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In vitro Propagation of *Rubia cordifolia* Linn., A Medicinal Plant of the Western Ghats

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Abstract: An efficient micropropagation protocol was developed for direct shoot induction from the seedlings of *Rubia cordifolia*, a species sparsely distributed in the Western Ghats of India. Plant regeneration was achieved using shoot tip, nodal and split nodal half explants on Murashige and Skoog medium. Effect of plant growth regulators like 6-Benzyladenine, Kinetin, 6-Benzyladenine + Indole-3-acetic acid and 6-Benzyladenine + α-Naphthalene acetic acid on shoot multiplication and Indole-3-acetic acid, Indole-3-butyric acid and α-Naphthalene acetic acid on rooting was studied. *R. cordifolia* shoots showed abundant proliferation and rooting capacity, both of which are significantly influenced by the varying concentrations of the different plant growth regulators. The optimum number of shoot obtained were 5.9 and 5.2 per explants in 2 weeks on the medium supplemented with 1 mg L^{-1} Benzyladenine and 0.02 mg L^{-1} Indole-3-acetic acid in nodes and split vertical halves of the node respectively. The results suggest that the nodal explants (intact and split vertical halves) are better sources of shoot formation than the shoot tip explant. Shoot multiplication was rapid and consistent for 4 subcultures with 0.5 mg L^{-1} Benzyladenine. The best root induction (98%) and survival was achieved on 1 mg L^{-1} Indole-3-butyric acid followed by 1 mg L^{-1} Indole-3-acetic acid. Rooted plantlets were successfully transferred to green house conditions with 89% survival. Micropropagated plants displayed normal phenotypes in *ex situ* conditions. These plantlets can be used to replenish declining populations in the wild, for the extraction of bioactive compounds and reducing pressure on wild stocks.

Key words: Conservation, regeneration, split nodal halves, plant growth regulators, *ex situ* adaptability.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. *In vitro* regeneration of medicinal plants hold tremendous potential for the production of high-quality plant-based medicine (Tripathi and Tripathi, 2003). Less side effects, better compatibility and only accessible cure for some diseases makes the herbal drugs an ideal remedy for treatment of the diseases (Karim *et al*., 2011). *Rubia cordifolia* Linn. (manjishtha) belonging to family Rubiaceae is a well known ayurvedhic herb, popularly known as Indian Madder. It is a perennial herbaceous climbing plant with very long roots, cylindrical, flexuous with a thin red bark. It is distributed throughout the lower hills of Indian Himalayas in the North and Western Ghats in the South, and Japan, Indonesia, Ceylon, Malay, Peninsula, Java and tropical Africa in moist temperate and tropical forests, up to an altitude of 3500 m (Khare, 2004).

The plant contains substantial amounts of anthraquinone especially in the roots (the coloring matter present in the root is a mixture of purpurin (trihydroxy anthraquinone) and manjistin (xanthopurpurin-2-carboxylic acid) having antitumor properties. It also have radioprotective (Tripathi and Singh, 2007), hepatoprotective (Mohana *et al*., 2006), anticancer (Son *et al*., 2008), antihyperglycemic (Patil *et al*., 2006), antistress, antimicrobial (Singh *et al*., 2005), anti-inflammatory (Antarkar *et al*., 1983), antioxidant (Son *et al*., 2008), astringent and antidysentric properties. The roots are very effective in purifying blood and are used as laxative, analgesic, lactogogue, emmanogogue, diuretic, and are used in eye sores, paralysis, lethargy, enlargement of spleen, pains in the joints, rheumatism and uterine pains. The stem is described as a cure for snake bite and scorpion sting (Deshkar *et al*., 2008). Apart from its medicinal value, this plant has also been used as natural food colorants and as natural hair dye. Various chemical constituents like iridoid glycoside, naphthoic acid esters, bicyclic hexapeptide and a novel antitumour bicyclic hexapeptide-RA-XVII (Hitotsuyanagia *et al*., 2004) along with anthraquinones have been isolated and

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identified from *R. cordifolia*. Owing to the enormous medicinal potential of this species, it is essential to apply *in vitro* approaches to augment the availability of plant material to facilitate a steady supply of biomass for production of its valuable medicinal compounds and to aid the conservation of its valuable germplasm for maintenance of biodiversity. This study was undertaken to develop the complete *in vitro* propagation protocol of *R. cordifolia,* as it is a potential candidate for further pharmacological investigations.

MATERIALS AND METHODS

Source of explant: *Rubia cordifolia* seeds were collected at the middle of November 2008 from the Karadi para of Munnar hills and planted in the Field Gene Bank repository of TBGRI. Emerging shoots (seedlings) with 3-5 nodes (Fig. 1a) collected from the plants were used as source of explant. After leaf excision, the cuttings each with 3-5 nodes were treated with 1% Labolene detergent (Glaxo India Ltd., Mumbai) in a beaker for 10 min., followed by continous washing in running tap water for 10-20 min. Surface decontamination of the cuttings were done by immersion under stirring in 0.1% (w/v) $HgCl_2$ for 5-10 min., followed by five rinses in sterile distilled water. Shoot tips (0.5- 0.8 cm), split nodal explants (vertical halves-1-1.5 cm) and single nodes (1.0-1.5 cm) were dissected from the cuttings and damaged cut ends if any were removed.

Media and culture conditions: All the explants were rinsed once in sterile distilled water to remove the yellow exudates before transfer to MS medium (Murashige and Skoog, 1962) with 3% sucrose as a source of carbohydrate. The plant growth regulators were added to the medium prior to autoclaving. The pH of the medium was adjusted to 5.8 before 0.5% (w/v) agar (CDH India Ltd., New Delhi) was added and autoclaved at 121°C under 15 kg cm^2 pressure. The explants were implanted into 15 mL aliquots of agar MS medium in 25×150 mm culture tubes, in such a way that the node was

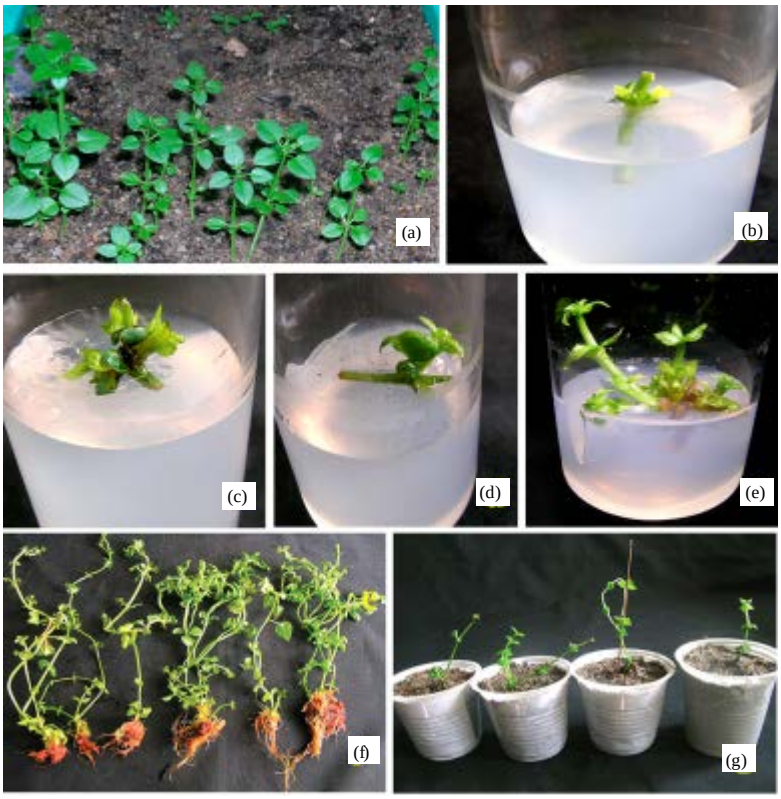

Fig. 1: *In vitro* propagation of *Rubia cordifolia* (a) Plants raised from seeds (b) Initiation of shoot buds from nodal explants-MS + 1.0 mg L^{-1} BA. (c) Multiple shoot formation from the nodal explants after 3 weeks (d) Multiple shoot proliferation in vertical half of the node- MS + 1.0 mg L^{-1} BA + 0.02 mg L^{-1}IAA (e) Axillary shoot proliferation in node after five weeks- MS+1.0 mg L^{-1} BA + 0.02 mg L^{-1} IAA (f) Microshoots rooted on MS + 1.0 mg L^{-1} IBA and (g) *In vitro* shoots established in pots

in contact with the surface of the medium. All the cultures were incubated in a culture room at 25±2°C with a relative humidity of 50-60% and 12 h photoperiod at a photon flux density of 50-60 $\mu Em^{-2}\ s^{-1}$ from day light fluorescent tubes (Philips India Ltd., Bombay).

Shoot initiation: The shoot initiation experiments were conducted in two steps. The initial experiment involved selecting suitable explants using varied concentrations of cytokinins, 6-Benzyladenine (BA) and Kinetin (Kn) and combinations of BA with IAA (Indole -3- acetic acid) and NAA (Naphthalene acetic acid). Each experiment consisted of 40 replicates with one explant per culture tube and was repeated twice.

Shoot multiplication: Multiplication experiments were carried out after 6 weeks of shoot culture initiation. Regeneration potential of shoot tip, split nodal halves and nodal explants in terms of shoot number and length was evaluated by culture on MS medium supplemented with cytokinins, 6-Benzyladenine (BA-0.1-5 mg L^{-1}) and Kinetin (Kn-0. 1-5 mg L^{-1}) and combinations of BA (1-2 mg L^{-1}) and Indole -3- acetic acid (IAA-0.01 mgL^{-1}), BA (1-2 mg L^{-1}) and α-Naphthalene acetic acid (NAA-0.01 -1 mg L^{-1}). Subsequently mass multiplication was achieved by repeated subculture of the shoot tips, split nodal halves and nodal explants of shoot cultures in the medium containing 0.5 mg L^{-1} BA at 6 weeks intervals.

Rooting and acclimatization: Terminal cuttings (3-5 cm) excised from the shoot cultures were used for root initiation in agar gelled MS medium containing different concentrations of different auxins, IAA (0.05- 2 mg L^{-1}), IBA-Indole-3-butyric acid (0.05-2 mgL^{-1}) and NAA (0.05-2 mgL^{-1}). Six weeks after root initiation and 2 weeks of hardening, the rooted plants were weaned from the culture vessels, washed gently to remove agar medium and sucrose traces to discourage infection by fungal contaminants and transplanted in pots containing different potting mixtures of river sand and soil (1:1), river sand, soil and farmyard manure (1:1:1) and river sand alone and kept in a mist chamber. The plants were well irrigated for 4 weeks in a mist chamber maintained at 35°C and 90% relative humidity. Established plants were transfered to pots with fresh potting mixture and kept under nursery conditions for further growth.

Each treatment contained minimum of 5 replicates and the experiments were conducted in randomly random design. All the data were taken after 6 weeks incubation, analysed by ANOVA ($p \leq 0.5$) and the means were compared using least significant Difference (LSD) test.

RESULTS

Initial experiments carried out with the nodal and shoot tip explants revealed that the percentage of infection and survival solely depend on the time of exposure in $HgCl_2$. Nearly 70% were lost due to microbial contamination when they were treated with 0.1% $HgCl_2$ for 5 min. High rate of survival (80%) and low percentage of microbial contamination was achieved when the explants were immersed in 1% (v/v) Labolene for 5-10 min. and disinfection with 0.1% $HgCl_2$ for 7 min. Sterilization of young shoot tips and nodes in 0.1% $HgCl_2$ for 5 min, high percentage (90%) of survival was noticed.

Axillary bud break and the emergence of single shoot from shoot tip explants were noticed in the first week. Among the cytokinins tested, BA was more effective in inducing multiple shoot formation than Kn in nodal explants. When BA alone was used, better results were obtained only at certain optimum concentrations in all the three types of explants. The highest frequency of shoot formation was recorded in nodes (88%) in an optimum concentration of 1.0 mg L^{-1} BA and 0.02 mg L^{-1} IAA with an average number of 5.9 shoots per node, with a mean shoot length of 3.7cm (Table 1). The quality of shoots and the over all growth response in terms of average number of nodes per shoot was better in this combination. The second highest frequency of shoot formation was recorded in split nodal halves (79%), in an optimum concentration of 1.0 mg L^{-1} BA and 0.02 mg L^{-1} IAA, with an average number of 5.2 shoots per explant, with a mean shoot length of 3.9cm (Table 2). In case of shoot tip explants, the highest frequency of shoot formation (77%) was recorded with an optimum concentration of 0.5 mg L^{-1} BA and 0.01 mg L^{-1} IAA with an average number of 1.6 shoots per explant with an average shoot length of 2.1 cm (Table 3). None of the combinations of BA and NAA stimulated shoot initiation more than individual concentrations of BA and BA with IAA, though callusing from the cut ends was pronounced. Although a synergistic effect of BA in combination with IAA was observed at certain concentrations, an increase in shoot multiplication rate was not observed with the same level of BA concentration and with increasing levels of IAA. Shoots obtained in the split nodal halves were far more than that developed from shoot tip explants. However addition of BA (1.0 mg L^{-1}) with IAA (0.02 mg L^{-1}) was beneficial for nodes (Fig. 1e) and split vertical halves (Fig. 1d) in initiation, multiple shoot formation and shoot elongation. A significant increase in shoot elongation (4.92) was observed particularly in medium containing 0.5 mg L^{-1} BA and 0.02 mg L^{-1} IAA (Table 3) in split nodal halves. None of the explants

Table 1: Shoot initiation in isolated young nodes of *Rubia cordifolia* in MS medium supplemented with various concentrations of BA and Kn and combinations of BA and auxins (IAA/NAA). Nodes of 3rd to 5th position from the top of the shoot were used for the experiments. Observations were made after 6 weeks of culture initiation

PGR (s)(mg L^{-1})		Percentage response	Mean No. of shoots/node±SE	Mean shoot length (cm)±SE	Mean No. of node/shoots±SE	Callusing
BA						
0.1		55	1.1±0.20	2.2±0.2	3.1±0.1	-
0.5		70	1.8±0.40	2.3±0.3	3.1±0.2	-
1.0		88	2.4±0.20	2.4±0.4	2.9±0.3	-
2.0		79	3.6±0.01	3.6±0.3	3.6±0.1	-
3.0		69	2.5±0.10	2.4±0.2	2.4±0.1	-
4.0		72	2.0±0.20	2.9±0.3	2.4±0.2	-
5.0		78	1.2±0.01	2.2±0.1	2.4±0.2	-
Kn						
0.1		66	1.1±0.01	2.4±0.1	2.9±0.3	-
0.5		69	1.2±0.20	2.4±0.3	2.8±0.2	-
1.0		78	1.2±0.20	2.7±0.3	2.7±0.1	-
2.0		71	1.2±0.03	2.4±0.2	2.8±0.2	-
3.0		70	1.1±0.30	2.4±0.4	2.4±0.4	-
4.0		67	1.1±0.20	2.1±0.1	2.6±0.1	-
5.0		68	1.1±0.20	2.4±0.2	2.4±0.3	-
BA						
1.0	0.01	51	2.3±0.20	2.7±0.3	2.9±0.1	-
1.0	0.02	88	5.9±0.30	3.7±0.1	3.7±0.2	-
1.0	0.05	67	2.3±0.01	2.4±0.2	2.7±0.3	-
1.0	0.1	65	2.2±0.20	2.3±0.2	2.2±0.5	-
2.0	0.01	61	2.1±0.02	2.2±0.3	2.2±0.1	-
2.0	0.02	63	2.0±0.01	2.2±0.1	2.1±0.2	-
2.0	0.1	56	1.8±0.03	1.6±0.1	2.1±0.3	++
BA	**NAA**					
1.0	0.01	65	2.0±0.20	2.4±0.1	2.5±0.1	-
1.0	0.02	61	2.3±0.10	2.4±0.2	2.5±0.2	-
1.0	0.05	63	2.1±0.10	2.2±0.2	2.4±0.3	-
1.0	0.1	65	2.3±0.20	2.1±0.4	2.2±0.4	-
2.0	0.01	66	2.4±0.20	2.2±0.3	2.1±0.2	+
2.0	0.02	67	2.0±0.02	2.2±0.2	2.0±0.3	+
2.0	0.05	68	2.0±0.01	2.4±0.3	2.1±0.2	++
2.0	0.1	55	2.0±0.10	2.1±0.1	2.2±0.2	++

Mean values (n = 50) - Sign represents no callusing. + Sign represents low callusing. ++ Sign represents high callusing

Table 2: Influence of different concentrations of BA and combinations of BA and IAA on shoot multiplication during subculture of shoot culture derived from shoot tip explants of *Rubia cordifolia* cultured in MS agar medium. Data were recorded after 5 weeks of culture

PGR(s) (mg L^{-1})		%Response	Mean no. of shoots/ explant±SE	Mean shoot length (cm)±SE
BA				
0.5		67	2.3±0.3	2.1±0.2
1.0		63	2.2±0.2	2.3±0.3
2.0		61	2.0±0.1	2.2±0.1
3.0		67	1.9±0.1	2.0±0.3
4.0		66	1.9±0.1	1.9±0.2
5.0		66	1.5±0.01	1.2±0.4
BA	**IAA**			
0.5	0.01	77	1.6±0.2	2.1±0.1
0.5	0.02	61	1.3±0.1	2.1±0.01
0.5	0.1	62	1.5±0.1	1.3±0.01
1.0	0.01	46	1.3±0.01	1.5±0.1
1.0	0.02	30	1.2±0.01	1.3±0.1
1.0	0.1	23	1.3±0.1	1.2±0.2

Mean values (n = 50)

Table 3: Influence of different concentrations of BA and combinations of BA and IAA on shoot multiplication during subculture of shoot culture derived from split vertical halves of nodes of *Rubia cordifolia* cultured in MS agar medium. Data were recorded after 5 weeks of culture

PGR(s) (mg L^{-1})		% Response	Mean No. of Shoots/explant±SE	Mean shoot length (cm)±SE
BA				
0.5		60	4.7±0.1	3.7±0.2
1.0		78	5.6±0.2	3.9±0.1
2.0		55	4.1±0.2	3.3±0.03
3.0		67	2.3±0.1	2.9±0.02
4.0		43	2.5±0.1	2.4±0.1
5.0		45	2.2±0.01	2.1±0.1
BA	**IAA**			
0.5	0.01	45	3.4±0.2	3.2±0.1
0.5	0.02	56	3.6±0.1	4.9±0.4
0.5	0.1	57	2.3±0.2	2.3±0.4
1.0	0.01	65	4.4±0.2	3.7±0.2
1.0	0.02	79	5.2±0.03	3.9±0.2
1.0	0.1	45	2.7±0.01	2.2±0.1

Mean values (n = 50)

showed callusing in the basal medium and medium with different concentrations of plant growth regulators except with NAA. A compact red callus proliferated upon the cut ends of nodal explants cultured in media containing BA and NAA. The repeated subculture of the nodes and split nodal halves of shoot cultures up to 4 cycles at 2 week intervals using 0.5 mg L^{-1} BA enabled continuous production of shoots without loss of vigor, callusing and growth abnormalities.

When individual shoots of 3-6 cm length were separated and transferred to MS basal medium an initial swelling of the cut ends occurred and slender long roots

Table 4: Rhizogenic response of shoots transferred to MS medium containing different concentrations of auxins. Observations were made 6 weeks after transfer

Auxins (mgL^{-1})	Percentage Rooting	Mean no. of roots±SE	Mean root length (cm)±SE	Callusing
0.0	-	-	-	-
IAA				
0.05	68	2.3±0.1	2.4±0.01	-
0.1	67	2.7±0.01	2.5±0.1	-
0.5	66	4.6±0.2	3.4±0.1	-
1.0	67	6.2±0.3	3.9±0.2	-
2.0	64	2.1±0.1	3.1±0.2	+
IBA				
0.05	67	2.1±0.1	2.3±0.1	-
0.1	73	3.3±0.2	3.1±0.2	-
0.5	74	5.4±0.3	5.3±0.2	-
1.0	98	8.9±0.1	6.4±0.3	-
2.0	71	2.1±0.01	2.1±0.3	-
NAA				
0.05	67	2.1±0.1	1.3±0.1	-
0.1	61	2.4±0.2	2.3±0.1	+
0.5	64	2.1±0.2	2.1±0.1	++
1.0	65	2.2±0.2	2.1±0.2	++
2.0	61	2.2±0.1	2.0±0.2	++

Mean values (n = 40) - sign represents no callusing + sign represents low callusing ++ sign represents high callusing

Table 5: Influence of auxin type dependent rooting of *Rubia cordifolia* shoots on their establishment in pots after hardening in the mist chamber for 4 weeks. The plants were transferred to pots after 6 weeks of rooting and establishment rate was recorded after 4 weeks of hardening

Auxins	Potting mixture	% Establishment
IBA	River sand and soil	89
	River sand	74
	River sand, soil and farmyard	67
IAA	River sand and soil	80
	River sand	63
	River sand, soil and farmyard	62
NAA	River sand and soil	54
	River sand	45
	River sand, soil and farmyard	40

Each set consists of 40 *in vitro* derived plants

were formed after one week. Among the three auxins tested, the best root induction and survival was achieved in IBA followed by IAA and then NAA. The highest frequency (98%) of root regeneration and survival was observed in 1 mg L^{-1} IBA, with an average number of 8.9 thick roots having an average length of 6.4 cm per microshoot within five weeks after transplantation into the rooting medium (Fig.1f). An average of 6.2 short slender roots was obtained in 1 mg L^{-1} IAA (Table 4).

Hardening is also an unavoidable requirement of the rooted plants for their successful establishment in pots. The percentage survival of plantlets developed in IBA and IAA were 89 and 80% respectively in river sand and soil (Table 5). NAA treated microshoots produced the maximum amount of basal callus which resulted poor establishment in community pots. All the micropropagated plants exhibited uniform morphological characteristics without any growth defects.

DISCUSSION

The present investigation indicates the high regenerative potential of the nodal and split nodal half explants of *R. cordifolia* on MS medium supplemented with different plant growth regulators. It appears that organogenesis is dependent on the factors like explant type, physiological state of donor plant or organ and endogenous level of phytohormones (Thanh and Trinh, 1990). When BA was used alone, highest frequency of shoot formation was observed only at certain optimum concentrations and low frequency shoot formation was recorded both at lower and higher concentrations of BA. Similar results were reported in *Citrus* sps. (Baruach *et al*., 1995). The relative merit of using optimum concentration of BA compared to other cytokinins for shoot initiation in tissue culture of a number of species has been well documented. The effect of BA on *R. cordifolia* agrees with the results of Jain *et al.* (2009) in shoot bud induction from the nodes of *Withania coagulans*. The effect of Kn also showed good percentage of response, although it is very poor in multiple shoot induction. In this study, highest frequency of shoot formation was recorded in nodes when compared to shoot tips. Similar results were observed by Murkute *et al.* (2009) in *Poncirus trifoliate*. Second highest frequency of shoot multiplication was observed in split nodal halves in the presence of BA (1.0 mg L^{-1}) with IAA (0.02 mg L^{-1}). These results are in agreement with the greater number of shoots multiplied from half stem explants of *Brassica alboglabra*, where the large cutsurface area was thought to contribute enhanced regeneration (Pua *et al*., 1989). These results were contradictory to negligible increase in multiplication when the nodal halves were used in *Adhatoda beddomei* (Sudha and Seeni, 1994). The results suggest that the nodal explants (intact and split vertical halves) are better sources of shoot formation than the shoot tips, despite the presence of both the apical and axillary meristems in the latter. Differential response could be due to the

varying concentrations of the growth regulators used in the medium and the explant types which has also been observed in other systems (Rout and Das, 1997; Saxena *et al*., 1997; Patra *et al*., 1998).

Combinations of BA and IAA (1BA and 0.02IAA) gave better response than BA alone. As in the present study, other workers have also reported the effect of combination of cytokinins and auxins for inducing shoot regeneration, where the concentration of cytokinins were higher than that of auxins (Vidya *et al*., 2005). As reported by Zheng *et al*. (2001), the balance of auxin to cytokinin is a determining factor for efficient shoot regeneration. The synergistic effect of BA in combination with an auxin has been reported in other medicinal plants like *Adhatoda beddomei* (Sudha and Seeni, 1994), *Murraya koeningii* (Rout, 2005) and *Melia azadarach* L. (Husain and Anis, 2009).

Significant differences for the number, length and thickness of the roots were observed between the auxins. Among the three auxins, the best rooting was observed in IBA than IAA. IBA is a plant growth regulator most commonly used for the rooting of *in vitro* shoots. These findings are in agreement with those observed in other medicinal plants like *Adhatoda vasica* (Azad *et al*., 1999) and *Holostemma adakodien* (Martin, 2002). Rooting efficiency was poor on NAA supplemented medium compared to other auxins . Moreover, NAA induced profuse callusing from the cut ends. Plantlets were successfully hardened in the room temperature for 2 weeks, followed by establishment in pots. Hardened plants of *R. cordifolia* recorded 89% survival in the field, which is a good indication for the restoration of the species. All the established plants were apparently uniform and did not show any detectable variation.

This system suggest a feasible method for replenishing the wild population as the *in vitro* progenies accomplished their life cycle successfully in the field and makes a valuable contribution to the conservation of the species.

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ABBREVIATIONS

BA : 6- Benzylaminopurine
IAA : Indole-3-acetic acid
IBA : Indole-3-butyric acid
Kn : Kinetin
MS : Murashige and Skoog
NAA : α-Naphthalene acetic acid
PGR : Plant Growth Regulator

REFERENCES

Antarkar, D.S., T. Chinwalla and N. Bhatt, 1983. Anti-inflammatory activity of *Rubia cordifolia* Linn. in rats. Indian J. Pharmacol., 15: 185-188.

Azad, M.A.K., M.N. Amin and F. Begum, 1999. *In vitro* rapid regeneration of plantlets from cotyledon explant of *Adhatoda vasica* Nees. Plant Tissue Cult., 9: 121-126.

Baruach, A., V. Nagarajan and V.A. Parthasarathy, 1995. Micropropagation of three endangered citrus species. Ann. Plant Physiol., 10: 124-124.

Deshkar, N., S. Tilloo and V. Pande, 2008. A comprehensive review of *Rubia cordifolia* Linn. Pharm. Rev., 2: 124-134.

Hitotsuyanagia, Y., H. Ishikawaa, T. Hasudaa and K. Takeya, 2004. Isolation, structural elucidation and synthesis of RA-XVII, a novel bicyclic hexapeptide from *Rubia cordifolia* and the effect of side chain at residue1 upon the conformation and cytotoxic activity. Tertrahedron lett., 45: 935-938.

Husain, M.K. and M. Anis, 2009. Rapid *in vitro* multiplication of *Melia azadarach* L. (a multipurpose) woody tree. Acta Physiol. Plant., 31: 765-772.

Jain, R., A. Sinha, S. Kachhwaha and S.L. Kothari, 2009. Micropropagation of *Withania coagulans* (Stocks) Dunal: A critically endangered medicinal herb. J. Plant Biochem. Biotechnol., 18: 249-252.

Karim, A., M. Nouman, S. Munir and S. Sattar, 2011. Pharmacology and phytochemistry of Pakistani herbs and herbal drugs used for treatement of diabetes. Int. J. Pharmacol., 7: 419-439.

Khare, C., 2004. Encyclopedia of Indian Medicinal Plants-Rational Western Therapy, Ayurvedic and other Traditional Usage. Springer, Germany, ISBN: 3-540-20033-9.

Martin, K.P., 2002. Rapid propagation of *Holostema ada-kodien* Schult. a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. Plant Cell Rep., 21: 112-117.

Mohana Rao, G.M., C.V. Rao, P. Pushpangadan and A. Shirwaikar, 2006. Hepatoprotective effects of rubiadin, a major constituent of *Rubia cordifolia* Linn. J. Ethnopharmacol., 103: 484-490.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Murkute, A.A., S. Sharma and S.K. Singh, 2009. Micropropagation of trifoliate orange (*Poncirus trifoliate*). Ind. J. Plant Physiol., 14: 190-193.

Patil, R.A., S.C. Jagdale and S.B. Kasture, 2006. Anti hyperglycemic, antistress and nootropic activity of roots of *Rubia cordifolia* Linn. Indian J. Exp. Biol., 44: 987-992.

Patra, A., B. Rai, G.R. Rout and P. Das, 1998. Successful plant regeneration from callus cultures of *Centella asiatica* (Linn.) Urban. Plant Growth Regul., 24: 13-16.

Pua, E.C., T.H. Trinh and N.H. Chua, 1989. High frequency plant regeneration from stem explants of *Brassica alboglabra* Bailey *in vitro*. Plant Cell Tissue Organ Culture, 17: 143-152.

Rout, G.R and P. Das, 1997. *In vitro* organogenesis in ginger (*Zingiber officinale* Rosc.). J. Herbs Spices Medi. Plants, 4: 41-51.

Rout, G.R., 2005. Direct plant regeneration of curry leaf tree (*Murraya koenigii* Koenig.), an aromatic plant. *In vitro* Cell. Dev. Biol. Plant, 41: 133-136.

Saxena, C., S.K. Palai, S. Samantaray, G.R. Rout and P. Das, 1997. Plant regeneration from callus cultures of *Psoralea corylifolia* Linn. Plant Growth Regulat., 22: 13-17.

Singh, R., A. Jain, S. Panwar, D. Gupta and S.K. Khare, 2005. Antimicrobial activity of some natural dyes. Dyes Pigments, 66: 99-102.

Son, J.K., S.J. Jung, J.H. Jung, Z. Fang and C.S. Lee *et al*., 2008. Anticancer constituents from the roots of *Rubia cordifolia* Linn. Chem. Pharmaceutical Bull., 56: 213-216.

Sudha, C.G. and S. Seeni, 1994. *In vitro* multiplication and field establishment of *Adhatoda beddomei* C.B. Clarke, a rare medicinal plant. Plant Cell Rep., 13: 203-207.

Thanh, K.T. and T.H. Trinh, 1990. Organogenetic Differentiation. In: Plant Tissue Culture Application and Limitations, Bhowani, S.S. (Ed.). Elsevier, USA., pp: 34-53.

Tripathi, L. and J.N. Tripathi, 2003. Role of biotechnology in medicinal plants. Trop. J. Pharm. Res., 2: 243-253.

Tripathi, Y.B. and A.V. Singh, 2007. Role of *Rubia cordifolia* Linn. in radiation Protection. Indian J. Exp. Biol., 45: 620-625.

Vidya, S.M., V. Krishna, V.K. Manjunatha and K. Sankarmurthy, 2005. Micropropagation of *Entada pursaetha* DC: An endangered medicinal plant of Western Ghats. Ind. J. Biotechnol., 4: 561-564.

Zheng, S.S., H.Y. Yuan, L.J. Wang, C.C. An and Z.L. Chen, 2001. The tissue culture of medicinal plant *Trichosanthes kirilowii* and its protein analysis. Sheng wu Gong Cheng Xue Bao, 17: 420-422.