EUROPEAN ACADEMIC RESEARCH
Vol. II, Issue 7/ October 2014

Impact Factor: 3.1 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

In vitro regeneration and evaluation of genetic stability of *Rubia cordifolia* L.: An endangered medicinal plant of Pachamalai Hills, Tamil Nadu, India

L. VIVEKANANDAN[1]
Plant Physiology and Biotechnology Division
UPASI-TRF, Tea Research Institute, Nirar Dam PO., Valparai, Coimbatore, India
AMZAD BASHA KOLAR
Department of Molecular Biology
Bangalore University, Bangalore, India
E. EDWIN RAJ
Plant Physiology and Biotechnology Division
UPASI-TRF, Tea Research Institute, Nirar Dam PO., Valparai, Coimbatore, India
N. SISUBALAN
P.G. and Research Department of Botany
Jamal Mohamed College (Autonomous), Tiruchchirappalli India
M. GHOUSE BASHA
P.G. and Research Department of Botany
Jamal Mohamed College (Autonomous), Tiruchchirappalli India

Abstract:

Rubia cordifolia L. (Rubiaceae) popularly known as 'Indian Madder' has been used in Indian medicine, especially the roots which have tremendous medicinal values. The present study is focused to develop an efficient in vitro regeneration and subsequent conservation of the endangered species of R. cordifolia from Pachamalai Hills which is a part of the Eastern Ghats in India. Node, internode and leaf explants of R. cordifolia were cultured on Murashige and Skoog (MS) medium supplemented with a combination of 2,4-dichlorophenoxy

[1] *Corresponding author: vivek.kaushi@gmail.com

acetic acid (2,4-D) and α-naphtaleneacetic acid (NAA) for callus induction. Highest percentage (85%) of callus was induced from nodal explants on MS media fortified with 2 mg L^{-1} 2,4-D and 2.5 mg L^{-1} NAA. Calli derived from the nodal explants were used for shoot induction on MS medium supplemented with BAP and AdS. 4 mg L^{-1} BAP and 5 mg L^{-1}AdS significantly induced higher frequency of shoots (60%) and shoot length (5.4±0.3 cm). The optimal medium for rooting was on MS with 2 mg L^{-1} IBA, produced a higher percentage of root induction (60%), frequency of roots (8.0±0.2) and root length (4.6±0.4 cm). The genetic fidelity of the in vitro and mother plants was analysed using random primers. 10 out of 20 OPU primers produced reproducible bands, where no genetic variation was observed. The in vitro regeneration system so developed, can be successfully employed for the large-scale multiplication vis-â-vis conservation of this endangered species and germplasm without inducing any genetic variability.

Key words: 2,4-Dichlorophenoxy acetic acid, 6-Benzylamino purine, Adenine sulphate, genetic stability, Indian Madder, Indole-3-butyric acid, medicinal plant, RAPD, α-Naphthalene acetic acid.

Introduction

Rubia cordifolia L. (Rubiaceae) is a well-known medicinally and economically important Ayurvedic herb popularly known as Indian Madder (English) or Manjitti (Tamil) (Ved *et al* 2002). It is a perennial climber with long, cylindrical, flexuous roots with a thin red bark and with a long, rough and grooved stem. The plant is distributed throughout the lower hills of Himalayas, Western and Eastern Ghats, Assam, Sikkim, Arunachal Pradesh and Manipur. It is also dispersed in temperate and tropical forests up to an altitude of 3500m MSL (Khare, 2004). Almost all the parts of the plant have been used as medicine and are found to be effective in various ailments that have been described in Indian and Tibetan systems of medicine whose efficacy and safety have been proved by clinical trials

(Chatterjee *et al* 2005). Several classes of compounds like anthraquinone (Alizarin), hydroquinone, iridoids, triterpenoids, naphtoic acid, esters, bicyclic hexapeptides, etc., have been reported in *R. cordifolia* (Deshkar *et al* 2008). Alizarin is reported to show various pharmacological and biological activities including anticancer (Son *et al* 2008; Zhang *et al* 2007), antioxidant (Galindo *et al* 2008), anti-proliferative, radio protective, spasmolytic, antimalarial (Bringmann *et al* 2008), antimicrobial (Lenta *et al* 2007; Singh *et al* 2006; Xiang *et al* 2008) and wound-healing activities (Deshkar *et al* 2008). Economically, *R. cordifolia* is one of the most important dye-yielding plants with high commercial value that has been used for the extraction and preparation of dyes from roots, stem and leaves utilising indigenous process since ancient times (Oyen, 1991).

Commercial harvesting of *R. cordifolia* started about a century ago when the roots became a valuable trading commodity (Plate-1b). During while harvesting was uncontrolled and there was no replanting after the uprooting of the native population. Deforestation reduced the availability of suitable habitats and accelerated even further the demographic decline of wild populations of *R. cordifolia*. The Indian system of medicine is predominantly dependent upon plant-based raw materials in most of their preparations and formulations, thereby, widening the gap between demand and supply and thus putting further pressure on the species. Poor seed viability coupled with over-exploitation by dyeing and pharmaceutical industries and subsequent clearing of forest covers has led to the plant being threatened. The plant has now been categorised as one of the endangered plant species of Pachamalai Hills and on the verge of extinction (Soosairaj *et al* 2007).

An effective *in vitro* propagation system for regenerating this plant is the need of the hour to elucidate its potential medicinal values and for the conservation of its natural habitats. It has also been observed that many scientific workers

and researchers have not tried to employ micropropagation for this valuable plant, even though tissue culture provides a promise for the production of more robust plants compared to conventional methods. To our knowledge, limited experiences in the *in vitro* propagation of *R. cordifolia* have been described (Radha *et al* 2011; Khadke *et al* 2013; Ghatge *et al* 2011). Specifically, no earlier report on the *in vitro* regeneration of *R. cordifolia* with reference to Pachamalai Hills. Therefore, the present study was aimed to decipher the most appropriate basal culture and growth hormones for the *in vitro* induction of the plant using different explants and the genetic stability of the regenerated plants. Since the occurrence of somaclonal variation in the *in vitro* cultures is reported in several plant species (Al Khateeb *et al* 2013; Bhatia *et al* 2011; Goto *et al* 1998; Martins *et al* 2004), it is important to ascertain the genetic fidelity of the resultant *in vitro* conserved germplasms. Apart from morphological assessment, the genetic stability of the *in vitro* plants regenerated should be assessed by using PCR based molecular markers. Dominant DNA-based markers such as AFLP, RAPD and ISSR have been used to investigate the extent of clonality both in the wild and *in vitro* regenerated plants. For the present study, RAPD markers were chosen based on earlier reports (Moosikapala and Te-Chato 2010; Kumar and Tarun 2012; Soliman *et al* 2012; Soumen *et al* 2012; Rajaseger *et al* 1999; Rubén *et al* 2010; Martins *et al*2004).

Materials and Methods

Plant material and surface sterilisation

The present study used explants of *R. cordifolia* collected from Pachamalai Hills, a part of the Eastern Ghats in the Indian state of Tamil Nadu (Plate-1a). The plant material was identified and authenticated by the Botanical Survey of India (BSI), Southern circle, Coimbatore (Gamble and Fischer, 1935). Voucher specimens were submitted to the herbarium to the

Department of Botany, Jamal Mohamed College (*Autonomous*), Tiruchchirappalli, Tamil Nadu. Visibly healthy emerging shoots with 3-5 nodes collected from the single mother plant were used as explants (Plate-1a). The excised explants were thoroughly washed with tap water for 30min to remove the adhered soil particles. They were treated with 0.2% bavistin (w/v) for 10min and 0.04% streptomycin followed by washing five to six times with distilled water. The explants were then taken to aseptic chambers where they were sterilised with Savlon (1.5% v/v chlorhexidinegluconate solution and 3.0% w/v cetrimide), disinfected with 0.05% (w/v) mercuric chloride for 1-2 min and then washed with sterilised distilled water. Finally, they were soaked in 70% ethanol for 30 sec and rinsed with sterilised distilled water for three to four times.

Media and culture conditions

The surface sterilised explants were trimmed into smaller segments and inoculated on MS medium (Murashige and Skoog, 1962) containing 0.8% agar and 3% sucrose fortified with different concentrations and combinations of growth hormones. The pH of the medium was adjusted between 5.6 to 5.8 before sterilisation by autoclaving at 121 °C for 20 min. The cultures were incubated in a growth chamber at a temperature of 25±2°C and illuminated (2000 lux) with fluorescent light (Philips TL 34, 25 μmol m^{-2} sec^{-1}) in a 16/8 h photoperiod.

Callus, shoot and root induction

The surface sterilised explants were inoculated on MS medium enriched with different concentrations of 2,4-D and NAA for *in vitro* callus induction. Growth regulator free MS basal medium served as a control for the all experiments. Subculturing was done regularly after every 2 weeks to avoid browning of the culture medium. The data were recorded after 7 days of initial culture. Parameters such as frequency, percentage and colour of calli were recorded. Since the internodal explants produced

higher frequency of calli, internode derived calli were only used subsequently. The node derived calli was cut into small pieces and inoculated on the MS basal medium supplemented with various concentrations of BAP and AdS for shoot induction. Subculturing was carried out regularly for every 2 weeks. About 3.0±0.5 cm long shoots were excised and implanted on the medium supplemented with different concentrations of IBA (0.5-2.0 mg L^{-1}) for root development. Data on the number of shoots per explants, percentage of shoot induction, shoot length, number of roots per shoot, percentage of root induction and root length were recorded.

Hardening

Completely regenerated plantlets with the enough rooting system were taken out of the culture vessels and washed several times with sterile distilled water to remove traces of the medium. The *in vitro* regenerated rooted plantlets were then transplanted into small plastic cups containing sterilised vermiculite, garden soil and farmyard soil (1:1:1) mixture. Each plastic cup was covered with polythene bags with small holes to maintain humidity and kept in the culture room. The plantlets were irrigated with MS half-strength salt solution once in three days. The cups were taken out of the culture room after two weeks and were exposed to ambient conditions for two to three hours daily. After five weeks of hardening, the plants were finally transferred to the field.

DNA isolation and RAPD

Genetic stability of the *in vitro* propagated plants was tested using RAPD markers (Rajaseger *et al* 1999). Total genomic DNA was isolated from twelve randomly collected young and immature leaves of field grown and *in vitro* regenerated plants following CTAB method (Murray and Thompson, 1980) with minor modifications. DNA was amplified in a Corbett Research PCR™ JH BRO® model CG1-96 (Australia) containing

10×thermostable PCR assay buffer, 25 mM $MgCl_2$, 0.2 mM dNTPs in equimolar ratio, 1 unit Taq polymerase (Sigma, USA), 2.5 µL of forward and reverse primer (Operon, Alameda, USA) and 100 ng template DNA made up to a final volume of 25 µL. Twenty OPU primer series procured from Operon technologies were used to amplify the genomic DNA. Initial denaturation was at 94 °C for 2 min, followed by 94 °C for 60 sec, 37 °C for 2 min and 72 °C for 2min. The cycle was repeated 40 times, followed by a 60 sec extension at 72°C. The amplified DNA was then electrophoresed through 1.5% agarose gel under constant voltage of 50 V/50 mA for 40 min. 100 bp DNA marker was used to assign the molecular weight of individual RAPD bands. The gel images were documented by Alpha Quant™ Gel documentation unit and analysed with help of AlphaEase®FC, Version 6.0.

Statistical analysis

Experiments were carried out in a randomised design and repeated three times with each treatment having fifteen independent replicons. The effect of different treatments on various parameters was quantified and the level of significance was determined by analysis of variance (ANOVA) using SPSS Version 20 (Stull, 1994). Mean values were presented with S.E. values and level of differences between the treatments were assessed by Duncan's New Multiple Range Test (DMRT) at $P \leq 0.05$ level.

Results and Discussion

Callus induction

Callus is an unorganised mass of plant cells and its formation is controlled by growth regulating substances present in the medium (Shah *et al* 2003). The specific concentration of plant regulators needed to induce callus, varies from species to species and even depends on the source of the explants

(Charriere *et al* 1999). In the present investigation, callus induction was achieved from different explants of *R. cordifolia* on MS medium fortified with 2,4-D and NAA within 60 days of culture. The swelling of the explants was observed at the cut end of all the explants after the first week of inoculation and callus induction occurred at the end of the third week while the degree of response varying between the explants type (Plate-1c). Correspondingly, explants showed dose dependent significant callus induction with respect to hormone concentration. A lower concentration of 2,4-D (1.5-2.0 mg L^{-1}) and NAA (1.0-1.5 mg L^{-1}) poorly support the callogenesis and growth of the callus, whereas higher concentration supports the growth to an extent. The optimal concentration for higher frequency of callus production varied among the explants, where internodal and leaf explants registered a higher degree of callus at 3 mg L^{-1} 2,4-D and 2.5 mg L^{-1} NAA while nodal explants required 2.5 mg L^{-1} 2,4-D and 2.0 mg L^{-1} NAA (table-1, Plate-1d). Among the different explants, higher percentage of callus induction was noticed in nodal explants (85%) followed by leaf (80%) and internodal explants (70%). Interestingly, the leaf explants proliferated quickly and produced profuse yellowish brown callus. Whereas the colour of nodal and leaf calli ranged from yellow to yellowish brown and yellowish brown to brown respectively (Plate-1e and f). Statistical analysis indicated a significant difference among hormone concentrations, wherein hormone free medium was not included in the analysis. Callogenesis of explants cultured on MS medium enriched with 2,4-D and NAA was previously established by Martins *et al* (2004), Munshi *et al* (2007), Saravanan *et al* (2007), Apurva and Thakur (2009), Siva *et al* (2009), Mohan and Saritha (2012), and Khadke *et al* (2013). They reported the significant amount of callus induction was achieved in leaf, internode and node explants. The result further strengthens our finding that 2,4-D and NAA are essential for callus induction in *R. cordifolia*. The protocol

developed using nodal explants cultured could be a suitable medium and noble approach to produce a maximum percentage of callus induction. Moreover, browning of the media was not observed in either the hormone containing or hormone-free MS medium.

Multiple shoots induction

Multiple shoots were initiated by inoculating callus on MS medium supplemented with different concentrations of BAP and AdS. Formation of multiple shoots from the callus was observed after three weeks of culture (Plate 1g, h). There was a statistically significant and higher percentage of responding culture (60%) was observed in the MS media with 4 mg L^{-1} BAP and 5 mg L^{-1}AdS (table 2). Callus treated with the optimal concentration gave highest 5.4±0.5 cm shoots length significantly different from shoot length obtained from callus receiving either lower or higher hormone concentration. Moreover, the same hormone concentration produced higher frequency of shoots (1.9 per culture), but it was not statistically significant. Our findings are supported by the studies of Sanghamitra *et al* (1998) and Arias *et al* (2010), who have reported that the inclusion of BAP and AdS in the culture medium increases the shooting capacity of the explants. Ghatge *et al* (2011) and Radha *et al* (2011) have shown the most satisfactory effect on promoting the proliferation of multiple shoots through direct organogenesis in *R. cordifolia*. Where, maximum shoot induction 45 and 88% with an average shoot length of 3.25 and 3.7 cm on MS medium containing 3 mg L^{-1} BA alone and 1.0 mg L^{-1} BA and 0.02 mg L^{-1} IAA respectively. However, Khadke *et al* (2013) has recorded highest 71.8 and 61.7% shoot induction with 24.4 and 11.2 shoots having 4.01 and 3.8 cm leaf and intermodal explants on MS medium with 4 mgL^{-1} TDZ. But the results of the present study recorded better result than the findings of Ghatge *et al* (2011), Radha *et al* (2011), and Khadke *et al* (2013) using nodal explants derived

calli through indirect organogenesis. The study further strengthens the fact that BA is very much needed for shoot induction in the plant as evidence in our finding that MS medium containing 4 mg L^{-1} BAP and 5 mg L^{-1} AdS showed significant results. Moreover, the combination of BAP and AdS in the culture medium did not produce any callus while culturing even after three weeks of culture.

Rooting

In vitro rooting of the micro shoots was observed by treating different concentration of IBA in the culture medium. There was a significant difference in the percentage of culture responded, the number and length of the roots induced after three weeks of culture (table 3). Highest 60% of root induction was observed at an average of 8.0±0.2 roots per shoot when 2 mg L^{-1} IBA was used in the rooting medium (Plate-1i and j). However, lower (0.5-1.5 mg L^{-1}) hormonal concentration poorly supported the root induction and it was found that 2 mgL^{-1} IBA is suitable for rooting. Among the auxins, IBA is the most frequently applied chemicals for rooting (Harry and Thrope, 1994). Our results also show that IBA was the best to be used for root formation in *R. cordifolia*. Similar results about the response to IBA have been observed in *Boscia senegalensis* (Khalafalla *et al* 2011), *Prosopis ceneria* (Kumar and Singh, 2009), *Teucrium fruticans* (Frabetti *et al* 2009), *Pappea capensis* (Mngomba *et al* 2007), *Eclipta alba* (Dhaka and Kothari, 2005), *Azadirachta indica* (Chaturvedi, Razdan, and Bhojwani, 2004), *Paederia foetida* (Alam *et al* 2010) and *Centella asiatica* (Tiwari *et al* 2000). The result of the study is corroborated with Radha *et al* (2011) who induced rooting in *R. cordifolia* using, which revealed that 1 mg L^{-1} IBA produced 98% of rooting at the rate of 8.9 per micro shoot and an average length of 6.4 cm, within five weeks after transplantation into the rooting medium. Similar observation has been made by Khadke *et al* (2013) who achieved a highest rooting response of 93.7% with 1 mg L^{-1} IBA

with a mean number of 4.9±0.7 roots per shoot and a mean length of 4.7±0.2 cm in *R. cordifolia.* Furthermore, Ghatge *et al* (2011) reported that 3 mg L^{-1} IBA induced 100% root induction in the *in vitro* grown micro shoots of *R. cordifolia.* Further, they noticed that the concentration of the IBA in the culture medium did not influence the number of roots per shoot. In fact, the number was reduced to 4.5 roots per shoot as compared to 5.3 roots per shoot with 2 mg L^{-1}IBA. In the present investigation, there was also an appreciable difference in the root length as compared to the media with other concentrations. Decrease in the hormone concentration of IBA, lead to decreasing trend in rooting. The present study uses 2.5 mg L^{-1} to bring about rooting at a rate on par with Radha *et al* (2011) in terms of the number of roots. However, the average number of roots is more as compared with the report of Khadke *et al* (2013) and Ghatge *et al* (2011). Even though there was a decrease in the rooting percentage, the number of roots per shoot was better than earlier studies.

Acclimatisation and *ex vitro* establishment

For a safe acclimatisation to the rigour of the natural environment, a careful hardening method was followed. Well-developed plantlets were removed from the agar medium, washed thoroughly with tap water and dipped in MS basal medium for an hour. The plantlets were transferred to the plastic cups containing sterilised vermiculite, sand and garden soil in the equal ratio with a survival rate of 60% and established well in the field conditions (Plate 1k).

Genetic fidelity of *in vitro* regenerated plants

The aim of conservation of plant genetic resource was not only to ensure the availability of the plant resource through biotechnological tools but also to produce invariant regenerantsduring conservation. The study has found that *in vitro* conserved plantlets were morphologically different from

the field grown plants, which was probably caused by the effect of slow growth condition in cultures. The morphological markers were found to be inefficient for detection of variations. Molecular markers are of great importance in this case to examine genetic variation at the DNA level (Moosikapala and Te-Chato, 2010). The amplification profiles were screened for the presence of polymorphisms among the samples. A total of 20 random primers were used to confirm the genetic fidelity from which out of ten primers resulted in a total of 207 scorable bands (table-4). A variable number of bands were observed for each primer ranging from 5 (OPU-07) to 11 (OPU-11) bands per primer. Furthermore, RAPD analysis confirms that the *in vitro* regenerated callus and the plantlets showed an identical banding pattern with the wild mother plant (fig-1). These results indicate that regenerated plants were similar and no genetic variation was detected after *in vitro* culture. All the RAPD profiles were found to be monomorphic among the *in vitro* regenerated plants and mother plant. The genetic stability of *in vitro* propagated plants has been confirmed in many numbers of species like *Albizia lucida* (Shah, Jabeenand Llahi, 2003), *Commiphora wightii* (Kumar and Tarun, 2012), *Ocimum gratissimum* (Soumen *et al* 2012), *Gerbera jamesonii* (Bhatia *et al* 2011) and *Centaure aultreiae* (Rubén *et al* 2010).

Conclusion

In vitro requirement is an efficient means of *ex situ* conservation of plant diversity which assists in the sustainable maintenance of present day dwindling germplasm on a long term basis, especially for endangered medicinal plant. The study established a genetically stable and successful *in vitro* propagation system for *R. cordifolia* with high proliferation and survival rate. Callus with regeneration potential reported in this study could be useful in raising large-scale production, especially raw drug based industries. Based on the results, we

concluded that this protocol could be used efficiently as a means of rapid propagation of this endangered dye-yielding plant. Considering the therapeutic prospective of this plant, further conservation strategies can be implemented to bring out a good bioavailability of the plants and high quality of herbal products.

Acknowledgements

Authors are thankful to the Department of Science and Technology, New Delhi, for providing the Fund for Improvement of Science and Technology (FIST)-sponsored project to the P.G. and Research Department of Botany, Jamal Mohamed College (Autonomous). The authors are also grateful to the University Grants Commission, New Delhi, for providing financial support through Major Research Project.

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Table 1: Effect of MS medium with different concentrations of 2,4-D with NAA for callus induction from explants of *Rubia cordifolia*

PGR (mg L^{-1})		Internode			Node			Leaf		
2,4-D	NAA	X	Y	Z	X	Y	Z	X	Y	Z
0.0	0.0	-	-	-	-	-	-	-	-	-
1.5	1.0	+	20±1.0[a]	YL	+	25±1.3[b]	YL	-	-	-
2.0	1.5	+	25±1.1[a]	YB	++	45±2.0[c]	YL	+	35±1.8[a]	YB
2.5	2.0	+	35±1.4[b]	YB	+++	85±3.4[d]	YL	++	50±2.3[b]	YB
3.0	2.5	++	70±3.2[d]	YB	++	45±2.0[c]	YB	+++	80±3.2[d]	YB
3.5	3.0	++	65±3.6[c]	BR	+	15±0.8[a]	YB	+++	70±3.2[c]	BR

X= Degree of callus: - No callus; + Scanty; ++Moderate; +++ Profuse; Y= %explants forming callus; Z= Colour of callus: YL=Yellow; YB=Yellowish brown; BR=Brown. Each value represents the Mean ± S.E of 15 replicates per treatment and repeated 3 times. Values with the same superscript are not significantly different at P<0.05 level according to DMRT.

Table 2: Effect of MS medium with different concentrations of BAP with AdS for *in vitro* shoot regeneration from callus of *Rubia cordifolia*

PGR (mg L^{-1})		% of responding culture	No. of shoots/ culture	Shoot length (cm)
BAP	AdS			
0.0	0.0	-	-	-
1.0	2.0	0.0 [a]	0.0±0.0[a]	0.0±0.0[a]
2.0	3.0	13.0±0.7[b]	1.0±0.0[b]	3.1±0.1[b]
3.0	4.0	33.3±1.5[c]	1.0±0.0[b]	4.3±0.2[c]
4.0	5.0	60.0±3.0[e]	1.9±0.1[c]	5.4±0.3[e]
5.0	6.0	40.0±1.6[d]	1.7±0.2[c]	4.9±0.3[d]

Each value represents the Mean ± S.E of 15 replicates per treatment and repeated 3 times. Values with the same superscript are not significantly different at P<0.05 level according to DMRT.

Table 3: Effect of MS medium with different concentrations of IBA on *in vitro* rooting of *Rubia cordifolia*

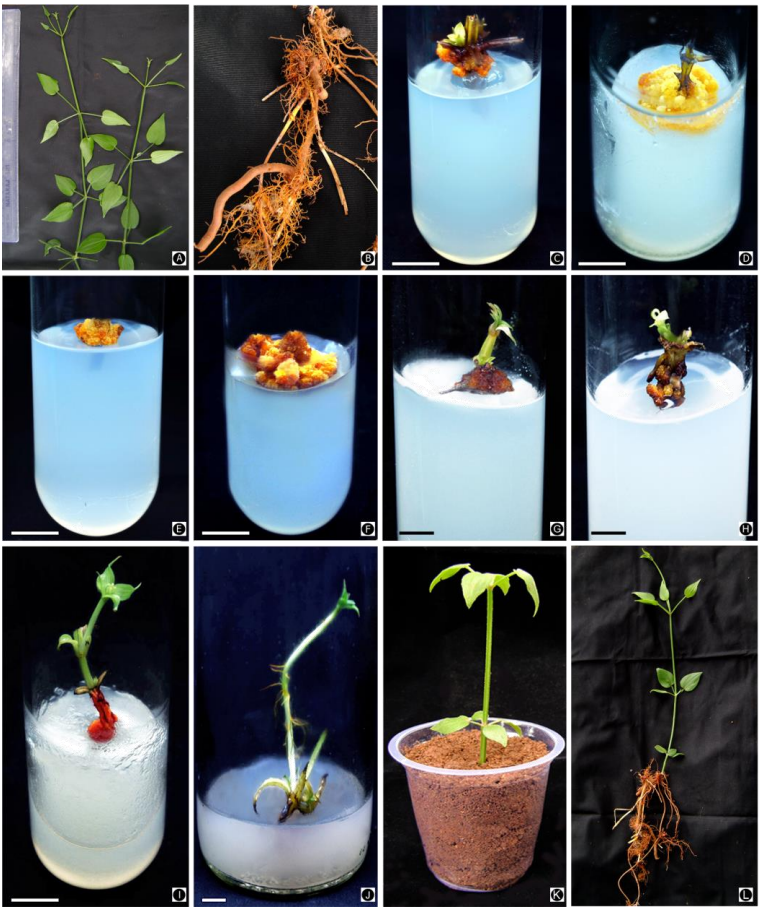
IBA (mg L^{-1})	% of root induction	No. of roots/shoot	Root length (cm)
0.0	-	-	-
0.5	0.0[a]	0.0±0.0[a]	0.0±0.0[a]
1.0	20.0±1.0[b]	2.7±0.3[b]	3.2±0.3[b]
1.5	33.3±1.5[c]	4.4±0.2[c]	3.5±0.4[c]
2.0	60.0±2.4[e]	8.0±0.2[e]	4.6±0.4[e]
2.5	40.0±1.8[d]	5.5±0.2[d]	4.2±0.3[d]

Each value represents the Mean ± S.E of 15 replicates per treatment and repeated 3 times. Values with the same superscript are not significantly different at P<0.05 level according to DMRT.

Table-4: List of primers, their sequences, number and size of the amplified fragments generated by inter-simple sequence repeat (RAPD) primers

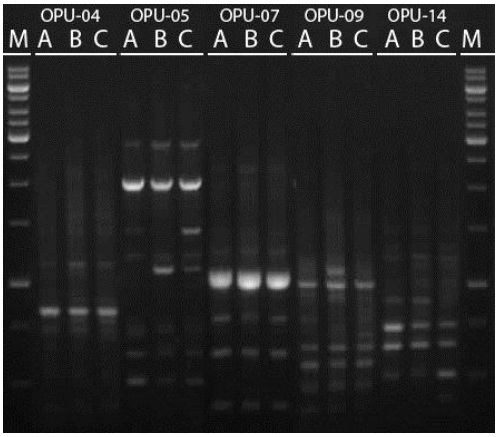
Primer name	5'–3' sequence	GC %	Number of scorable bands/primer	Total number of bands amplified	Polymorphic Bands
OPU-01	ACGGACGTCA	60	0	0	0
OPU-02	CTGAGGTCTC	60	0	0	0
OPU-03	CTATGCCGAC	60	9	27	0
OPU-04	ACCTTCGGAC	60	6	18	0
OPU-05	TTGGCGGCCT	70	7	21	0
OPU-06	ACCTTTGCGG	60	6	18	0
OPU-07	CCTGCTCATC	60	5	15	0
OPU-08	GGCGAAGGTT	60	6	18	0
OPU-09	CCACATCGGT	60	11	33	0
OPU-10	ACCTCGGCAC	70	0	0	0
OPU-11	AGACCCAGAG	60	6	18	0
OPU-12	TCACCAGCCA	60	6	18	0
OPU-13	GGCTGGTTCC	70	0	0	0
OPU-14	TGGGTCCCTC	70	7	21	0
OPU-15	ACGGGCCAGT	70	0	0	0
OPU-16	CTGCGCTGGA	70	0	0	0
OPU-17	ACCTGGGGAG	70	0	0	0
OPU-18	GAGGTCCACA	60	0	0	0
OPU-19	GTCAGTGCGG	70	0	0	0
OPU-20	ACAGCCCCCA	70	0	0	0
	Total		69	207	0

Plate-1: *In vitro* regeneration of *Rubia cordifolia* through nodal explants.



A and B: wild grown *R. cordifolia* twig and economically important root; C and D: callus induction from node explants cultured on MS medium enriched with 2 mg L^{-1} 2,4-D and 2.5 mg L^{-1} NAA, after three and four weeks old culture respectively; E and F: yellowish brown coloured subcultured callus; G and H: node derived callus cultured on MS medium supplemented with 4 mg L^{-1} BAP and 5 mg L^{-1} AdS for multiple shoot induction; I: micro shoots cultured on MS medium with 2 mg L^{-1} IBA for root induction; J: plantlet with adventitious aerial root system; K: acclimatised plantlet transplanted into artificial soil mixture after two weeks; L: *ex situ* conserved plant. Scale bar in figures are 1 cm.

Figure-1: DNA fingerprinting pattern generated by OPU random primers of *in vitro* regenerated and mother plants of *Rubia cordifolia*



M: Marker; A: *in vivo* plant; B and C: *in vitro* plants; B: Callus; and C: Hardened plant.