x10^{-5} M) continued to form somatic embryos when transferred to low Kn medium i.e. NAA (1x10^{-5} M) and Kn (1x10^{-6} M) for at least one year. On the other hand, hypocotyl derived callus when maintained on MS medium containing 2,4-D (9.0 μM) and Kn (2.3 μM) lost its morphogenetic potential considerably and failed to form somatic embryos after 6-8 subcultures even with gradual reduction in 2,4-d level and finally complete withdrawal of it from the medium containing Kn (1x10^{-6} M).
Table - 21

Flow Chart - Somatic Embryogenesis in T. anom

Explants → Cotyledonary leaf → Hypocotyl cell suspension → Shoot cell suspension

Callus induction → NAA ($1 \times 10^{-5} M$) + 2,4-D (9 mM) + 2,4-D (5 mM) → 2,4-D (2.25 μM)

Gradual Removal of 2,4-D

Embryo formation → NAA ($1 \times 10^{-5} M$) + Kn (1 mM) + ABA (0.3 mM) or CM (3.5%)

MS basal

Germination → Kn (1 mM) → Kn (1 mM) → Kn (1 mM) → Kn (1 mM)
D. BIOCHEMICAL STUDIES:

Detection for Thymol in seeds, plant parts, callus and differentiated shoots.

The benzene extract obtained from various plant tissues was subjected to TLC. The standard thymol band was obtained at a Rf value 0.75. Similar bands (with same Rf value) from plant material were obtained.

Thymol was found to be present in various conc as could be seen by degree of colour development on TLC plates (Fig. 62) in various seed sources. The thymol was found to be present in undifferentiated callus mass and in differentiated shoots (Fig. 63). When various regenerated plantlets were screened for the presence of thymol, they too exhibited varied conc of thymol presence on TLC plates.

The order of contents as indicated by degrees of coloration on TLC plates was found highest in seeds followed by regenerated shoots and lowest in regenerated roots. Presence of thymol in callus was observed minimum.

Since most of thymol demands in our country (50 metric tonnes) are at present met by chemical synthesis, there seem to be no compelling reasons to isolate high thymol producing cell lines
Plate 16

Fig. 62  Thymol cone in various seed sources of *T. ammi* seen by degree of colour development on TLC plate

S  Standard thymol
1  Thymol from different seed sources

Fig. 63  Thymol detection in callus and regenerated plantlets seen on TLC plate in various cone

S  Standard thymol
C  Thymol in callus
R1  Thymol in regenerated plantlets
R2  Thymol in regenerated plantlets
of T. ammi. It would be more rewarding to improve, quantitatively as well as qualitatively, the seed yield to enhance Ajowan oil production for which there is increasing demand in perfumary industries and also for medicinal uses.
DISCUSSION

Micropropagation through apical/axillary meristem in *T. ammi*.

Shoot tip (meristem) culture has become an important technique for micropropagation. The application of the method in plant tissue culture offers true to type pathogen free plants in large numbers. The success arises partly from the fact that the apices of many virus-infected plants remain free of infection, although many other factors such as culture conditions may be involved. Though there are several reports on plant regeneration through shoot tip culture from ornamental and horticultural plant species, not many attempts are made yet in plants belonging to Apiaceae. Hence, studies were undertaken on this line with *T. ammi*.

Explant size and orientation were found critical in the present investigations with *T. ammi* shoot tip cultures. Explants measuring 2 cm produced shoot buds from the apical or axillary meristems, while 1 cm long explants induced callus only irrespective of their position in cultures. Large shoot tips, with its apparent availability of stored food reserve and endogenous growth regulators in the stem is preferred for the initiation of new growth (Anderson; 1980).

Vertically placed explants responded to induction of shoot buds from the apical or axillary meristems but horizontally inoculated explants failed to show any morphogenetic response. Perhaps, this reflects the *in vivo* system where similar phenomena is pronounced.
The results also revealed that high auxin (NAA/2,4-D) to low Kn MS medium could stimulate shoot bud formation from the apical or axillary meristems (Table 19). This is in contrast to the reports seen in *Picrorhiza kurroa* (Nandlal *et al.*, 1988), *Plantago ovata* (Barna and Wakhlu, 1988) and *Glehnia littoralis* (Hiraoka and Miho Oyanagi, 1989), where low NAA (0.01 - 0.3 μM) and high Kn/BAP (15 - 25 μM) containing medium proved effective to induce multiple shoots from the apical/axillary meristems. In *Aconitum carmichaeli* (Hatano *et al.*, 1988), only BAP (23.25 μM) in MS medium was required for shoot buds formation from axillary meristems of the explant.

Medium supplemented with NAA (1x10^{-5}M) and Kn (1x10^{-6}M) produced one or two plantlets from the apical meristem in 90% of the cultures (Fig. 44); while increased Kn level (5x10^{-6}M) in NAA (1x10^{-5}M) containing medium triggered 3-5 shoot buds from the axillary meristem (Fig. 45) in 80% of the cultures with scanty callusing from the cut end of the explant. Similarly, 3-5 shoot buds were also induced from the apical meristem in the medium supplemented with 2,4-D (4x10^{-6}M) and Kn (2x10^{-6}M) with more callus at the cut end of the explant in 70% of the cultures (Fig. 46), whereas NAA/2,4-D (1x10^{-5}M) and Kn (2.5x10^{-6}M) containing medium showed poor morphogenetic response by inducing 2-3 shoot buds from the apical meristem in 60% of the cultures with more callus at the cut end of the explant (Fig. 47). It seems Kn conc in high auxin containing medium is a limiting factor in achieving multiple shoot buds from
apical/axillary meristems. At the same time high NAA/2,4-D inhibited further growth of regenerated shoots. On transfer to medium containing NAA (1x10^{-6} or 1x10^{-7}M) and Kn (1x10^{-6}M) the shoots developed to maturity (Fig. 48). This suggests that low conc of NAA and Kn in the medium is promotory for shoots development.

IBA (2.5x10^{-6}M) in half strength MS medium triggered root induction on the shoots to form complete plantlets after 10 days of incubation (Fig. 49). On the other hand, NAA/IBA (5x10^{-6}M) induced cullusing at the cut ends of the shoots; while NAA + IBA (2.5x10^{-6}M) each yielded no response (Table 20) in T. ammi. Clearly, high auxin (IBA/NAA) in the medium is inhibitory for rooting the shoots, whereas low IBA (2.5x10^{-6}M) in the medium proved promitory for root induction on shoots.

In contrast to this, IBA (5x10^{-6} or 1x10^{-5}M) in Raphanus sativus (Kee Yoeup Pack et al., 1987), IBA (5x10^{-6}M) + NAA (5x10^{-7}M) in Plantago ovata (Barna and Wakhlu, 1988), and IBA (1x10^{-6}M) in Glehnia littoralis (Hiraoka and Miho Oyanagi, 1989) induced rooting on regenerated shoots.

At the multiplication rate of 3 to 5 shoots per 4 wk passage, 3000 to 5000 plantlets can be obtained in 6 months time. However, the rate of multiplication is reported to increase with subsequent passages in cultures. After an optimal number of subcultures, it may be possible to achieve 10 fold multiple index, which on future
projection, may yield over 50,000 plants in six months. If the projected yield is confirmed by future work, it would be commercially feasible to produce such economically useful plants of high and uniform quality by tissue culture methods.

Somatic Embryogenesis in T. ammi and P. ovata

In P. ovata regeneration of plants was obtained through direct organogenesis from explants. In case of T. ammi regeneration is achieved indirectly by somatic embryogenesis. The latter is fairly common as indicated in the Introduction (Chapter 1). In the present studies with T. ammi, a number of cultural parameters were found to have pronounced effect on the induction of somatic embryos from vegetable tissues. The results obtained in the investigation are discussed in light of relevant literature.

Somatic embryogenesis has been reported to be influenced by 50 substances belonging to 10 classes of organic and inorganic compounds, naturally occurring plant juices, plant extracts and some physical factors. Extensive studies on somatic embryogenesis have indicated that two important parameters, namely auxin and source of nitrogen influence the process most (Rangaswamy, 1986). However, what makes a cell competent for embryogenesis is still empirical.
Auxins:

For most cultures, an external auxins supply is necessary for the induction and maintenance of embryogenic cultures and its removal leads to embryo maturation (Ammirato, 1984). Rare instances of induction of somatic embryogenesis without exogenous auxin may represent systems that have optimal level of endogenous auxin (Vardi et al, 1975; Hu et al, 19780. Like in zygotic embryogenesis polarised distribution of endogenous auxin in tissue culture seems to be prerequisite for induction of embryogenesis. Though systematic investigation of auxin specificity in embryogenesis has not been carried out for most species, a variety of individual auxins have been reported to induce embryogenesis (Tisserat et al, 1979).

Of all the auxins tried, 2,4-D has proved extremely useful, having been employed in nearby 58% of successful embryogenic cultures (Evans et al, 1981). In a number of plants, the basic protocol followed to obtain embryoids involves induction of callus growth in an auxin enriched medium and then somatic embryogenesis upon transfer of callus to a medium free of auxin (Tisserat and Murashige, 1977). It is often argued though that auxins have no direct effect on promoting embryogenesis except by promoting callus growth which normally preceeds embryogenesis (Fujimura and Komamine, 1980).

In the present studies with Trachyspermum ammi also somatic embryos were induced from the hypocotyl and shoot after gradual withdrawal of 2,4-D from the medium. Direct transfer of cultures
from callus inducing high 2,4-D medium to very low, 2,4-D medium a
failed to induce embryogenesis. Stepwise reduction in 2,4-D conc in
presence of appropriate level of Kn was very important as shown in
the Flow Chart (Table 21).

Rappaport et al (1980) and Fuji (1982) have also reported plant
regeneration in callus and suspension cultures of celery cv Tall Utah
52-70 R after removal of 2,4-D from the callus medium. Similar
results are also reported in Foeniculum vulgare (Miura et al, 1987),
Pimpinella anisum (Ernst and Oesterhelt, 1984), Solanum carolinense
(Thomas Reynolds, 1986), Vitis longi (Gray and Mortensen, 1987) and
Papaver somniferum (Hsu and Pack, 1989).

It is clearly seen from most studies including the present one that
presence of 2,4-D is essential for the production of somatic embryos
either from the callus or from explants directly. However, for
further growth and development of embryos into plantlets, auxin is
not required. Low conc (1.0 μM) of Kn was found critical for embryo
development into plantlets in case of T. ammi (see Flow Chart,
Table 21).

There are several reports showing similar results such as Santalum
album (Lakshmi Sita et al, 1980), Cymbopogon (Jagadishchandra and
Sreenath, 1982), Dioscorea (Ammirato, 1984), Oryza sativa (Toshinori
and Yuzo Futsuhara, 1985), Papaver somniferum (Ilahi and Jabeen,
1986), Sugarcane (Gupta and Durzan, 1986), Clery (Williams and

In case of *P. ovata* leaf callus studied here the case was very different in that presence of 2,4-D was required not only for induction but also for maturation of somatic embryos. Similar observations have been made with Chinese celery (Zee and Wu, 1979), *Panicum maximum* (Chin-Yi, Lu and Vasil, 1981), *Saccharum officinarum* (Wai-Jane Ho and Vasil, 1983), *Solanum melongena* (Gleddie et al., 1984), *Foeniculum vulgare* (Hunault, 1984; Miura et al., 1987), *Manihot esculenta* (Stampe, 1987; Stamp and Hanshaw, 1987), *Gossypium hirsutum* (Normal Trolinder and Goodin, 1988) and *Vigna mungo* and *V. radiata* (Eapen and George, 1990).

An interesting situation was noticed with *T. ammi* cotyledonary leaf callus induced on high NAA (1x10^-5 M) and Kn (5x10^-6 M). It was not the reduction in NAA conc but that in Kn conc (1x10^-5 M) that turned the callus embryogenic. Complete removal of Kn in presence of NAA induced few roots but not embryoids. Thus, retention of NAA seemed essential in this system and its acting synergistically with Kn to elicit somatic embryogenesis is significant (see Flow Chart, Table 21).
Matsuoka and Hinata (1979) had reported maturation of eggplant somatic embryos to the cotyledonous stage in the presence of NAA. In another species, however, while auxins induced the embryogenic stage, the inhibit embryo development beyond the immature globular stage. This is the case for carrot (Halperin and Withrell, 1964; Fujimura and Komamine, 1975; Sung, 1979), Nigella (Banerjee and Gupta, 1976), Celery (Al-Abta and Collin, 1978), Antirrhinum (Sangwan and Harada, 1975), Citrus (Kochba and Spiegel-Roy, 1977) and Brassica (Pareek and Chandra, 1978). In all these species embryo formation required a short inductive phase on auxin followed by its removal or reduction in conc.

On the other hand, in case of inflorescence axis callus of P. ovata induced on NAA + Kn medium, it was gradual reduction in NAA (1x10^-7 M) and rise in Kn conc (2.5x10^-5 M) that induced embryo formation. Similar interactions of NAA + Kn have been reported in Atropa (Cosch et al., 1975), alfalfa (M. Coy and Walker, 1984), Achrus sapota (Sachdeva and Mehra, 1986) and Arachis hypogaea (Natraja and Patil, 1987).

Unlike above cited cases where either 2,4-D or NAA is essential for the induction of embryogenesis, there are a few instances in which both the auxins (NAA, 2,4-D) and Kn are required to form embryoids, e.g. Aesculus hippocastanum (Rosa Marina Dameri et al., 1986), Trifolium repens (Pederson, 1986) and Arachis hypogaea (Hazra et al., 1989).
Somatic embryogenesis is presumably controlled both at the transcriptional (Fujimura and Komanine, 1982) and translational (Raghavan, 1983) levels. However, the control of synthesis of tissue specific proteins is an event far removed from the primary action of auxin. Because the same hormone can evoke varied molecular events in different tissues (Wareing, 1971; Zeroni and Hall, 1980), future research on auxin mechanism in somatic embryogenesis must be directed to discover a primary auxin action such as binding with a receptor or insertion into a membrane (Vanderhoef and Kosuge, 1984) and in turn a control of ion fluxes. Auxin may modify the 3 dimensional structure of acyl lipids in membrane and thus influence the transport and/or detection at the membrane level of chemical signals (Warren and Fowler, 1979) essential for somatic embryogenesis.

Cytokinins:

Although there is no compelling evidence for a universal requirement of cytokinins for somatic embryogenesis, they do play an important role in the induction, especially for maturation and germination of somatic embryos (Fujimura and Komamine, 1980). Data on the nature and amounts of cytokinins used for induction of somatic embryogenesis has been compiled by Evans et al (1981).

In the present studies with T. ammi and P. ovata, Kn proved essential for somatic embryogenesis. Whether the auxin (2,4-D) was gradually removed (as in case of T. ammi hypocotyl callus and cell
suspension, or cell suspensions of shoot explants and leaf callus of
P. ovata) or NAA was retained (as in the case of cotyledonary leaf
callus of T. ammi and inflorescence axis cell suspensions of P.
ovata, the conc of Kn and its quantitative interaction with auxins
was found critical not only for the induction of somatic embryos but
also for their germination into plantlets (see Flow Chart,
Tables 16, 21).

Cytokinins have been implicated as a necessary factor for somatic
embryogenesis in some other species (Mullins and Srinivasan, 1976.
Sondahl and Sharp, 1977); Kavathekar and Johri, 1978; Desai et al,
1986; Maheswaran and Williams, 1986; Bauchan, 1987; George and

On the other hand, when Kn (2.5x10^{-5}M) was replaced with BAP
(5x10^{-6}, 1x10^{-5}, 2x10^{-5} and 2.5x10^{-5}M) in liquid medium containing
NAA (1x10^{-7} and 1x10^{-6}M) no induction of somatic embryos occured in
inflorescence axis cell suspensions of P. ovata though the callus
turned green and formed a few roots. Similarly, in Nigella (Banerjee
and Gupta, 1976), citrus (Kochba and Spiegel-Roy, 1977), Daucus
carota (Kamada and Harada, 1979), Panax (Chang and Hsing, 1980)
and Perilla (Tonimoto and Harada, 1980) embryogenesis was inhibited
by cytokinins.

This is in contrast with soapnut cultures where both Kn and BAP had
promotory effect on embryogenesis (Desai et al, 1986). Similarly,
somatic embryogenesis was reported in BAP containing auxin-free medium in Albizzia richardiana tissue cultures (Tomar and Gupta, 1988). While all the cytokinins tested failed, Zeatin and Zeatin riboside formed somatic embryos in Hyoscyamus niger (Raghavan and Nagmani, 1988).

*In T ammi* the somatic embryos germinated and developed into plantlets only on transfer to MS medium containing Kn (1 μM). Somatic embryo of *Sorghum almum* (George and Eapen, 1988) also germinated into plantlets upon transfer to MS basal medium containing Kn (0.5 mg/l) or BAP (1 mg/l) and developed good root system on NAA containing medium. Embryoids of *Pennisetum americanum* (Vasil and Vasil, 1981) developed into robust seedlings on transfer to MS medium plus 1 mg/l 2iP or Zeatin. Dale et al. (1981) grew germinated Italian rye-grass embryos on MS + 0.2 mg/l Kn before they were transferred to soil. On the other hand, Gray and Mortensen (1987) found BAP alone effective to stimulate germination of somatic embryos into plantlets in grape. In *Dysoma pleiantha* (Meng–Jin Chuang and Web-Chin Chang, 1987), the embryoids derived from young leaves and rhizomes germinated into plantlets in MS/B5 medium containing BAP (1 mg/l) and GA3 (1 mg/l).

Interestingly in Dioscorea, embryos subcultured in groups of five developed shoots and roots on unsupplemented medium, but if transferred singly they would only grow if the medium was supplemented with Zeatin (0.2 mg/l) and glutamine (500 mg/l).
(Ammirato, 1984). However, plantlets were formed on hormone-free
basal medium in *Digitalis lanata* (Kuberski and Scheibner, 1984),
*Medicago scutellata* (Barwale et al, 1986) and *Musa ornata* (Sandra et
al, 1988). In case of *T. ammi*, on the other hand, transfer to MS
basal medium failed to support healthy growth of somatic embryos.

Besides auxins and cytokinins, in several taxa especially in
Apiaceae plants, coconut milk (CM) has promoted somatic
embryogenesis when used alone or with hormones. The promotory
effect of CM to foster embryogenesis is well documented in *Conicium
maculatum* (Steward et al, 1970; Netien and Raynaud, 1972),
*Coriandrum sativum* (Joshi and Raghuvanshi, 1966; Steward et al,
1970) *Anethum graveolens* (Johri and Sehgal, 1965; Ratnamba and
Chopra, 1974), *Sium suave* (Steward et al, 1970), *Pimpinella anisum*
carota* (Ammirato, 1984) and *Urginea* (Jha and Sen, 1986).

In present studies with *T. ammi* also CM at 3.5% (v/v) conc in
liquid hormone-free medium stimulated somatic embryogenesis.
However, increase/decrease in conc from 3.5% proved inhibitory to
foster somatic embryogenesis; but promoted callus proliferation with
few roots.

The effective conc of CM for somatic embryogenesis varied from
plant to plant; 2.5% in *Panicum maximum* (Chin-Yi, Lu and Vasil,
1981), 5% in sugarcane (Wai Jane Ho and Vasil, 1983) and in
Zea mays (Swedlund and Locy, 1988), while 10% CM fostered somatic embryogenesis in Achrus sapota (Sachdeva and Mehra, 1986). However, all conc of CM tried in MS medium failed to induce normal somatic embryos in leaf and inflorescence axis cell suspension cultures of P. ovata.

According to Kovoor (1962) the stimulating factor of CM has characteristic of indole compounds as well as nucleic acid bases; while Letham (1974) demonstrated that the cytokinin of CM was ribosylzeatin which was verified by Van Staden and Drewes (1975).

Instead of growth regulators/promoters, a growth inhibitor ABA was also employed in present investigation with T. ammi hypocotyl cell suspensions in hormone-free liquid MS medium. Embryogenesis was observed only at 0.3 µM conc in the range 0.1 to 1.0 µM ABA tested, whereas the rest of ABA conc promoted callus proliferation and a few roots.

In embryogenic cultures of caraway ABA curtailed such aberrations as pluricotyl, secondary regeneration and precocious germination of embryos and promoted a normal course of ontogeny and maturation of somatic embryos (Ammirato, 1974). The normalizing influence of ABA on somatic embryogenesis has been reported for certain other systems also (Kochba et al., 1978; Kamada and Harada, 1981; Vasil and Vasil, 1981). On the other hand, its inhibitory effect too is observed in some cases (Tisserat and Murashige, 1977b; Kamada and Harada,
Increasing the conc of ABA (upto 10 mM/l) caused progressive decrease in number of somatic embryos formed (Tisserat and Murashige, 1977b).

Timely addition of ABA to embryogenic culture increased the number of heart-shaped embryos (Phillips and Collins, 1981). The frequently observed absence of heart-shaped somatic embryos in cultures of several dicotyledonous species points to a stage-development role of naturally occurring growth inhibitors such as ABA in normal embryogenesis.

Moreover, continued presence of ABA is inhibitory for somatic embryos to develop into plantlets; for the plantlets were formed from the embryoids in T. ammi hypocotyl cell suspensions when the latter were transferred to Kn containing medium devoid of ABA. In contrast to this, presence of ABA (0.4 mM) in the medium not only promoted somatic embryogenesis but the embryoids also developed into plantlets in Hordeum vulgare (Rengel, 1986); while in Brassica juncea (Eapen et al., 1989) somatic embryos germinated into plantlets when medium was supplemented with GA3 and ABA.

In case of P. ovata in the present studies, ABA (0.3 mM), with/without CM in the medium, promoted callusing and root formation instead of normal somatic embryods in inflorescence axis cell suspensions.
The role of nitrogen compounds in embryogenesis has been studied extensively. Tazawa and Reinert (1969) had observed that embryogenesis in vitro could be induced by both inorganic and organic compounds. When either NH$_4^+$ or NO$_3^-$ was sole source of nitrogen in the culture medium, somatic embryogenesis did not occur or incidence was infrequent. When the ratio of NO$_3^-$ to NH$_4^+$ was 2:1, as in Murashige and Skoog medium, somatic embryogenesis is reported to occur with the highest frequency in eggplant (Gleddie et al., 1983). In the present investigations with *P. ovata* and *T. ammi*, MS (1962) medium is used without altering the conc of individual components for successful somatic embryogenesis.

In many systems it is found that casein hydrolysate (CH) and amino acids stimulate somatic embryogenesis (Stuart and Strickland 1984; Stuart, 1985; Mauro et al., 1986; Swedlund and Locy, 1988). For instance, 1 or 2 g/l CH or 4.4 mM glutamine with 3.1 mM proline caused 20-30% increase in the number of embryoids in *Medicago sativa* (Meiger and Brown, 1988). However, in the present studies with *T. ammi* and *P. ovata*, incorporation of CH over a range of conc in MS medium failed to induce somatic embryogenesis.

Among the amino acids used as sole sources of nitrogen, glutamine or its products is critical for embryogenesis (Wetherell and Dougall, 1976; Kamada and Harada, 1979b; Sharp et al., 1980). Glutamine most readily promoted embryogenesis in carrot tissue cultures.
(Kamada and Harada, 1979b), when added to a medium containing NH$_4^+$ nitrogen. However, in the present studies with _P. ovata_ it was found ineffective for somatic embryogenesis.

Proline too is known to enhance somatic embryogenesis in Maize (Armstrong and Green, 1985) and pollen embryogenesis in cereals (Sozinov et al., 1981); while tryptophan favoured somatic embryogenesis in some cultivars of rice (Siriwardana and Nabors, 1983). A tryptophan analogue, 5 methyl tryptophan was found effective in preventing recallusing and precocious germination of somatic embryos in soapnut (Desai et al., 1986). However, in inflorescence axis cell suspensions of _P. ovata_ studied here, 5 methyl tryptophan proved ineffective to induce normal somatic embryogenesis and to prevent precocious germination of embryos.

**Carbon/Energy Source:**

Sucrose (2-3%) is proved to be the best carbon source in most investigations, although glucose (Homes, 1967; Eapen and George, 1990), fructose (Callebaut et al., 1987) and galactose (Kochba et al., 1978b) also promote somatic embryogenesis in some instances.

Sucrose requirements for somatic embryogenesis varies from plant to plant, for example, 1% in _Musa ornata_ (Sandra et al., 1988), 2% in _Glycine max_ (Hammatt and Davey, 1987) and _Abies alba_ (Schuller et al., 1989), 2% for leaves and 6% for embryos in _Zea diploperennis_ (Swedlund and Locy, 1988), 5% in _Sinocalamus latifolia_ (Meel-Ling-Yeh
and Wol Chin Chang, 1987), 9% in *Triticum aestivum* (Chu and Hill, 1988), 6-10% in *Saccharum officinarurn* (Wai Jane Ho and Vasil, 1983), 13% in *Brassica napus* (Chuong and Beversdorf, 1985). Efficiency of embryogenesis increased as sugar concentration decreased from 12 to 3% in *Glycine max* (Lazzeri et al., 1987).

In the present studies with *T. ammi*, 3% sucrose is used for somatic embryogenesis. However, in *P. ovata*, in leaf cell suspensions 2% and in inflorescence axis cell suspensions 4% sucrose were used for somatic embryogenesis.

Though somatic embryos usually germinate in the medium containing 2-3% sucrose higher than normal concentration can sometimes favor embryo growth. Somatic embryos of Citrus were found by Kochba and Button (1974) to develop best on Murashige and Tucker (1969) medium containing 6% sucrose, and Lu et al. (1982) used MS medium with 12% sucrose for the induction and growth of maize embryoids.

In rice sorbitol or manitol in combination with sucrose was found beneficial for inducing differentiation in long term cultures (Kavi Kishor, 1987). Mannitol (3%) produced largest number of singular somatic embryos in celery cultures (Barry et al., 1989). Not many detailed studies are made to determine as to how much sugar is required as carbon/energy source and how much to provide suitable osmoticum.
Like morphogenesis in general, embryogenesis occurs more readily in short term cultures, and this ability decreases gradually with the increasing duration of the cultures (Steward et al., 1967; Smith and Street, 1974; Reinert et al., 1977). The decline has been attributed partly to the genetic changes induced during prolonged culturing. Increase in the percentage of aneuploid cells is known to accompany the decline in embryogenic potential (Smith and Street, 1974). It is proposed that the embryogenic potential can be prolonged by altering the ratio of N and auxin (Reinert, 1973) or by adjusting osmoticm of the medium (Kavi Kishor, 1987).

Aberrant Embryos:

Somatic embryos resemble to zygotic embryos in major morphological features, but vary in Ontogeny. It has been observed that embryos induced by NAA typically exhibit clear bipolarity (Lazzeri et al., 1987). The most frequent abnormalities seen are the loss of one or more cotyledons or fusion to the parent tissue. Somatic embryos induced on 2,4-D are in general horn-shaped, with indistinct or fused cotyledons.

Arcioni et al. (1982) reported the formation of some embryos of Medicago glutinosa with two cotyledons while others had fused cotyledons. Sauner and Bingham (1972) also observed the formation of embryos with fused cotyledons in Medicago sativa. Somatic embryos were white and exhibited frequent pluricotyly and tended to be larger than zygotic embryo in Vitis longii (Gray and Mortensen,
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1987). Globular somatic embryos elongated and formed numerous cotyledons in a repetitive and true to type polyembryonic process in sugarpine embryo cultures (Gupta and Durzan, 1986).

In the present studies with *T. ammi*, the frequency of atypical somatic embryos was very low. Two anomalous types were observed. The first is, the unipolar embryoid in which polarity of root development was strong, but the shoot did not develop at all. The second is multicotyledonary embryoid. Normal embryoids of *T. ammi* have two equal sized cotyledons like the sexual embryos. However, a few embryoids that developed in vitro had three to four cotyledons. (Fig. 61). This may be due to prolong exposure of cultures to 2,4-D and Kn. 2,4-D is known to cause aberration in vitro cultures during developing events.