

## Somatic embryogenesis in *Pimpinella tirupatiensis* Bal. and Subr., an endangered medicinal plant of Tirumala hills

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**Hypocotyl segments were excised from 4-week-old aseptic seedlings of *Pimpinella tirupatiensis*, a medicinal plant and were cultured on MS medium with TDZ (1 mg/l) and NAA (0.5 mg/l), which gave rise to friable, pink callus after 4 weeks of culture. Embryogenic callus on transfer to MS medium containing TDZ (1 mg/l) produced somatic embryos after 8 weeks having dark green shoots and white hairy roots. On MS + TDZ (1 mg/l) + BA (1 mg/l), somatic embryo formation was enhanced. Embryos isolated and germinated in the presence of MS + TDZ (1.0 mg/l) and GA<sub>3</sub> (1.0 mg/l) showed normal flowering without any morphological variation on transplantation to soil.**

*PIMPINELLA tirupatiensis* Balk and Subr. (1960)<sup>1</sup>, locally known as 'adavikothimeera' (forest coriander) is a herbaceous medicinal plant, distributed on Tirumala Hills (1000 m above msl) of Chittoor district, Andhra Pradesh<sup>2</sup>. It is a narrow endemic species (Umbelliferae) of seasonal occurrence with underground tuberous root system<sup>3</sup>.

Dried roots of *P. tirupatiensis* are administered along with few other ingredients to cure colic and rheumatic ailments in cattle<sup>4</sup>. The local Yanadhi tribal community uses the tuberous roots of *P. tirupatiensis* to cure severe ulcers of stomach, throat and genital organs and also as aphrodisiac<sup>5</sup> and as abortifacient agents<sup>6</sup>. Fruits are used to cure asthma and are considered as an effective remedy for 'flatulent colic'<sup>5</sup>.

Over the last few years, extensive damage caused to the natural habitats of rare species of Tirumala hills due to several reasons<sup>7</sup> has depleted wild strands of many endemic species in this region. Indiscriminate forest fires, overgrazing, illegal collection of tubers and seeds for various medicinal uses are certain major causes for the rapid depletion of *P. tirupatiensis*. Once described as 'the queen of herbaceous vegetation' of Tirumala Hills<sup>8</sup>, now the distribution of this rare species is regrettably confined to very few areas. *P. tirupatiensis* is at present under

endangered status<sup>9</sup>. Conventional propagation methods through seed and root tubers for cultivation of *P. tirupatiensis* are beset with limited planting material and poor fruit setting. The availability of the seed is also very less due to its dispersal by wind, on attaining maturity. In the present investigation, a regeneration protocol through somatic embryogenesis is attempted to conserve this rare species of Umbelliferae for posterity.

Seeds of *P. tirupatiensis* collected from Tirumala forest region during the month of January were surface sterilized by rinsing them for 5 min in 1% (v/v) Teepol and 5 min in 0.05% (w/v) HgCl<sub>2</sub> followed by 5–6 rinses in sterile distilled water. Surface-sterilized seeds were blotted on pre-sterilized filter papers and were germinated on Murashige and Skoog (MS)<sup>10</sup> medium containing GA<sub>3</sub> (0.1–2 mg/l) in 30 cm × 125 cm culture tubes. Different explants obtained from the seedlings like leaf, internode and hypocotyl segments were inoculated onto media containing various concentrations of TDZ (0.5, 1.0, 1.5 and 2.0 mg/l). All media were solidified with 0.8% agar after adjusting the pH to 5.8 and cultures were incubated in the light (16 h, 3000 lux) at 25 ± 2°C.

Friable, dark, pink regions (200 mg FW) of the proliferating hypocotyl callus obtained on MS medium containing TDZ (0.1–2 mg/l) and NAA (0.5 mg/l) were transferred onto MS medium with different combinations of TDZ, BA and 2,4-D (Table 1). Callus was selectively subcultured onto fresh medium containing TDZ alone and in combination with BA. The embryogenic capacity was determined at the end of 8 weeks of culture as the mean number of epicotyl and hypocotyl formed per explant.

MS basal salts, TDZ and BA were used alone and in combination with GA<sub>3</sub> to enhance frequency of maturation of somatic embryo to plants. All other culture conditions were maintained the same. Matured plantlets were soaked in water for an hour and transferred in a mixture of soil and vermiculite (1 : 1) in 6 cm plastic pots and covered with polythene bags for two weeks under culture-room conditions. They were later gradually exposed to low humidity by removing the polythene cover and transferred to a shade-house. Percentage of survival was calculated at the end of six weeks of transplantation.

Germination of seeds was not noticed in water agar medium as well as MS medium free of any growth regulators. However, seeds inoculated on MS medium containing GA<sub>3</sub> (1.0 mg/l) germinated (65%) within three weeks. Hypocotyl segments were found to induce high frequency of embryogenic callus among various seedling explants and hence further studies were restricted only to hypocotyl callus.

Among the various combinations of TDZ and NAA tested (Table 2), callus induction with a maximum of 95% was observed from hypocotyl explants in presence of TDZ (1.0 mg/l) and NAA (0.5 mg/l). The type of explant can influence<sup>11</sup> the effective concentration of TDZ for promoting morphogenic response *in vitro*. Friable and

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**Table 1.** Effect of growth regulators on embryogenic callus induction from hypocotyl segments of *P. tirupatiensis* (after 8 weeks of culture)

Concentration of growth regulator (mg/l)			Nature of callus	Percentage calli forming embryos	*Mean number of cotyledonary-stage embryos
TDZ	BA	2,4-D			
–	–	–	No response	–	–
0.5	–	–	White, nodular, embryogenic	53.3	3.1 ± 0.4
1.0	–	–	White, nodular, embryogenic	75.3	6.1 ± 0.1
2.0	–	–	White, nodular, embryogenic	68.3	4.0 ± 0.6
–	0.5	–	White, friable	–	–
–	1.0	–	White, friable	–	–
–	2.0	–	Green, nodular, embryogenic	44.0	2.3 ± 0.7
0.5	0.5	–	White, nodular, embryogenic	67.3	4.0 ± 0.6
1.0	1.0	–	Green, nodular, embryogenic	74.0	7.8 ± 0.5
–	–	0.5	White, compact	–	–
–	–	0.5	White, friable	–	–
–	–	0.5	White, friable	–	–

\*Mean of 12 replication ± S.E. (standard error).

**Table 2.** Effect of different concentrations of BA and NAA on callus formation from various explants derived from aseptic seedlings of *P. tirupatiensis*

BA (mg/l)	NAA (mg/l)	Leaf		Internode		Hypocotyl segments	
		Freq. (%)	Deg.	Freq. (%)	Deg.	Freq. (%)	Deg.
0.0	0.0	0.0	–	0	–	0	–
0.5	0.1	47	+	25	+	55	++
1.0	0.1	40	+	49	++	60	++
2.0	0.1	–	–	–	–	35	+
0.5	0.5	30	++	60	++	78	++
1.0	0.5	45	+	65	++	95	+++
2.0	0.5	42	+	73	++	80	++

Values are mean of 20 replicates after four weeks of culture. Freq., Frequency of callusing. Deg., Degree of callusing: less (+), moderate (++) and high (+++).

white hypocotyl callus, when subcultured onto embryo-induction medium containing TDZ, developed dark pink regions as a result of accumulation of anthocyanin pigments (Figure 1 a) which were selectively isolated and further subcultured onto MS medium containing various concentrations of TDZ in combination with BA and 2,4-D.

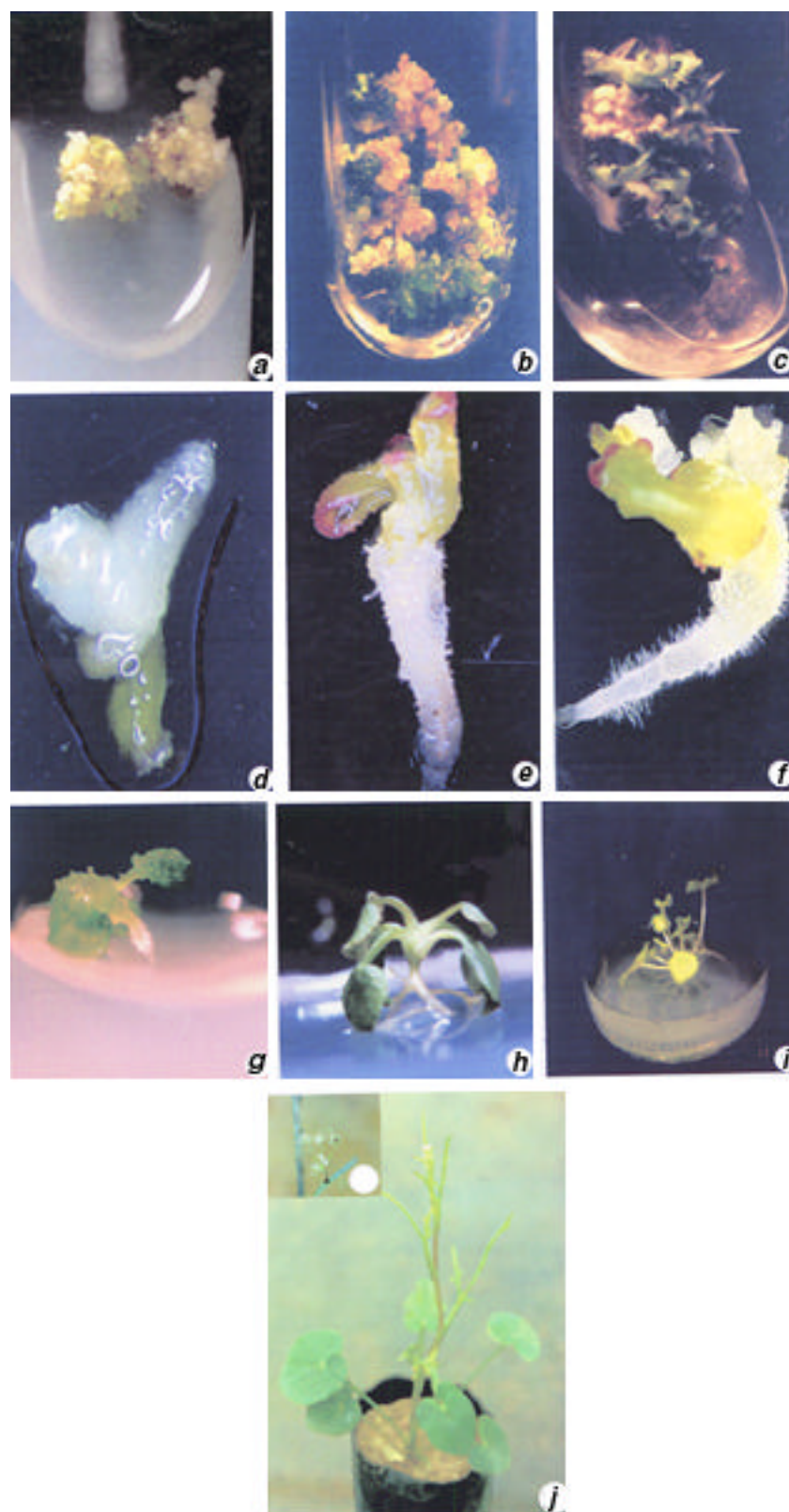
Hypocotyl callus showed varied responses when subcultured under different growth regulator regimes (Table 1). The callus transferred to MS medium with 2,4-D was white, friable and did not show any embryogenic response. On media supplemented with low concentrations of BA (0.5, 1.0 mg/l) as a single growth regulator, the callus remained white, friable and proliferative in nature, without any signs of embryo formation till 8 weeks of culture (Figure 1 b). However, higher concentrations of BA (2.0 mg/l) induced a mean of 2.3 (± 0.7) somatic embryos with lowest frequency (44%) of embryo induction, observed among all tested combinations. The callus transferred to MS medium containing TDZ alone and in combination with BA turned nodular and embryogenic,

after two subcultures with a passage of four weeks (Figure 1 c). TDZ (1 mg/l) alone induced a maximum of 6.1 (± 0.1) somatic embryos with a high frequency of (75%) embryo induction.

A combination of TDZ (1.0 mg/l) with BA (1.0 mg/l) in MS medium increased the mean number (7.8 ± 0.5) of somatic embryos formed with an embryo induction frequency of 74%. At this stage, somatic embryos of 3–5 mm in length were developed and characterized with two well-developed cotyledons and an elongated hypocotyl root axis showing axial polarity (Figure 1 d–f). According to Krishnamurthy<sup>12</sup>, somatic embryos are unipolar in development and such roots are only adventitious by origin. The high frequency of somatic embryo induction in our results suggests that it might influence the endogenous level of cytokinin, auxin and abscisic acid, so as to induce the positive embryogenic response of the activated tissue<sup>13,14</sup>.

Although somatic embryos resembling zygotic embryos through concomitant formation of shoot and root poles, were induced on a TDZ-supplemented medium, the shoots failed to elongate and were often fasciated when further subcultured on the same medium combination. Fasciation of the shoots on TDZ-supplemented medium, has been reported in several other species such as *Malus*<sup>15</sup> and *Rhododendron*<sup>16</sup>; the possible cause was attributed to the phenyl group present in TDZ<sup>17</sup>.

To promote development of somatic embryos in *P. tirupatiensis*, MS medium containing TDZ and BA in combination with GA<sub>3</sub> was tested. Except in growth regulator-free medium, maturation of somatic embryos was noticed in all other combinations. BA in combination with GA<sub>3</sub> showed a maximum of 54% cultures with epicotyl formation and 45% cultures with radicle formation and only 18% of the cultures survived with high rate of mortality. However, in MS medium supplemented with TDZ (0.5 mg/l) and GA<sub>3</sub> (0.5 mg/l), 80% of the cultures



**Figure 1.** *a*, Induction of callus with pink regions (proembryogenic mass) from hypocotyl segments of *P. tirupatiensis* on MS medium with TDZ (1 mg/l) + NAA (0.5 mg/l); *b*, White, friable, proliferative callus obtained on MS medium containing BAP alone; *c*, Formation of dark-green embryogenic callus on MS medium with BAP (1 mg/l) + TDZ (1 mg/l); *d-f*, Development of embryos with shoot and root primordia after 8 weeks of culture on MS medium containing BA (1 mg/l) + TDZ (1 mg/l); *g*, Maturation of somatic embryos upon transfer to MS medium with TDZ (1.0 mg/l) + GA<sub>3</sub> (1.0 mg/l); *h*, Somatic embryos showing formation of cotyledonary leaves and hairy roots; *i*, Plantlets regenerated through somatic embryogenesis show formation of rosette leaves after four weeks; *j*, Acclimatized plantlet showing normal flowering after 8 weeks. (Inset) Close view of the inflorescence with six umbels.

**Table 3.** Effect of various growth regulators on development of somatic embryo in *P. tirupatiensis*

Concentration of growth regulator (mg/l)			Frequency of epicotyl formation (%)	Frequency of radicle formation (%)	*Survival (%)
TDZ	BA	GA <sub>3</sub>			
–	–	–	–	–	–
–	0.1	–	25	15	8
–	0.5	0.5	40	12	12
–	1.0	1.0	54	45	18
0.1	–	–	65	65	70
0.5	–	0.5	80	80	63
1.0	–	1.0	86	75	89

\*After 6 weeks of field transfer.

showed epicotyl and hypocotyl formation (Figure 1 g). Results of GA<sub>3</sub>-promoted germination of somatic embryos were already observed in *Lavatera thuringiaca*<sup>18</sup> and in *Iris germanica*<sup>19</sup>. In the present investigation, TDZ (1.0 mg/l) in combination with GA<sub>3</sub> (1.0 mg/l) was found to be comparatively more effective than BA for somatic embryo maturation, where a maximum of 89% of the embryos was successfully regenerated into plantlets (Table 3).

Of the 300 plantlets transferred to soil, 260 (86.6%) survived. Regenerated plantlets through somatic embryos showed normal growth (Figure 1 i) and flowering pattern (inset, Figure 1 j) in 90% of the cultures. The method described here is useful for producing large number of plantlets of *P. tirupatiensis* for reintroduction in this region.

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## *In vitro* micropropagation of *Citrus aurantifolia* (lime)

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**This paper describes a micropropagation technique for lime, *Citrus aurantifolia* Christm. Swing., using nodal explants of mature trees. Nodes were cultured on Murashige and Skoog medium containing indole-3-butyric acid (IBA) at 0, 0.5 and 1 mg l<sup>-1</sup> combined with 6-benzylaminopurine (BAP) at 0, 0.25, 0.5, 1 and 2 mg l<sup>-1</sup>, in combination with 6-furfurylaminopurine (kinetin) at 0, 0.5 and 1 mg l<sup>-1</sup>. Best results for multiple shoot formation, 8 shoots per node, were obtained with 1 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> kinetin. The concentration of IBA had little effect on shoot multiplication. Shoot elongation appeared to favour 0.25 mg l<sup>-1</sup> BAP combined with 1 mg l<sup>-1</sup> kinetin. Shoot elongation and leaf size were inhibited in response to high levels of BAP. Transfer of shoots to a rooting medium induced the highest percentage of rooting, 56%, on 1 mg l<sup>-1</sup> IAA. Plantlets survived in soil and exhibited normal growth in a greenhouse.**

LIME, *Citrus aurantifolia* Christm. Swing. (family Rutaceae) is a commercially important crop in the tropical and subtropical regions. *Citrus* regeneration and genetic transformation have been the focus of numerous studies in the past<sup>1–3</sup>. Indirect and direct shoot regeneration of lime has been demonstrated<sup>4–9</sup>. However, in the previous regeneration studies of lime as well as other *Citrus*

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