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An Improved Plant Regeneration System for High Frequency Multiplication of *Rubia cordifolia* L.: A Rare Medicinal Plant

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ABSTRACT

Rubia cordifolia (Rubiaceae), a rare and highly medicinal plant of Western ghats of India, needs to be conserved as its primary gene pool is threatened by deforestation and extensive exploitation by pharmaceutical industries which has resulted in disappearance of natural habitats of this species. Therefore, rapid multiplication and conservation of *R. cordifolia* is of paramount importance. An *in vitro* multiplication protocol for this plant has been developed by using axillary bud culture. MS basal medium supplemented with a synthetic cytokinin, thidiazuron (1 mg L^{-1}) induced highest number of shoots per tube (12.67) with 85% of response, as compared with other cytokinins. Rooting of micropropagated shoots were achieved on half strength MS basal medium supplemented with NAA (2 mg L^{-1}). Hardening of plants was accomplished by transfer of rooted plants to a mixture of soil, sand and compost (1: 2: 1) and then plantlets were transferred subsequently to the field. Using this axillary bud technology from single bud, more than 1000 plants were raised. This has proven to be the best tool for conservation of *R. cordifolia* and its rapid multiplication as well.

Key words: *Rubia cordifolia*, medicinal plant, axillary bud culture, MS basal medium, thiodiazuro

INTRODUCTION

In India, medicines based on herbal origin have been the basis of treatment and cure of various diseases (Biswas *et al.*, 2004). *Rubia cordifolia,* a member of family Rubiaceae, possesses various medicinal properties with respect to its plant parts. The roots are major source of its medicinal properties. Root extracts possess hepatoprotective activity, antineoplastic property and are considered to be useful for the disintegration and elimination of urinary stones (Gilani and Janbaz, 1995; Divakar *et al.*, 2010). It has an immunomodulatory property (Joharapurkar *et al.*, 2003) and antiproliferative activity against epidermal keratinocytes (Tse *et al.*, 2006). In Ayurveda, the roots are used in Oedema, disorders of blood, gout, diarrhoea, leprosy, erysipelas, wounds, polyuria, gynaecological disorders, eye diseases, dysuria and ear diseases. Similarly, stem of this plant is used as an antidote to snake bite and scorpion sting (Talapatra *et al.*, 1981).

In Chinese Pharmacopoeia, dried roots of *R. cordifolia* are listed as a herbal medicine for the treatment of arthritis, dysmenorrhea, hematorrhea, hemostasis, as a tonic and for wound healing (Anonymous, 1992). In addition to this, the plant has been used for curing menstrual pain, rheumatism and urinary disorders (Hocking, 1997). It has been reported that the plant contains naturally occurring chemo preventive agents (Chang *et al.*, 2000). It also possesses antipyretic and analgesic activities (Gupta *et al.*, 2008). It is used as an antiseptic for wounds and known to be used in folk medicines for the treatment of cancer, skin diseases like eczema, dermatitis and skin ulcers (Karodi *et al.*, 2009).

R.cordifolia is known to contain substantial amounts of anthraquinones specially in the roots (Singh *et al.*, 2004). It contains antitumor bicyclic hexapeptides (Itokawa *et al.*, 1992a; Itokawa *et al.*, 1992b), manjistin and purpurin (Mischenko *et al.*, 1999), rubiadin (Rao *et al.*, 2006), rubierythrinic acid, alizarin and pseudoperpurin (Tripathi *et al.*, 1997).

Due to its high medicinal potential, extensive exploitation is being occurred from its natural resources leading to the depletion of its population. This situation of mass extinction has lead for its rarity. Biotechnological tools are important for multiplication of medicinal plants by adopting techniques such as in vitro regeneration (Tripathi and Tripathi, 2003). Therefore, a rapid *in vitro* multiplication protocol has been developed for this plant using axillary bud culture, for their sustainable use.

MATERIALS AND METHODS

The nodal explant was harvested from a one-year old plant of *R. cordifolia* (Fig. 1a) growing in the polyhouse of the Department of Botany, Shivaji University, Kolhapur. Healthy nodal stem segments were washed in running tap water for 30 min. The nodal explants were thoroughly rinsed with commercial detergent (labolene) for 4-5 times and then again washed in running tap water. Under aseptic conditions these explants were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 7 min followed by several repeated washings of sterilized double distilled water. Further, explants were cut into appropriate sizes (~1 cm) with a single node and placed on sterile nutrient medium.

In the study of Murashige and Skoog (1962), medium; containing different concentrations and combinations of growth hormones was used for the multiple shoot induction studies. The medium was supplemented with 3% sucrose and pH of the medium was adjusted to 5.8±0.1 prior to the addition of agar (0.8%; w/v). The agarified medium was dispensed in culture tubes (15 mL tube), plugged with non-absorbent cotton and autoclaved at 1.06 kg^{-1} cm^2 pressure for 20 min.

For the root induction, half strength of MS basal medium supplemented with different concentrations of auxins was used. All the cultures were maintained at 25±2°C with 16 h light 8 h dark photoperiods (Philips TL 34, 25 μmol m^{-2} sec^{-1}) and the relative humidity of the culture room was maintained >80%.

Rooted shoots were transferred directly to small plastic pots using different potting mixtures containing variable proportions of sterile sand, soil and compost mixtures. The hardened plantlets were transferred subsequently to the field.

Statistical analysis of data: Analysis of variance and comparison of means were carried out on all data using Statistical Analysis System (SAS). The effects of different hormones on the induction of shoots, as well as on induction of *in vitro* rooting, were compared by one-way ANOVA. Differences between means were assessed for significance at $p<0.05$ and $p<0.001$ using Tuke-Kramers multiple comparison test.

All the experiments were repeated at least twice. For each of the treatments stated, twenty replicates in case of multilple shoot induction and twelve replicates in case of *in vitro* rooting experiments were maintained. The data was collected after 4 weeks interval.

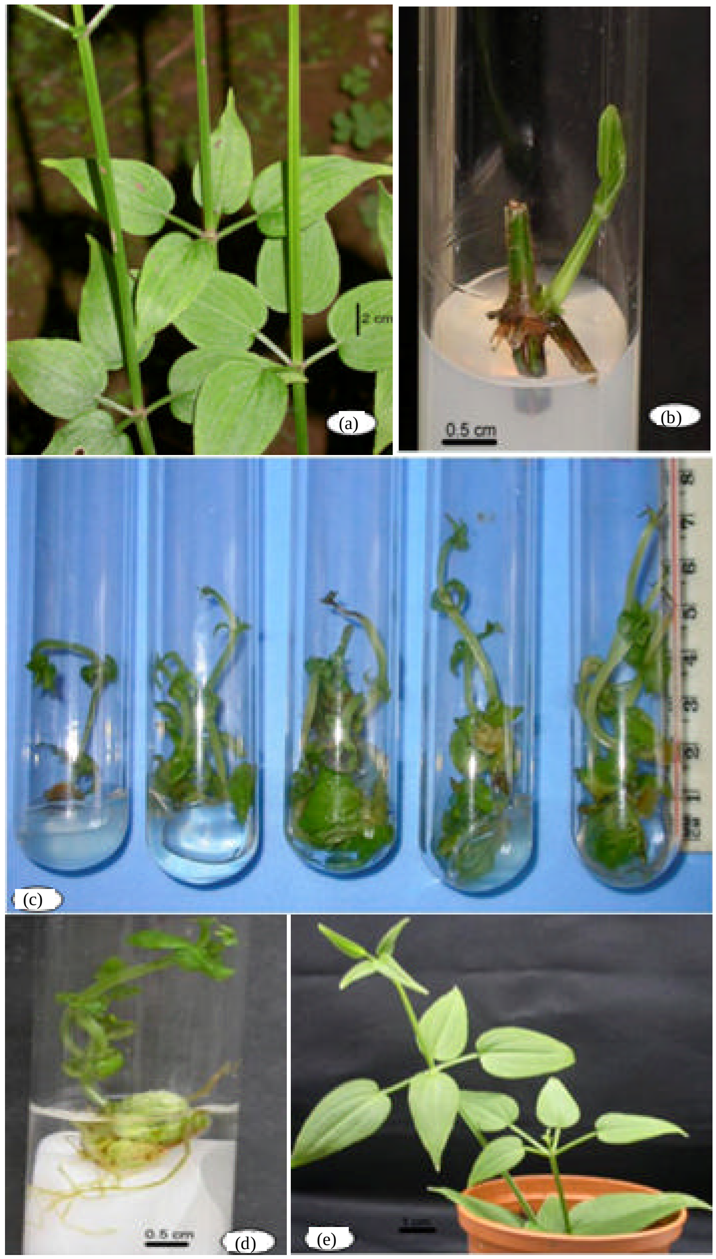

Fig. 1: (a) *Rubia cordifolia* (b, c) shoot multiplication on MS basal medium supplemented with TDZ (1 m g^{-1}) (d) *in vitro* rooting of micropropagated shoots on half strength MS basal medium supplemented with NAA (2 mg L^{-1}) with callusing at base (e) hardened plant after 30 days

RESULTS AND DISCUSSION

Multiple shoots were initiated from the nodal segments (Fig. 1b) on all media combinations tried in present investigation except MS basal medium (Table 1, Fig. 1c). Among the different cytokinins

Table 1: Effect of different concentrations of growth hormones on shoot multiplication in cultured nodal explant of *Rubia cordifolia*

Medium	(%) Response	Mean shoot±SE
MS basal	--	--
MS basal + BA 0.5 mg L^{-1}	15	1.67±0.41
MS basal + BA 1.0 mg L^{-1}	25	2.27±0.91
MS basal + BA 2.0 mg L^{-1}	55	7.25±0.69[b]
MS basal + BA 3.0 mg L^{-1}	45	3.25±0.68[a]
MS basal + TDZ 0.5 mg L^{-1}	60	4.67±0.89[b]
MS basal + TDZ 1.0 mg L^{-1}	85	12.67±0.79[b]
MS basal + TDZ 2.0 mg L^{-1}	75	9.75±0.74[b]
MS basal + TDZ 3.0 mg L^{-1}	65	4.33±0.34[b]
MS basal + kinetin 0.5 mg L^{-1}	15	1.42±0.40
MS basal + kinetin 1.0 mg L^{-1}	20	2.33±0.54
MS basal + kinetin 2.0 mg L^{-1}	35	2.75±0.56
MS basal + kinetin 3.0 mg L^{-1}	30	2.58±0.45
MS basal + zeatin 0.5 mg L^{-1}	15	1.92±0.63
MS basal + zeatin 1.0 mg L^{-1}	35	2.83±0.57
MS basal + zeatin 2.0 mg L^{-1}	65	6.08±0.62[b]
MS basal + zeatin 3.0 mg L^{-1}	45	3.25±0.41[a]
MS basal + TDZ 1.0 mg L^{-1} + kinetin 0.5 mg L^{-1}	40	1.25±0.45
MS basal + TDZ 1.0 mg L^{-1} + kinetin 1.0 mg L^{-1}	60	7.08±0.63[b]
MS basal + TDZ 1.0 mg L^{-1} + kinetin 2.0 mg L^{-1}	30	2.17±0.57
MS basal + TDZ 1.0 mg L^{-1} + kinetin 3.0 mg L^{-1}	15	1.08±0.62

Value represents Mean±Standard error of twenty replicates per treatment and all the experiments were repeated at least twice. Data were statistically analysed using Tukey-Kramer multiple comparison test of ANOVA where a indicates a significant difference at a level of $p<0.05$ and b indicates difference at a level of $p<0.001$

tested, TDZ was found to be the most effective in shoot induction (85%) and subsequent shoot proliferation with an average of 12.67 shoots per axillary bud. Effectiveness of TDZ in shoot multiplication has been previously reported in *R. cordifolia* with 0.5 mg L^{-1} concentration (Shrotri and Mukundan, 2004). In the present investigation, 1 mg L^{-1} TDZ was found most effective in shoot multiplication in *R. cordifolia*. The average number of shoots is less as compared with reports of Shrotri and Mukundan (2004) where they reported 15.6 shoots per node. BA (2 mg L^{-1}) and zeatin (2 mg L^{-1}) singly were also found effective in shoot multiplication in *R. cordifolia* (7.25 shoots per node and 6.08 shoots per node respectively). However, the number of multiple shoots in BA concentrations was found less as compared with those in TDZ. Use of BA singly in the medium neither accelerated the percent response, nor could increase the number of shoots per explants. These reports are in contradiction with the recent studies performed by Radha *et al.* (2011) in *R. cordifolia*, where BA alone gave better results at certain optimum concentrations.

The overall effect of BA in shoot multiplication studies in *R. cordifolia* was found better than that of kinetin and zeatin. The effect of zeatin (2 mg L^{-1}) showed good percentage of response, although it is very poor in increasing the number of multiple shoots (Table 1). However, hormone TDZ showed a promising effect with respect to the multiplication of axillary buds.

In the present study it was found that TDZ had an imperative role in shoot induction and multiplication as well, than in somatic embryogenesis in *R. cordifolia*. The noteworthy effect of TDZ on shoot multiplication was reported previously by several workers in number of plant species like *Arachis hypogea* (Murthy *et al.*, 1995), *Artemisia judaica* (Liu *et al.*, 2003), *Hevea brasiliensis*

Table 2: Effect of MS basal medium supplemented with various concentrations of auxin on *in vitro* rooting in *Rubia cordifolia*

Concentrations	(%) Response	Mean root±SE
MS* Basal	83.33	3.75±0.70
MS* + IBA 0.5 mg L^{-1}	83.33	2.33±0.58
MS* + IBA 1.0 mg L^{-1}	91.67	3.67±0.76
MS* + IBA 2.0 mg L^{-1}	91.67	5.33±0.82
MS* + IBA 3.0 mg L^{-1}	100.00	4.58±0.77
MS* + IAA 0.5 mg L^{-1}	58.33	1.58±0.47
MS* + IAA 1.0 mg L^{-1}	66.67	2.17±0.74
MS* + IAA 2.0 mg L^{-1}	41.67	0.92±0.40
MS* + IAA 3.0 mg L^{-1}	33.33	0.75±0.35
MS* + NAA 0.5 mg L^{-1}	83.33	1.83±0.53
MS* + NAA 1.0 mg L^{-1}	91.67	3.42±0.79
MS* + NAA 2.0 mg L^{-1}	100.00	8.75±0.91[b]
MS* + NAA 3.0 mg L^{-1}	100.00	5.75±1.01[a]
MS * + IBA 2.0 mg L^{-1} + IAA 0.5 mg L^{-1}	58.33	2.17±0.55
MS * + IBA 2.0 mg L^{-1} + IAA 1.0 mg L^{-1}	66.66	4.08±0.42
MS * + IBA 2.0 mg L^{-1} + IAA 2.0 mg L^{-1}	41.66	2.08±0.82
MS * + IBA 2.0 mg L^{-1} + IAA 3.0 mg L^{-1}	25.00	1.08±0.27
MS * + IBA 2.0 mg L^{-1} +NAA 0.5 mg L^{-1}	58.33	3.25±0.51
MS * + IBA 2.0 mg L^{-1} + NAA 1.0 mg L^{-1}	83.33	5.00±0.67
MS * + IBA 2.0 mg L^{-1} + NAA 2.0 mg L^{-1}	41.66	3.08±0.53
MS * + IBA 2.0 mg L^{-1} + NAA 3.0 mg L^{-1}	25.00	1.08±0.12
MS * + IAA 1.0 mg L^{-1} + NAA 0.5 mg L^{-1}	50.00	1.50±0.29
MS * + IAA 1.0 mg L^{-1} + NAA 1.0 mg L^{-1}	50.00	2.00±0.34
MS * + IAA 1.0 mg L^{-1} + NAA 2.0 mg L^{-1}	75.00	6.08±0.57
MS * + IAA 1.0 mg L^{-1} + NAA 3.0 mg L^{-1}	41.66	2.42±0.78

*MS basal medium (Murashige and Skoog, 1962) containing half strength major, minor, vitamins, FeEDTA, inositol and Sucrose (1.5%). Value represents Mean±Standard error of twelve replicates per treatment and all the experiments were repeated at least twice. Data were statistically analysed using Tukey-Kramer multiple comparison test of ANOVA where a indicates a significant difference at a level of $p<0.05$ and b indicates difference at a level of $p<0.001$

(Seneviratne and Flagmann, 1996), *Kigelia pinnata* (Thomas and Puthur, 2004), *Morus alba* (Thomas, 2003), *Primula* sp. (Schween and Schwenkel, 2002), *Saintpaulia ionantha* (Mithila *et al.*, 2003) and *Selenicereus megalanthus* (Pelah *et al.*, 2002) which are well in coordination with the present results. In addition to this, high concentrations of TDZ resulted in stunted shoots with hyperhydric condition which is in concurrence with the preceding studies accounted in *Pyrus pyrifolia* (Kadota and Niimi, 2003) and *Musa acuminate* (Farahani *et al.*, 2008). Indeed, various studies showed that TDZ was effective and more active than BA and zeatin especially in the micropropagation of woody plants (Lu, 1993).

In the *in vitro* rooting studies, NAA (2.0 mg L^{-1}) was found highly effective (100% root induction) with an average of 8.75±0.91 roots per shoot (Table 2, Fig. 1d). However, there was presence of unwanted callus at the base of the shoot. Increase in the NAA concentration (3 mg L^{-1}), does not affect the rooting percentage but the number of roots per shoot declined (Table 2) considerably. It was also observed that increased auxin concentration favours callus induction at the shoot base. Phytohormone IBA (3 mg L^{-1}), was also found to induce 100% root induction in the *in vitro* grown shoots but the concentration in any way, did not support the increase in number of roots per shoot. Infact, the number was reduced to half of that (4.58±0.77) which were recorded in

Table 3: The survival percentage of micropropagated plantlets of *Rubia cordifolia* in different potting mixtures

Soil : Sand: Compost mixture	Percent survival*
1: 1: 1	40
1: 2: 1	65
1: 3: 1	30
1: 4: 1	20
1: 5: 1	09
2: 1: 1	35
2: 2: 1	55

*Data collected after 30 days of transplantation

NAA (2 mg L^{-1}). Among three different auxins tested, IAA was found least effective. Efficacy of an auxin is very well known for induction of rooting in several plant species such as *Aloe polyphylla* (Abrie and Van Staden, 2001), *Cunila galioides* (Fracaro and Echeverrigaray, 2001), *Myrtus communis* (Shekafandeh, 2007) and *Morus alba* (Thomas, 2003). Similarly, the role of NAA in root induction of *in vitro* derived shoots has been well documented in *Grevillea robusta* (Rajsekaran, 1994), Safflower (Radhika *et al*., 2006), *Spondius mombin* (Carvalho *et al*., 2002) and *Vitex nigundo* (Kannan and Jesrai, 1998). Combination of two auxins did not show any indicative effect for the increment in number of roots per shoot. In all the combinations of auxins tested (each combination contained two auxins), half strength MS basal medium supplemented with IBA (2 mg L^{-1}) and NAA (1 mg L^{-1}) indicated 83.33% of rooting with an average of 5.0 roots per shoot. These findings are in contradiction to the reports of Rahman *et al*. (2004) in *Elaeocarpus robustus* where they posed that when auxins are used in combination, it enhances the average number of roots/plant, percent root induction and the average length of the roots. Effectiveness of two or more auxins in the *in vitro* rooting has been previously reported in *Baliospermum montanum* (Johnson and Manickam, 2003), *Ophiorrhiza mungo* (Jose and Satheeshkumar, 2004), *Prunus* hybrid (Vaez-Livari and Salehi-Soghadi, 2006) and *Rosa indica* (Soomro *et al*., 2003).

Direct transfer of tissue culture raised plants to field or wild is not possible due to high rate of mortality, as the regenerates in the culture condition has been cosseted in an environment with very high humidity, varied light and temperature conditions (Deb and Imchen, 2010). Direct transfer to sunlight also causes charring of leaves and wilting of plants (Hiren *et al*., 2004; Lavanya *et al*., 2009). The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment (Deb and Imchen, 2010). Hardening and acclimatization are the crucial stages of tissue culture (Chabukswar and Deodhar, 2005). In this study, it was observed that the potting mixture (soil: Sand: compost; 1: 2: 1) showed high survival rate (65%) of the *in vitro* derived plants (Table 3) at relative humidity 80-90% and exhibited normal growth within 30 days (Fig. 1e). This may probably be due to high sand content in the mixture which favours the adequate aeration for roots. In the present study, high content of sand was found useful because the *in vitro* grown plants had very delicate and fragile roots which otherwise would have been damaged by hard soil. Micropropagated plants established in potting mixture were uniform and identical to donor plants with respect to growth characteristics and vegetative morphology.

In conclusion, using axillary bud culture technique more than 1000 plants were raised from single axillary bud within one year. Due to its tremendous medicinal potential, there is rapid depletion and exploitation of this plant from its natural resources. Thus the present *in vitro* multiplication protocol developed for *R. cordifolia* will significantly contribute not only towards its conservation but also for its sustainable use.

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