Rapid *in vitro* propagation of *Premna serratifolia*, a medicinally important declining shrub, India

Ravinder Singh Chinnappan[1*], Nelson Ruthar[2] & Siva Subramanian Sethu[3]

[1]*Department of Biotechnology, Vivekanandha College of Arts and Science for Women, Thiruchengodu – 637 205, Tamil Nadu, India*

[2]*Department of Botany, Government Arts College, Ariyalur- 621 713, Tamil Nadu, India*

3 Department of Botany, J J College of Arts and Science, Pudukkottai 622 422 Tamil Nadu, India

*Corresponding author e-mail: chinnaravinder@yahoo.co.in

SUMMARY

A protocol for micropropagation of the medicinally important plant *Premna serratifolia* was developed due to its increasing rarity as a result of over-exploitation and poor natural regeneration within its range of occurrence in the Indian sub-continent. Plantlets were regenerated through shoot tip explants. Shoot tip explants were cultured on different media (MS, SH, Y3 and B5). Anti-oxidants (activated charcoal, citric acid, polyvinylpyrrolidone) and the effect of seasonal changes (through out the year) were analyzed for reducing explant browning and better shoot multiplication. The highest number of shoots was developed from MS medium supplemented with BAP (3.0 mg/l), IAA (0.5 mg/l) and activated charcoal (10.0 mg/l) between November and March. Best rooting was achieved from the medium supplemented with NAA (1.0 mg/l). Complete regeneration was achieved in about 21 days. The plantlets, thus developed were maintained under controlled conditions in the green house for 40 days. They were then planted out into a nursery where growth has been good and survival, up to 1 year, has been high (95%).

BACKGROUND

In Asia *Premna serratifolia* L. (Lamiaceae) is an important medicinal shrub. Roots, leaves and stem bark are used to treat various ailments including cardiac disorders, constipation, obesity, asthma, leprosy, skin diseases and diabetes (Sudo *et al.* 2000). In India *P. serratifolia* is one of 10 plant ingredients of 'Dasamula' used in the Indian Ayurvedic system of medicine.

Whilst is *P.serratifolia* is a common and widespread coastal plant in south-east Asia and the Pacific, in the Indian sub-continent it is dwindling at an alarming rate due to over-exploitation by the pharmaceutical industry and low seed viability. *P. serratifolia* is listed in the Red Data book of Andhra Pradesh, India (Pullaiah *et al.* 2004). Hence, *in vitro* methods to propagate the species are desirable to assist in the tissue culture protocols to conserve the species within its Indian range. To the best of our knowledge, this is the first report of an *in vitro* protocol for micropropagation of *P. serratifolia* using shooting tips. The ultimate aims are to increasing regeneration capacity and reintroduce *in vitro*-reared plants back into the wild within India.

ACTION

Materials and methods: Young fresh shoot tip (1-2 cm in length) material was collected from the vicinity of Keelathanyam (Pudukkottai District) in south-east Tamil Nadu state, during January 2007. Explants were washed in running tap water for 30 min, and subsequently washed in 3-4 drops of Teepol (a commercial liquid detergent) dissolved in 100 ml distilled water for 20 min

followed again by washing in tap water. The explants were then rinsed in distilled water and subjected to surface sterilization in 70% ethanol for 30 seconds, followed by treatment with 0.1% (w/v) solution of mercuric chloride for 3-4 minutes and rinsed for 5-10 min in distilled water. The surface sterilized shoot tip explants were aseptically inoculated into culture tubes containing different sterilized media, namely MS (Murashige & Skoog 1962), SH (Schenk & Hildebrandt 1972), WPM (Lloyd & McCown 1980) and B5 (Gamborg *et al.* 1968) with 3% sucrose and solidified with 0.8% agar. The effect of these different hormones, seasonal variations and anti-oxidants (CA, AC and PVP) were studied, aiming to establish an efficient protocol for mass propagation.

Culture conditions: All cultures were incubated at 25.0º± 1.0ºC under white florescent light (3,000 lux) with a photo period cycle of 12 hours light and 12 hours darkness.

Statistical analysis: Data on multiple shoot regeneration were scored after 35 days after inoculation. Mean values were compared using Duncan's multiple range test (DMRT).

Acclimatization and field establishment: Healthy rooted plants were carefully removed from the culture medium and washed in tap water to remove agar gels. These were transferred to plastic pots containing mixture of sterilized soil, sand and compost in the ratio 2:1:2. Sterilized soil is considered a good transplant medium, whilst also minimizing the cost of transplantation, as documented by several authors (e.g. Anand *et al.* 1997). These plants were maintained at 30ºC under a 16 h photoperiod (white fluorescent tube lights; 30µ mol $m^{-2}s^{-1}$) for 14 days at high humidity. Finally, the regenerated plants were acclimatized in a glass house and transplanted out into field conditions (in a nursery).

CONSEQUENCES

Explants browning and influence of anti-oxidants: One of the major problems associated with *P. serratifolia* micropropagation was found to be the browning of explants due to oxidation of phenolic compounds released from the cut end of the explants by poly-phenoloxidases, peroxidases, as this led to cell death. Propensity to browning was anti-oxidant specific and the presence of an antioxidant greatly minimized the browning rate. To overcome phenolic exudation, CA, AC and PVP were investigated. Among the various anti-oxidant tested, AC (10 mg/l) completely inhibited explant browning by controlling phenolic oxidation and it enhanced shoot proliferation. AC proved to be the best of the anti-oxidants tested (Fig. 1).

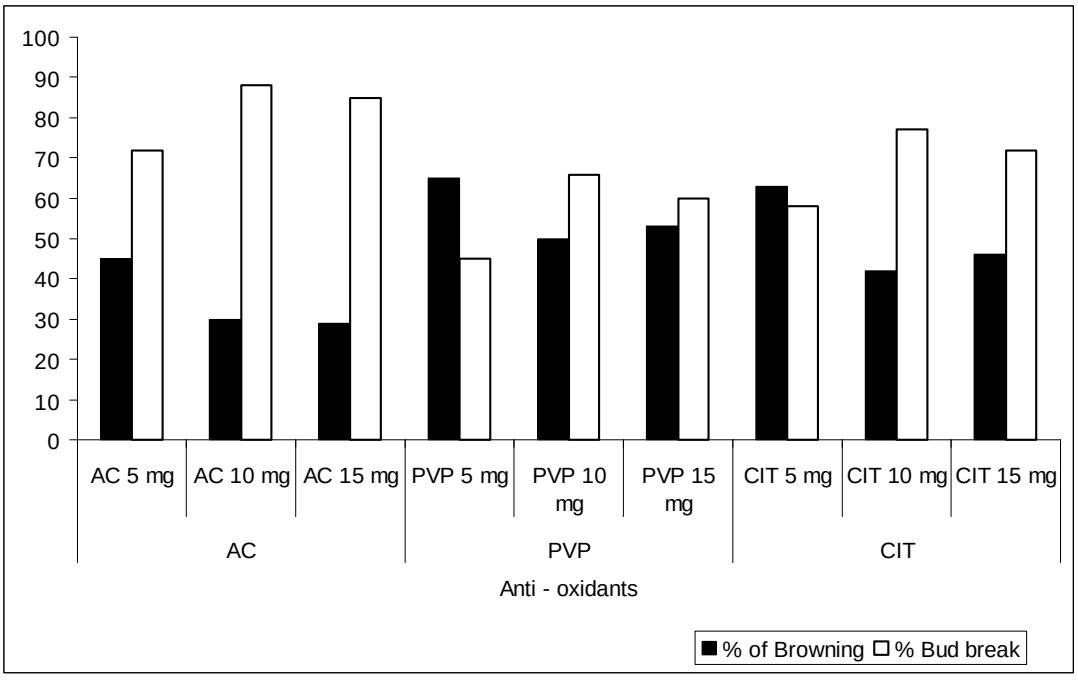


Figure 1. Effect of anti-oxidants on browning and bud break in *P. serratifolia*.

CA and PVP added to the culture medium reduced the number of shoots per explants and did not reduced phenolic exudation. Reduction in shoot number may be due to adsorbtion of essential components besides the inhibitory actors (Weatherhead *et al.* 1979).

Influence of media and seasonal changes: Shoot tips remained green and fresh but failed to sprout on some media e.g. B5, SH, WPM and Y3. Among the different media tested, MS was found to be the best basal medium for bud sprouting (65%), number (2.0±0.7) and length (3.4 ±1.0cm) followed by B5, WPM, SH and Y3 media Table1. The shoot buds sprouted on B5 medium showed only limited development even though they were maintained for a longer period of time. Shoot regeneration was observed within 7 days of culturing. Evaluation of the regeneration rate of explants were collected at various times year round, regeneration was at its maximum during November and March (90.5%) and lowest in May to August (Fig. 2).

Table 1. Effect of different media on adventitious shoot induction of *Premna serratifolia* (n = 80 explants per treatment).

Serial Number	Media	Shoot-tip		
		Response (%)	Number of shoots/explants culture	Shoot length (cm)
1	B5	42	1.7 ± 1.5^{ab}	3.0 ± 1.0^{ab}
2	WPM	35	1.2 ± 1.0^{b}	2.5 ± 1.2^{b}
3	MS	65	2.0 ± 0.7^{a}	3.4 ± 1.0^{a}
4	SH	0	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
5	Y3	0	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}

Values are mean ± SE of three repeated experiments. Mean within a column followed by the same letters are not significantly different at 5.0 % probability level according to Duncan's multiple range test (DMRT).

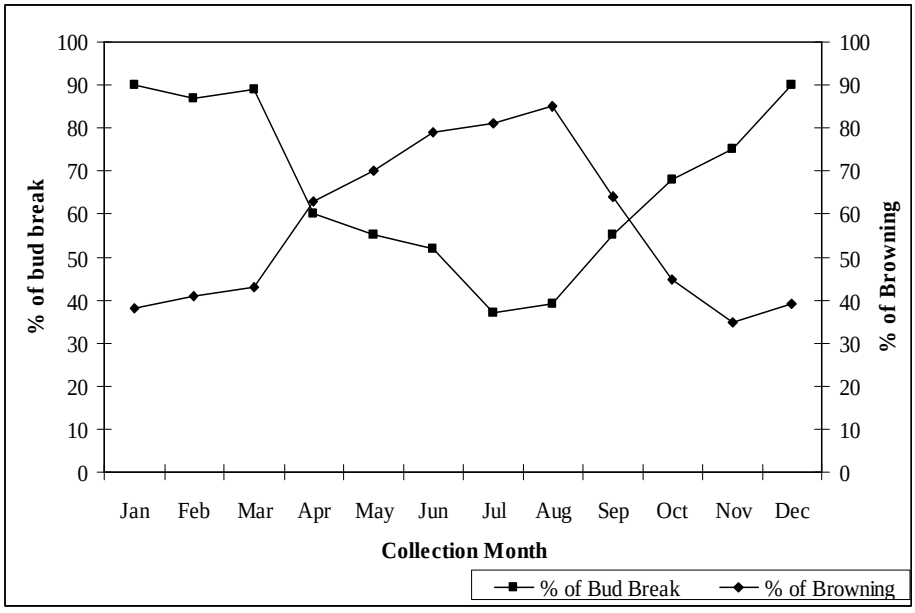


Figure 2. Effect of collection season on browning and bud break in *Premna serratifolia*.

Effect of hormones on shoot multiplication: The aseptic transfer of shoot tip explants on to hormone-free MS medium did not lead to multiplication and elongation. The effect of cytokinins on shoot multiplication is summarized in Table 2. The shoot tip explant culture in different cytokinins individually and in combinations (BAP, KN, 2-ip and Zeatin) ranged from 0.5 mg/l to 5.0 mg/l. Among the tested combinations, regeneration of multiple shoots (84.2%; Table 3) was achieved on MS medium supplemented with BAP (3.0 mg/l) and KN (0.5 mg/l) (illustrated in Fig. 3).

Adventitious shoot production and subsequent proliferation were decreased with increasing concentrations of both BAP and KN. High number of adventitious buds were produced in the presence of cytokinins, especially BAP, and is consistent with the general knowledge that cytokinins overcome apical dominance, release lateral buds from dormancy and promote shoot formation (George 1993, De Bruyn & Ferreira 1992). Our observations also show that addition of different auxins (IAA, IBA and NAA) along with cytokinin produced maximum numbers of microshoots. The highest (8.5 ±1.1) numbers of shoot were produced when the MS medium was supplemented with BAP (3.0 mg/l) and IAA (0.5 mg/l). Shoot tip explants cultured in the medium containing other combinations (e.g. BAP+NAA, Zeatin+IAA, Zeatin+NAA, KN+IAA and KN+NAA) showed comparatively low response (Table 4).

Rooting of *in vitro* regenerated shoots: Newly raised shoots 2-3 cm long were excised from the clumps just below the node, the leaves were removed from the lower nodes and the shoots were transferred to MS medium supplemented with 0.5-2.0 mg/l IAA, IBA and NAA. NAA (1.0 mg/l) was the best (89%) rooting hormone (Table 5).

Table 2. Effect of cytokinins on adventitious shoot induction from shoot-tip explants of *Premna serratifolia*.

Growth regulators (mg/l)	Shoot-tip		
	% of response	Number of shoots /explants	Shoot length (cm)
BAP (mg/l)			
0.5	66.2	2.7 ± 1.4^{de}	3.8 ± 0.5^{c}
1.5	71.0	3.5 ± 1.2^{c}	4.4 ± 1.4^{b}
3.0	80.5	5.8 ± 1.0^{a}	5.2 ± 1.0^{a}
5.0	72.0	4.1 ± 0.7^{bc}	4.0 ± 1.5^{bc}
KN (mg/l)			
0.5	65.3	2.1 ± 2.4^{e}	3.4 ± 1.6^{cd}
1.5	67.8	3.5 ± 1.0^{c}	4.1 ± 1.8^{bc}
3.0	64.7	3.0 ± 1.2^{d}	4.0 ± 1.0^{bc}
5.0	60.0	2.6 ± 1.3^{de}	3.7 ± 1.0^{c}
2-ip (mg/l)			
0.5	65.5	2.3 ± 0.7^{e}	3.5 ± 1.6^{cd}
1.5	69.0	3.0 ± 1.5^{d}	3.8 ± 0.9^{c}
3.0	71.1	3.6 ± 1.2^{c}	4.0 ± 0.7^{bc}
5.0	75.0	4.3 ± 1.0^{b}	4.5 ± 0.8^{b}
Zeatin (mg/l)			
0.5	65.9	2.5 ± 1.0^{de}	3.7 ± 0.8^{c}
1.5	70.0	3.3 ± 1.7^{cd}	4.0 ± 1.0^{bc}
3.0	77.4	4.9 ± 0.5^{ab}	4.8 ± 1.2^{ab}
5.0	74.0	4.0 ± 0.7^{bc}	4.5 ± 1.0^{b}

Values are mean ± SE of three repeated experiments. Mean within a column followed by the same letters are not significantly different at 5.0 % probability level according to Duncan's multiple range test (DMRT).

Table 3. Effect of cytokinins combinations on multiple shoot induction from different explants of *Premna serratifolia.*

Growth regulators (mg/l)		Shoot-tip		
		% of response	Number of shoots /explants	Shoot length (cm)
BAP + KN 0.5				
0.1		79.7	5.6 ± 1.6^{c}	2.4 ± 0.0^{b}
1.5	0.3	81.0	6.1 ± 1.2^{b}	1.8 ± 0.1^{ab}
3.0	0.5	84.2	6.6 ± 1.1^{a}	1.1 ± 0.0^{a}
5.0	0.7	80.0	6.3 ± 1.0^{ab}	1.9 ± 0.0^{ab}
BAP + 2-ip				
0.5	0.1	68.0	4.8 ± 1.0^{d}	0.9 ± 0.2^{b}
1.5	0.3	71.0	5.9 ± 0.6^{bc}	3.5 ± 0.1^{b}
3.0	0.5	73.0	5.0 ± 1.0^{cd}	1.8 ± 0.2^{ab}
5.0	0.7	69.7	4.7 ± 1.2^{d}	2.6 ± 0.5^{b}
Zeatin + KN 0.5				
0.1		77.9	4.2 ± 1.1^{e}	1.3 ± 0.0^{bc}
1.5	0.3	79.0	4.8 ± 1.0^{d}	0.7 ± 0.1^{b}
3.0	0.5	82.0	4.5 ± 0.2^{de}	0.9 ± 0.7^{b}
5.0	0.7	75.0	4.0 ± 1.8^{e}	1.4 ± 0.5^{b}
Zeatin + 2-ip				
0.5	0.1	71.1	2.0 ± 0.5^{h}	1.2 ± 0.4^{c}
1.5	0.3	75.8	3.0 ± 0.9^{ef}	1.5 ± 0.7^{b}
3.0	0.5	77.0	2.6 ± 0.0^{g}	2.5 ± 0.0^{b}
5.0	0.7	73.0	2.3 ± 1.3^{gh}	1.4 ± 0.0^{b}

Values are mean ± SE of three repeated experiments. Mean within a column followed by the same letters are not significantly different at 5.0 % probability level according to Duncan's multiple range test (DMRT).

Acclimatization and field establishment: Complete regenerated plantlets with sufficient roots were carefully removed from culture tubes and the medium adhered to roots was gently washed off in water with the help of fine brush. The plantlets were then transplanted into small earthen pots containing sterilized soil and sand mixture (3:1) (Fig. 3). Each pot was covered with a polythene bag (with small aeration holes) to maintain high humidity and kept in the culture room to acclimatize. The plantlets were initially irrigated with half strength MS medium without sucrose on alternate days. The plantlets were exposed to 3-4 hours daily to conditions of natural humidity after 10 days of transfer. After 30 days the plants were transferred to larger pots in a green house and were maintained under natural conditions of day length, temperature and humidity. After 40 days in the greenhouse, the plants were planted out in a medicinal garden (nursery) in Tamil Nadu. Of the transplants, survival (up to one year) has been 95%. No plants have exhibited any great variation in morphology or growth characteristics compared to the donor plants.

Conclusions: Plantlets were successfully regenerated through shoot tip explants. The highest number of shoots was developed from MS medium supplemented with BAP (3.0 mg/l), IAA (0.5 mg/l) and activated charcoal (10.0 mg/l) between November and March. Best rooting was achieved from the medium supplemented with NAA (1.0 mg/l). Complete regeneration was achieved in about 21 days. The plantlets were grown on in a greenhouse for 40 days and then planted out in a nursery, where growth has been good and plant survival high.

Table 4. Effect of cytokinins and auxin combinations on multiple shoot induction from shoot tip explant of *Premna serratifolia*.

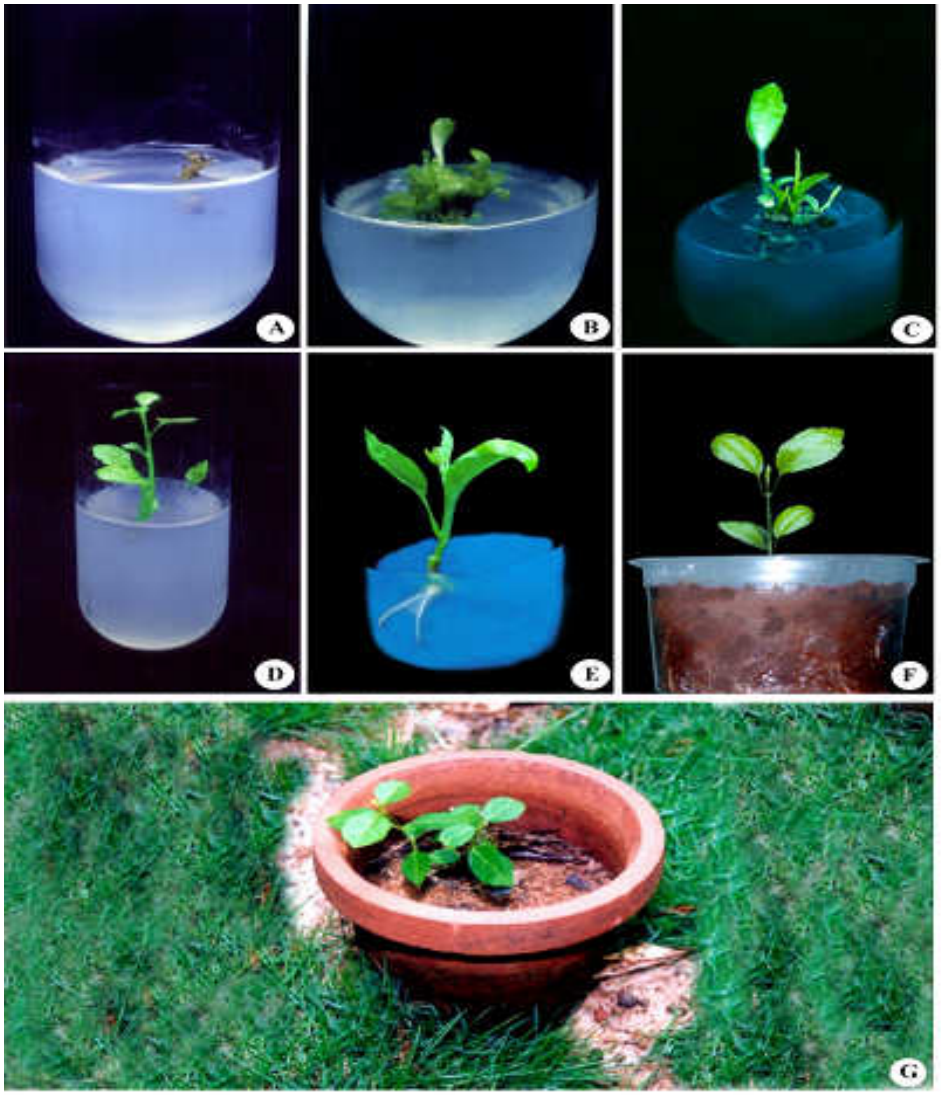
Growth Regulators (mg/l)		Shoot-tip		
		% of response	Number of shoots /explants	Shoot length (cm)
BAP + IAA (mg/l)				
0.5	0.1	68	5.9 ± 2.0^{cd}	3.4 ± 0.0^{c}
1.5	0.3	79	7.7 ± 2.3^{ab}	1.5 ± 0.7^{bc}
3.0	0.5	90.5	8.5 ± 1.1^{a}	1.5 ± 0.0^{bc}
5.0	0.7	80	7.4 ± 1.9^{ab}	1.5 ± 0.5^{bc}
BAP + NAA (mg/l)				
0.5	0.1	64.0	5.6 ± 1.5^{cd}	1.3 ± 0.0^{cd}
1.5	0.3	75.0	6.6 ± 1.4^{bc}	4.4 ± 1.2^{c}
3.0	0.5	78.0	7.5 ± 1.0^{ab}	3.6 ± 1.0^{b}
5.0	0.7	72.0	7.1 ± 1.3^{b}	1.5 ± 0.8^{bc}
Zeatin + IAA (mg/l)				
0.5	0.1	60.0	4.4 ± 1.0^{de}	1.3 ± 0.7^{cd}
1.5	0.3	65.0	5.8 ± 1.8^{cd}	1.5 ± 0.0^{bc}
3.0	0.5	79.0	6.1 ± 1.2^{c}	1.4 ± 0.3^{c}
5.0	0.7	74.0	6.0 ± 0.7^{c}	1.4 ± 0.0^{c}
Zeatin + NAA (mg/l)				
0.5	0.1	46.0	3.8 ± 0.9^{e}	3.3 ± 0.7^{cd}
1.5	0.3	58.0	4.9 ± 0.6^{de}	1.5 ± 0.2^{bc}
3.0	0.5	55.0	5.5 ± 0.7^{cd}	2.7 ± 0.1^{ab}
5.0	0.7	47.0	5.2 ± 1.7^{d}	1.2 ± 0.4^{a}
KN + IAA (mg/l)				
0.5	0.1	66.0	5.3 ± 0.9^{d}	1.4 ± 0.4^{c}
1.5	0.3	75.5	6.7 ± 1.4^{bc}	1.5 ± 0.0^{bc}
3.0	0.5	80.0	7.3 ± 1.0^{b}	1.5 ± 0.7^{bc}
5.0	0.7	75.0	6.5 ± 1.0^{bc}	0.9 ± 0.8^{cd}
KN + NAA (mg/l)				
0.5	0.1	61.2	4.6 ± 1.7^{de}	1.5 ± 0.2^{bc}
1.5	0.3	74.0	5.0 ± 1.5^{de}	1.5 ± 0.0^{bc}
3.0	0.5	78.0	5.7 ± 3.7^{cd}	0.8 ± 0.1^{ab}
5.0	0.7	70.0	5.5 ± 1.2^{d}	1.5 ± 0.0^{bc}

Values are mean ± SE of three repeated experiments. Mean within a column followed by the same letters are not significantly different at 5.0 % probability level according to Duncan's multiple range test (DMRT).

Table 5. Effect of different concentrations of IBA, NAA and IAA added to half strength MS basal medium on elongation and rooting proliferations of *Premna serratifolia* shoot cultures, after 3 weeks.

Growth regulators (mg/l)	No. of shoots responded	% of rooting	Mean no. of roots/shoot	Root length (cm)
IBA				
0.5	17.2 ± 1.2^{bc}	57.3	$1.0 \pm 1.0_{c}$	0.7 ± 0.7^{cd}
1.0	20.7 ± 1.0^{ab}	69.0	1.2 ± 0.6^{c}	1.6 ± 0.3^{b}
2.0	19.0 ± 2.0^{b}	63.3	0.8 ± 2.6^{b}	1.1 ± 0.1^{bc}
NAA				
0.5	21.5 ± 1.0^{ab}	71.7	1.5 ± 1.5^{bc}	1.9 ± 0.7^{ab}
1.0	26.7 ± 1.8^{a}	89.0	3.4 ± 0.9^{a}	2.4 ± 0.0^{a}
2.0	25.0 ± 2.5^{a}	83.3	1.9 ± 1.0^{b}	2.0 ± 0.0^{ab}
IAA				
0.5	14.0 ± 0.5^{c}	46.7	0.5 ± 1.0^{d}	2.4 ± 0.4^{d}
1.0	19.5 ± 1.2^{b}	65.0	1.0 ± 1.4^{c}	1.6 ± 0.6^{cd}
2.0	20.0 ± 0.7^{ab}	66.7	0.8 ± 0.5^{b}	0.9 ± 0.8^{c}

Values are mean ± SE of three repeated experiments. Mean within a column followed by the same letters are not significantly different at 5 % probability level according to Duncan's multiple range test (DMRT).



A. Shoot-tip explant in MS medium, B. Shoot initiation from shoot-tip explant in MS medium containing BAP (3.0 mg/l), IAA (0.5 mg/l) C. Shoot proliferation in shoot-tip explant cultured in MS medium containing BAP (3.0 mg/l), IAA (0.5 mg/l)
D. Elongated shoots cultured on root induction medium containing NAA (1.0 mg/l)
E. Rooting of *in vitro* regenerated shoots on MS medium containing NAA (1.0 mg/l)
F. Plantlets transferred to plastic pots containing soil mixture, G. Plants growing in earthen pots containing soil mixture

Figure 3. Stages in micropropagation (regeneration of shoot-tip explants to fully-developed young plants) of *Premna serratifolia*.

ACKNOWLEDGEMENTS

Many thanks to Fr. S. John Britto, Director, Rabinath Herbarium, Tiruchirappalli, Tamil Nadu (India) for confirmation of plant material identification.

REFERENCES

Anand P.H.M., Harikrishnan K.N., Martin K.P. & Hariharan M. (1997) *In vitro* propagation of *Kaempferia rotunda L* "Indian crocus"- A medicinal plant. *Phytomorphology*, **47**, 281-286.

De Bruyn M.H. & Ferreira D.L. (1992) *In vitro* corm production of *Gladiolus dalenii* and *Gladiolus tristis*. *Plant cell tissue and organ culture*, **31**, 123-128.

Gamborg O.L., Miller R.A.& Ojimo K. (1968) Nutrient requirement of suspension cultures of soyabean root cells. *Experimental Cell Research*, **50**, 151-158.

George E.F. (1993) *Storing and distributing clonal material* In: *Plant Propagation by Tissue Culture*: *The Technology*. Exegetics Ltd., Edington, UK. p.181.

Lloyd G. & McCown B. (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Comb. Proc. International Plant Propagators Society*, **30**, 421-427.

Murashige T. & Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Plant Physiology*, **15**, 473-497.

Prajapati N.D., Purohit S.S., Sharma A.K. & Kumar T. (2003) *A hand book of medicinal plants, a complete source book.* Agrobios, India.

Pullaiah T., Murthy K.S.R. & Rani S.S. (2004) Floristic analysis of Andhra Pradesh, India, In: Rama Swami N. *et al.* (eds.) Gleanings in Plant Sciences p. 43-52.

Schenk R.U. & Hildebrandt A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures, *Canadian Journal of Botany*, **50**, 199-204.

Sudo H., Ide T., Otsuka H., Hirata E., Takusi A., Shinzato T. & Takeda Y. (2000) Magastigmane, benzyle and phenthyl alcohol glycosides and 4, 4'-dimethoxy-beta-treexinic eatapol distort from The Chemical and Pharmaceutical Bulletin (Tokyo), **48**, 542-6.

Weatherhead M.A., Burdon J. & Henshaw G.G. (1979) Some effects of activated charcoal as an additive plant tissue culture media. *Zeitschrift für Pflanzenphysiologie*, **94**, 399-405.