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In vitro micropropagation of *Pentatropis capensis* (L.f.) bullock (Apocynaceae)

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Abstract
Pentatropis Plantlets were produced *In vitro* from the shoot and a nodal segment with an effective procedure developed with higher frequency with rapid rejuvenation. *In vitro* culture tubes were initiated by meristematic explants on MS medium with various growth hormones individually as well as combinations. Among the two concentrations used Benzyl amino purine and Kinetin with 2, 4-D the best for callus induction is 95% at 2.0 + 1.5 mg/l concentration. This clearly shows combinations of both used in cytokinins Benzyl amino purine alone at 3 mg/l induced maximum number of shoot buds when compared with KIN alone. Well-developed shoots were excised from cultured tubes and cultured on MS media having different concentrations like 0.1 to 1.2 mg/l and NAA is more effecient than IBA at 0.6 mg/l. NAA is the best concentration for auxin by proper rooting where 85% shoots were able to rooted within six weeks.

Keywords: micropropagation, *Pentatropis capensis*, Apocynaceae

Introduction
Plants have been an important source of medicine for thousands of years ago. Many drugs have their source in medicinal plants. Even today WHO says up to 80 per cent of people still rely on folklore medicines plants are resources of new drugs. Continuous depletion of many species medicinal plants from the wild and substantial loss of their habitats for the past fifteen years may resulted in the decline of population which leads do many high value medicinal plant species over the years (Kala, 2003) [1]. The main threats to medicinal plants is biodiversity caused by humans (Rao *et al*, 2004) [2]. Other disturbances also causes medicinal plant species like narrow range of distribution of species, agriculture land used for disturbances, habitat specificity, alteration, seed dormancy and explotion of human population and degradation of population (Oostermeijer *et al*, & Kala 2005) [4]. Most medicinal plants are now under threat due to human interaction.
Pentatropis capensis is a twining herb of the family Apocynaceae. The local name of the species is ‘‘Uppilankodi’’. This species can be found in Deccan and Carnataka, in hedges and open forest land. The crude extract of the plant have een used as a folklore medicine for treating many ailments such as antifungal, anti rheumatic, anti-inflammatory and analgesic and also for treating cold and diarrhoea. From the earlier studies the alcohol and aqueous extract of the leaf reported to have a significant antimicrobial activity (Rama prabha and Vasantha, 2010) [5]. The plants also have been reported for the presence of salicyclic acid, sitosterol, cardiac glycosides (Indian medicinal plants). This plant was selected for evaluating different pharmacological actions through *In vitro, in vivo* and *in silico* studies and also to develop an effective and an efficient procedure for *In vitro* propagation of this rare plant.

Materials and Methods
Botanical information
Collection and authentication
Fresh, young (aerial) parts of *Pentatropis capensis* (Apocynaceae) were collected from in and around Coimbatore. The plant material was confirmed by comparing voucher specimen at Botanical survey of India, herbarium Coimbatore, Tamil Nadu. Fresh plant material was cleaned to remove dust and dried under shady places. The dried plants were ground mechanically to coarse powder. For microscopic, physico-chemical and phytochemical analysis this powder passed through a willy mill to get (60- mesh size). This sample are stored in a well grade plastic, airtight container & maintained at room temperature for further analysis (Harborne, 1973) [6].

Vernacular name
Tamil-Uppili, Uppilankodi: Hindi-Ambarvel: Malayalam-Parpparam, Paparam: Marathi-Shingrota: Sanskrit-Kakanasa, Kakanasika, Kakatundaphala, Shringariti: Telugu-Chekurtitiwa, Pulapala, Chekurtitiwa.

Plate 1

Synonyms
Pentatropis microphylla, Asclepias microphylla, Cynanchum capense.

Explants selection and mode of sterilization

The plants of Uppilankodi leaves and roots were removed and only shoots were washed in tap water for 20 minutes. Nodal region were taken as explants. This was further treated with two drops of liqoid surfactant and for 15 minutes and then 1% Bavistin repeatedly rinsed with distilled water and again 1% Bavistin for 2 minutes and it was sterilized further under aseptic conditions (Laminar air Flow). Explants also sterilized at 50% ethyl alcohol for 1 min (50% w/v) and then with $HgCl_2$ (3 min). At last these explants washed fully and also sterilized with distilled water and cut each explant in 1cm approximately using surgical blade (Lisyster, No.12) and explants were kept over the sterilized medium.

Culture medium

MS (Murashige and Skoog.1962) [7] media was used for this present investigation.

Medium preparation

For medium preparation dual distilled water was used. The nutrient medium consists of vitamins, organic supplements and inorganic nutrients. Stock solutions also prepared separately for macro, micro nutrients, kinetin, vitamins and Iron. These chemicals were weighed in an electronic balance. All the prepared solutions were kept in stoppered bottles and stored at 4ºC in a refrigerator. Specific quantities from the stock were pipetted into one litre beaker. Sucrose, complex additives and other organic supplements also added to the stock solution and the final volume was cope up with distilled water and pH was adjusted to acidic of 5.6-5.8 with Na OH (0.1N) or HCl (0.1N) using pH meter.

To this medium 0.8-0.9% agar also added and allowed to solidify. This medium (50 ml approximately poured into test tube and the test tubes were covered with aluminium foil and autoclaved at 121ºC for 20 minutes. The medium was then cooled and stored at 25ºC. The inoculation was made after four days after confirming that the test tubes are free from contamination.

Preparation of growth regulators

Auxins and Cytokinins are the two important growth regulators used and these regulators are stored at 4ºC.

Auxins

Auxins such as NAA (2-naphthalene acetic acid), 2, 4- D (2, 4-dichlorophenoxy acetic acid) and IBA (Indole-3-butyric acid) were mainly used. This was prepared by dissolving in 10 mg of auxins separately in 1 ml of ethanol and the volume is made upto 10 ml with sterile water. Also required amount of auxins were added to nutrient media prior to autoclaving.

Cytokinins

This was also prepared by dissolving 10 mg of 6- benzyl amino purine (BAP) and kinetin separately in 0.1N HCL of about 1 ml and final volume was made upto 10 ml with sterile water.

Culture conditions (Inoculation and Incubation)

For aseptic cultures, laminar airflow chamber were used. The chamber was cleaned with alcohol. The prepared media and the accessories were exposed to UV for 10-15 minutes.

Callus induction

Murashige and Skoog medium with auxins, cytokinins at various concentrations and combinations were used for callus induction. Callus induction % was recorded with six week old cultures with an interval of 4 weeks. It was also calculated by frequency of callus induction and was represented in percentage.

$$\text{Frequency of response}(\%)=\frac{\text{Number of explants responded}}{\text{Total no. of explants cultured}}\times 100$$

Multiple shoot induction

MS basal media containing different concentrations and combinations of cytokinins BAP and KIN were investigated for their effects on shoot multiplication from nodal explants. Subcultures were carried out at 4 weeks intervals, and shoot induction frequency was measured after 4 weeks. The frequency of multiple shoot induction was calculated as shown below and was represented as percentage

$$\text{Frequency of response}(\%)=\frac{\text{No. of explants responded}}{\text{Total no. of explants cultured}}\times 100$$

Shoot induction and subculture

Sub culturing was carried out at the regular intervals of 22-30 days. The *In vitro* developed micro shoots were cut (6.8-1cm in length) with single node. The nodal explants were cultured on MS medium with BAP, KIN and NAA in different concentrations and combinations. The frequency of shoot induction was calculated as shown below and was represented as percentage

$$\text{Frequency of response}(\%)=\frac{\text{Number of explants responded}}{\text{Total no. of explants cultured}}\times 100$$

Rooting

The emerged shoots were transferred to MS medium with IBA and NAA at different concentrations for root induction. The rooted plantlets were transferred for hardening. The frequency of root induction was calculated as shown below and was represented as percentage

Acclimatization

In vitro regenerated rooted plantlets were washed and then transplanted to small pots containing two types of mixtures, one with sand and soil in the ratio of 1:1 v/v and other with sand, soil and vermiculate in the ratio of (2:1:1, v/v/v), covered with a translusent plastic bag to ensure high humidity around the plants. These plantlets were irrigated with sterile water once a day to avoid desiccation and kept for three weeks under culture room conditions. There after the plantlets were transferred to greenhouse, plants were hardened for 5 weeks in green house. Some of the plants were grown with humidity control which was slowly decreased from 80% initial RH to ambient RH of 50% and others were grown without humidity control then healthy and hardened plants were transferred to field.

Results

Effect of growth regulators on callus induction

Earlier studies proved that the plant growth regulators are essential for induction of callus from explants and MS medium alone will not result in callus formation. For induction of callus nodal explants of *Pentatropis capensis* are inoculated at varied concentrations of 2, 4-D, KIN and

BAP separately. In the varied range of 2, 4-D maximum callus production was found in 2.0mg/l concentration with 90% response. At this concentration the callus was compact and yellowish green. Similarly in the varying concentrations of Kinetin used, the highest response (85%) was found with 2.5 mg/l concentration, at which concentration the callus was compact and yellowish green in colour. When the callus inducing basal medium was supplemented with varying concentration of BAP alone, high frequency of callus proliferation was observed at 1.5 mg/l concentration (80%) where the callus was compact and yellow. Among the two different combinations of growth regulator used (2, 4 D+BAP and 2, 4-D +KIN) the former combination was found to be the best for callus induction (95%) at 2.0 + 1.5 mg/l concentration. Comparatively the later combination of growth regulators (2, 4 D + KIN) showed little response for callus induction. Among all combinations tried, the highest rate of callus proliferation and multiplication was found on MS media supplemented with 2, 4-D +BAP at a concentration of 2.0+1.5mg/l (Table 2, Plate 2).

Effect of growth regulators (Cytokinins) on multiple shoots

Calli were cultured on MS medium containing different concentrations of cytokinins individually and in combinations for multiple shoot bud induction and data have been presented in Table-3.

Table 1: Effect of various concentrations and combinations of Cytokinins (BAP and KIN), Auxins (2, 4-D) on callogenesis in *Pentatropis capensis* from nodal explants after 4 weeks (10 replicates per treatment)

Plant growth regulators (mg/l)	Percentage of responding culture	Callus texture	Callus colour
2,4-D			
0.5	40	Compact	Brown
1.0	55	Compact	Yellow
1.5	75	Compact	Green
2.0	90	Compact	Yellowish green
2.5	80	Compact	Yellowish green
KIN			
0.5	50	Watery	Yellow
1.0	65	Compact	Yellow
1.5	70	Compact	Yellow
2.0	80	Compact	Yellow
2.5	85	Compact	Yellowish green
BAP			
0.5	60	Compact	Yellow
1.0	75	Compact	Yellow
1.5	80	Compact	Yellow
2.0	70	Compact	Yellow
2.5	55	Compact	Yellow
2,4-D+ BAP			
2.0 + 0.5	85	Compact	Yellow
2.0 + 1.0	80	Compact	Yellow
2.0 + 1.5	95	Compact	Yellow
2.0 + 2.0	85	Compact	Yellow
2.0 + 2.5	70	Compact	Yellow
2,4-D+ KIN			
2.0 + 0.5	50	Compact	Yellow
2.0 + 1.0	45	Compact	Yellow
2.0 + 1.5	55	Compact	Yellow
2.0 + 2.0	65	Compact	Yellow
2.0 + 2.5	55	Compact	Yellow

Table 2: Effect of various concentrations and combinations of Cytokinins (BAP and KIN) on multiple shoot induction from callus of *Pentatropis capensis*

BAP (mg/l)	Kn (mg/l)	% Response	No. of Shoots (Mean ± SE)	Shoot length in cm
0.5	--	70	2.3 ± 0.30	2.7 ± 0.26
1.0	--	75	2.2 ± 0.24	2.5 ± 0.34
2.0	--	80	3.1 ± 0.23	3.0 ± 0.33
3.0	--	90	4.3 ± 0.32	3.1 ± 0.23
5.0	--	70	3.0 ± 0.25	2.8 ± 0.24
--	0.5	60	1.8 ± 0.24	2.7 ± 0.21
--	1.0	80	2.8 ± 0.24	1.4 ± 0.16
--	2.0	75	2.1 ± 0.27	3.6 ± 0.26
--	3.0	70	1.7 ± 0.21	2.1 ± 0.27
--	5.0	78	1.9 ± 0.17	2.8 ± 0.24
0.5	0.5	80	3.9 ± 0.37	1.3 ± 0.26
1.0	1.0	85	2.7 ± 0.30	2.7 ± 0.21
2.0	2.0	75	2.8 ± 0.29	2.9 ± 0.31
3.0	3.0	70	1.9 ± 0.23	2.6 ± 0.22
5.0	5.0	72	2.2 ± 0.20	2.5 ± 0.26

Data are the average of three triplicates with 10 explants. Values represent the M ± S.E

Table 3: Effect of various concentrations and combinations of Auxin (NAA) and Cytokinin (BAP+KIN) on shoot induction of nodal explants of *Pentatropis capensis*

Hormone concentration (mg/l)	Response	Mean no. of shoot/explants	Mean no of shoot Length
BAP			
0.2	65	2.6 ± 0.32	9.2 ±0.48
0.4	70	2.8 ± 0.27	9.4 ± 0.67
0.6	60	2.2 ± 0.48	10.2 ± 0.24
0.8	80	3.6 ± 0.62	10.0 ± 0.48
1.0	90	4.8 ± 0.36	12.0 ± 0.37
1.2	75	2.7 ± 0.48	11.2 ± 0.64
KIN			
0.2	60	2.1 ± 0.42	5.8 ± 0.24
0.4	65	2.3 ± 0.25	6.2 ± 0.48
0.6	70	3.5 ± 0.48	6.3 ± 0.66
0.8	65	2.0 ± 0.37	5.0 ± 0.24
1.0	65	2.1 ± 0.42	4.3 ± 0.67
1.2	65	2.0 ± 0.42	4.9 ± 0.48
BAP + NAA			
1.0+0.2	70	3.8 ±0.74	9.0 ±0.37
1.0+0.4	75	3.4 ±0.48	9.2 ±0.48
1.0+0.6	65	2.6 ±0.65	9.5 ±0.67
1.0+0.8	95	4.8 ±0.48	12.21 ±0.28
1.0+1.0	75	3.4 ±0.67	10.2 ±0.48
1.0+1.2	60	2.2 ±0.40	11.1 ±0.48
KIN+NAA			
1.0+0.2	65	2.2 ±0.74	5.4 ±0.24
1.0+0.4	70	2.7 ±0.48	6.3 ±0.68
1.0+0.6	80	3.8 ±0.40	6.5 ±0.68
1.0+0.8	60	1.6 ±0.80	5.2 ±0.36
1.0+1.0	70	2.4 ±0.60	4.8 ±0.28
1.0+1.2	60	1.2 ±0.48	4.1 ±0.68

Table 4: Effect of various concentrations of auxins (IBA+NAA) on Root induction of *Pentatropis capensis*

Plant growth regulators (mg/l)	Percentage of responding culture	Mean no of root/explants	Mean no of root length (cm)
IBA			
0.2	60	1.8±0.74	2.0±0.12
0.4	75	2.3±0.48	2.7±0.48
0.6	70	2.2±0.74	3.5±0.67
0.8	60	1.3±0.67	2.2±0.18
1.0	60	1.1±0.67	2.6±0.37
1.2	55	1.0±0.28	2.3±0.28
NAA			
0.2	60	1.2±0.47	4.0±0.13
0.4	80	2.4±0.63	4.7±0.67
0.6	85	2.8±0.80	5.6±0.28
0.8	75	2.6±0.47	5.4±0.48
1.0	70	2.5±0.68	5.0±0.48
1.2	60	1.2±0.63	4.2±0.40

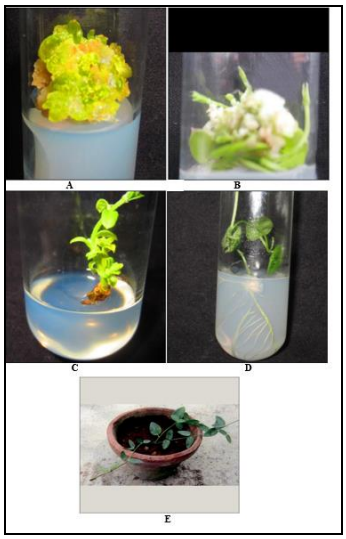

Plate-2: *In vitro* regeneration of *Pentatropis capensis*

When calli were cultured on MS medium containing different concentrations of Kinetin (0.5-5 mg/l), maximum number of shoots (2.8±0.24), with an average length of 3.6±0.26cm were produced on 1mg/l concentration. However when calli were cultured on MS medium fortified with different concentrations of BAP + Kinetin in different combinations for multiple shoot induction, highest number of shoots (3.9±0.37cm) with an average length of 2.9±0.31cm were induced on medium containing BAP and KIN at a concentrations of 1mg/l+ 1mg/l. The present investigation clearly indicates that, among different concentrations and combinations of cytokinins used (BAP and KIN), BAP alone particularly at 3 mg/l induced maximum number of shoot buds when compared to either KIN alone or combined with KIN in different concentrations (Table 2, Plate-2).

Effect of growth regulators on shoot induction

Shoots and buds were originated from nodal explants, when MS medium was supplemented with different

concentrations of BAP (0.2 – 1.2 mg/l) and Kinetin (0.2-1.2mg/l). Fifteen days after inoculation, the nodal explants showed slight swelling prior to the emergence of shoot buds that develop from the pre existing material. 20 days after inoculation shoot buds emerged and gradually about 2 to 4 shoots developed per explant (Table-3). After 4 weeks of incubation among the two different growth regulators, higher number of shoots (4.8±0.36) with 90% survival rate was observed in MS medium supplemented with 1.0 mg/l BAP. Maximum length of shoot (12.8cm) was observed at this concentration. When the MS medium was supplemented with KIN, the higher number of shoots (3.5±0.48) was produced in 0.6 mg/l concentration with 70% survival rate and the shoot length was found to be 6.3 cm. When MS medium supplemented with 1.0 mg/l BAP and different concentrations of NAA (0.2 to 1.2 mg/l) the maximum number of multiple shoot (4.8±0.48) was observed in 0.8 mg/l concentration of NAA and the shoot length was 12.21 cm. When MS medium supplemented with 1.0 mg/l KIN and different concentrations of NAA (0.2+ 1.2 mg/l), the maximum shoot proliferation (3.8±0.40) was observed in 0.6 mg/l NAA concentration and the shoots attained a length of 6.5 cm at this concentration (Table 3, Plate 2).

Effect of growth regulators on root induction

Well developed shoots were excised from cultured tubes and cultured on half strength MS media containing different concentrations of IBA (0.1 to 1.2 mg/l) and NAA (0.1 to 1.2 mg/l).The percentage of root frequency, number of root/shoot and length of roots were recorded after 4 weeks of culture. The rooting response to different auxin treatment is tabulated. Of the two types of auxins, NAA was found to be comparatively more effective than IBA. In cultures where IBA alone was used, the maximum number of root/explants (2.3±0.48) was observed at 0.4 mg/l with 75% survival rate and the root length at this concentration was 2.7±0.48 cm. NAA at 0.6 mg/l was found to be the best concentration of auxin for proper rooting where 85% of the shoots rooted within 6 weeks of culture. The highest mean number of roots (2.8 ±0.80) and highest average root length (5.6± 0.28) was also observed with NAA (Table 4, Plate-2).

Acclimatization

Trials indicated that 2:1:1 mixture of sand, soil and vermiculate was the best substrate as 70% plants could survive after transfer to field. There after the plantlets appeared morphologically uniform with normal leaf form, shape and growth pattern.80% of the plantlets survived and showed no sign of water stress and without any morphological abnormalities or variations (Plate-2).

Discussion

One of the objectives of this study was to establish a rapid micro propagation system for *Pentatropis capensis* through axillary shoot multiplication. Nodal explants were used in this experiment because they provide identical clones with desired traits, which is important for conservation of rare and endangered taxa (Gangaprasad *et al*., 2005) [8].

Effect of growth regulators on callus induction.

Many works have been carried out on the establishment of callus culture from the members of Asclepiadaceae. But this is the first time done on *Pentatropis capensis*. Callus is an undifferentiated cell mass produced from differentiated tissue and thus provides an important material for either directly regenerating plants or vegetative embryogenesis or suspension culture. Every differentiated plant tissue is totipotent, but the conditions to de-differentiate them vary from species to species and even tissues to tissue within same plant (Ezhova, 2003) [9]. For induction of callus, nodal explants of *Pentatropis capensis* are inoculated at varied concentrations and combinations of 2, 4-D, KIN and BAP. Among all combinations tried the highest rate of callus proliferation and multiplication (95%) was found on MS media supplemented with 2, 4 –D + BAP at 2.0 + 1.5 mg/l concentration. It has been demonstrated in many cases that 2, 4 D is usually the choice of auxin for callus induction and subculture of monocots and dicots (Evans *et al*., 1981 and Bhaskaran and Smith, 1990) [10, 13].

Effect of growth regulators on induction of multiple shoots

When calli were cultured on MS medium fortified with different concentrations of cytokinins (BAP and KIN) individually and also in combinations (BAP + KIN) for multiple shoot induction, the best results were observed with BAP. In the present study BAP supplemented MS medium promoted maximum multiplication shoot frequency; whereas KN showed lesser shoot multiplication frequency. Many authors have reported BAP as the best multiple shooting cytokinin for *Psudarthria viscida* (Vinothkumar *et al*., 2010) [24]. No significant variation was observed when these multiple shoots were sub cultured. This observed result is in contradiction with report on other Ascleploeidae members, such as *Hemidesmus indicus* (Sreekumar *et al*., 2000) [21] and *Decalepis arayalpathra* (Gangaprasad *et al*., 2005) [8].

Effect of growth regulators on shoot induction

Shoot buds developed from nodal explants when MS medium supplemented with different concentration of BAP and KIN. Among the two growth regulators experimented, higher number of multiple shoots and higher average shoot length were observed for BAP at 1.0 mg/l and KIN at 0.6 mg/l concentration. When these two optimal concentrations of BAP and Kinetins combined with varying concentrations of NAA, the best results were observed with BAP + NAA combination at 1.0+0.4mg/l concentration. An increase or decrease in the concentration of these growth regulators beyond optimum level resulted in decrease of shoot production and shoot length. Among all the growth regulators tried BAP in combination with NAA was found to be the best for shoot induction in *Pentatropis capensis*. The advantageous role of BAP on shoot bud induction was also advacated by Faisal and Anis (2003) [14] in *Tylophora indica* and Faisal *et al.* (2006) [15] in *Mucuna pruriens.*

Effect of growth regulators on root induction

Excised *In vitro* shoots from culture tubes were rooted only upon transfer to half strength MS medium containing auxins namely IBA and NAA. Half strength MS medium supplemented with different auxins at different concentrations showed varied effect on rooting. Of the two auxins tested, NAA was most effective for root induction. The effectiveness of NAA on rooting has been reported for an Asclepiadaceae species *Dacalepis hamiltonii* (Anitha and Pullaiah, 2002) [16] and *Tylophora indica* (Thomas and

Philip (2005) [22]. Many authors have reported IBA as the best rooting auxins for *Psudarthria viscida* (Vinothkumar *et al*., 2010) [24].

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