In Vitro Axillary Bud Multiplication Of An Important Medicinal Plant-*Rubia Cordifolia* L.

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Abstract
An efficient protocol was established for micropropagation *Rubia cordifolia* by multiplying axillary buds from nodal explants. The excised nodal segments were inoculated onto MS medium supplemented with various concentrations and combinations of plant growth regulators. The best combination for the shoot and root proliferation of *R. cordifolia* was Murashige and Skoog's medium fortified with 0.5 mg/L Kinetin. Each explant produced an average of 8 shoots within 80 days of incubation. The *in vitro*-developed plantlets were hardened and successfully established in pots, achieving a 95% survival rate. The organogenesis protocol developed in this study may enhance the clonal propagation and conservation of *R. cordifolia*, an important industrial and medicinal plant.

Introduction

Rubia cordifolia L., commonly known as Indian Madder or Manjishtha, is a small perennial climbing plant that thrives in damp tropical forests. It belongs to the Rubiaceae family and is typically found in moist tropical woodlands. Its thin red bark and tall, cylindrical, flexible roots characterise this plant. Its stems often have a lengthy, woody base that is rough and grooved. Plants in this family, especially those with roots, are known for containing high levels of anthraquinones (Deshkar et al., 2008; Patil et al., 2009).

Research has identified a variety of chemical constituents in *R. cordifolia*, with the majority being anthraquinones, naphthoquinones, anthraquinone glycosides, naphthoquinone glycosides, bicyclic hexapeptides, triterpenoids, and polysaccharides (Son et al., 2008; Itokawa et al., 1993; Rao et al., 2006; Gao et al., 2016; Natarajan et al., 2019; Wen et al., 2022b).

Traditionally, *R. cordifolia* is valued for its anti-inflammatory, astringent, and blood-purifying properties. It is a key ingredient in many Ayurvedic preparations and contains beneficial chemical compounds such as anthraquinones, iridoid glycosides, naphthoic acid esters, bicyclic hexapeptides, and triterpenes (Do et al., 2023b; Schmidt-Przewoźna and Kicińska-Jakubowska, 2023).

Among various botanical species with potential for dye extraction, *R. cordifolia* has been historically significant as the source of an anthraquinone-based dye. This dye was widely used in traditional dyeing techniques across Southeast Asia, the Indian subcontinent, and China. The dye is derived from the roots of R. cordifolia, which contain anthraquinone components that yield a range of red shades. Due to its anthraquinone content, this root extract exhibits antimicrobial, antioxidant, and anti-inflammatory properties (Yusuf et al., 2017; Meena et al., 2010; Eom et al., 2020).

The aim of this study was to establish an effective protocol for the rapid propagation of this significant medicinal plant using nodal segments.

Materials and Methods

Plant Material and Surface Sterilization

The mother plant, *R. cordifolia* L., was collected from Pookode in Wayanad, Kerala, India. Nodal sections from this plant were excised and used as explants for *in vitro* studies.

The nodal segments were washed with running tap water for 20 minutes, followed by treatment with Extran (5% v/v) for 10 minutes. They were rinsed with distilled water and surface-sterilized using a 0.1% (m/v) $HgCl_2$ solution for 3 minutes, then washed 3-4 times with sterile distilled water. Finally, the nodes were trimmed to 1 - 1.5 cm before being inoculated onto sterilized media.

Culture Medium

The investigation was conducted using the MS medium (Murashige & Skoog, 1962), supplemented with different combinations and concentrations of plant growth regulators (PGRs). The media was solidified using eight g/L agar, and the carbon source was 30 g/L sucrose. PGRs were employed in various combinations and concentrations, including Kinetin, BA, NAA, IAA, and IBA. The culture media were autoclaved at 1-atmosphere pressure and 121°C for 20 minutes to sterilize them after the pH of the media was brought to 5.8 using 0.1 M NaOH and 0.1 M HCl. Following inoculation, the cultures were

incubated at 25 ± 2 °C under white tube lights for 16 hours. Throughout the multiplication process, these requirements were upheld.

Shoot Multiplication

Explants were cultivated on MS media supplemented with varying concentrations of PGRs, such as kinetin, BA, NAA, IAA, and IBA, singly or combined, to promote axillary bud proliferation. PGRs and MS media were combined in 19 different ways. The optimal medium for axillary bud multiplication was chosen based on the number and length of shoots produced per explant.

Acclimatization

Wholly developed *in vitro*-grown plantlets were removed from the culture jars, cleaned of agar using sterile distilled water, and then placed in paper cups filled with autoclaved sand: soil (1:1). To preserve humidity and keep the plants from drying out, the cups were covered with clear polythene bags. After ten days, the polythene bag was taken off. The plantlets were then moved to the potting mixture-filled garden pots.

Statistical Analysis of Data

All experimental trials were conducted with 12 replicates for each treatment. Observations of the cultures were made regularly, and all morphological changes were recorded at set intervals. The results were analyzed statistically using SPSS Version 16.0, and data were compared using ANOVA and Duncan's multiple range test.

Result and Discussion

Nodal explants were cultured on MS media containing various concentrations and combinations of PGRs and the results were evaluated for 80 days of growth period (**Table 1, Figures 1**). Both shoot and root formed simultaneously from the nodal explant itself without any callus formation. 4– 6 shoots (7.428±0.07) were formed from each explant in MS basal media without any plant growth regulators (control). When cultured in media fortified with cytokinins increased growth rate and increase in the number of shoots (6- 8 shoots) per explant were observed. Best axillary bud multiplication was obtained when cultured on MS medium fortified with 0.5 mgL-1 Kinetin, this combination induced the maximum multiplication of shoots (8.692±0.03) than other concentrations and combinations of PGRs. Among the Kinetin concentrations, 0.5mg/L showed maximum shoot growth (9.7±0.34 cm) and root growth (8.24±0.36 cm), when compared to all other concentrations and control. Broad leaves were noticed here. Roots were numerous and clustered. Similar outcomes were showed by a study conducted by Bansal *et al.,* (2024). According to them*, R. cordifolia* nodal explants have shown a great deal of potential for regeneration, and the use of cytokinin like Kinetin and 2-isopentenyladenine for shoot induction has worked incredibly well. The best option was to augment MS medium with 1.0 mg/L of Kinetin, which produced (12.14 ± 1.58) shoots and (29.78 ± 1.93) axillary buds but in the above study 1mg/L Kinetin showed more root development than shoots. According to Sangeetha et al. (2014), cytokinin-supplemented medium was found to be essential for shoot regeneration in the Cucurbitaceae family same as that observed in case of *R. cordifolia.*

When MS media fortified with IAA, shoot development occurred first and roots were developed at a later period. Organogenesis was observed to occurs directly from the explant without callus formation. In the case of IAA 0.5 mg/L, less number of shoots were formed from the explant**.** No branching was observed. The length of the shoot (6.78±0.39 cm) was much higher when compared to other concentrations of IAA. Only a few shoots were developed in IAA 1mg/L, they were short, whereas in IAA 1.5mg/L, no roots were formed in most of the cultures. De Klerk et al. (1997) reported that the strong inhibition of root formation were occurred at supraoptimal auxin concentrations.

MS medium treated with NAA showed only root formation. Roots were developed from the callus. The maximum root formation was observed in NAA 1.5mg/L (2.8±0.42 cm). The small shoots developed from the axillary buds when grown in a medium containing 0.5 mg/L of NAA. No shoot formation occurs in the rest of the NAA treatments. The same results were noted in a work conducted by Radha et al. (2011), where nodal explants of *R. cordifolia* cultivated in conditions containing BA and NAA developed a compact red callus on their cut ends.

In case of BA treatment, the length of the shoot was small, but shoot multiplication was found to be greater. Shoot and roots were developed from the callus. Among all other BA concentrations, 1mg/L showed the second-highest shoot multiplication (6.29±0.06). A slight colour difference from dark green to yellowish green was noticed. The leaves were bent downwards and were wavy in nature when compared to control. Plants were short and stout but with a considerable thickness. Also, the roots were few but have greater thickness. Similar study shows that among cytokinin concentrations tested, BA was more effective in inducing multiple shoot formation than Kinetin in nodal explants of *R. cordifolia* (Radha et al., 2011). MS medium augmented with additives and 2.0 mg/L BAP was recorded optimum for shoot bud induction from the nodal meristems of *Oldenlandia corymbosa* (Revathi et al., 2018). The most gratifying effect on encouraging the proliferation of many shoots through direct organogenesis in *R. cordifolia* has been demonstrated by studies conducted by Swaroopa Ghatge et al. (2011) and Radha et al. (2011) where the results were same as that of present study.

In case of IBA treatment, the length of the shoot and multiplication was found to be substantially lower. But the thickness of shoots was comparatively high and the leaves are very broad. Among all other IBA concentrations, 0.5 mg/L showed maximum shoot multiplication (1.087±0.03) and length (1.2±1.54). An experiment conducted by Shahab et al. (2013) assessed the effects of different levels of IBA on Alstonia cuttings. The results showed that stem diameter was significantly influenced

by IBA treatment. The results showed that cuttings treated with 10% IBA had the largest stem diameter at 14.44 mm, while those without IBA treatment measured 8.53 mm.

When MS medium containing a combination of BA and Kinetin was used, comparatively less growth was observed. MS media containing Kinetin 0.5 mg/L and BA 1 mg/L as well as MS media containing BA 0.5 mg/L and Kinetin 1 mg/L were the two combinations used. In a combination of BA 1mg/L and Kinetin 0.5mg/L, Shoots and roots were originated from the callus. In certain cultures, leaves develop straight from the callus and were grouped in a clustered fashion without internodes. Roots were not formed here. The outcome was consistent with the finding of Saha et al. (2007), the regeneration capacity of bottle gourd, when treated with combinations of BA and Kinetin, was very low, and the emergence of shoot buds was delayed. BA promoted ethylene production, but the combined effect of kinetin and BA decreased ethylene production and contributed to a higher frequency of regeneration from cotyledon explants of bottle gourd (*Lagenaria siceraria*). Whereas when a combination of BA 0.5mg/L and Kinetin 1mg/L is used, two types of plants were developed. One type was with shoots only having short stem and small leaves and another one was with roots only which were formed from the callus**.**

Among the growth regulators tested, Kinetin was more effective in inducing multiple shoot and root formation than other treatments. when Kinetin alone was used, better results were obtained only at certain optimum concentration. The highest frequency of shoot formation was recorded in nodes in an optimum concentration of 0.5 mg/L Kinetin with an average number of 6-8 shoots per node, with a mean shoot length of 9.7±0.34 cm. The quality of shoots and the overall growth response in terms of average number of nodes per shoot was better in this concentration.

While analyzing the fresh and dry weight of each sample, it was observed that the medium with 0.5mg/L kinetin had maximum fresh weight (3.64±0.14 g) and dry weight (0.26±0.02) which were higher than the control (3.17±0.25 g and 0.24±0.04 g respectively).

A simple and dynamic protocol was developed for the micropropagation of *R. cordifolia* by testing various concentrations of growth regulators. Kinetin 0.5 mg/L to be the satisfactory media for the multiplication of shoots, roots and biomass production. Hence, the *in vitro* propagation of nodal segments of *R. cordifolia* using plant growth regulators was identified to be a rapid and cost-effective technique for the large scale production of this plant.

Figure 1: *In vitro* cultures of *R. cordifolia* in MS media containing various concentrations of plant growth regulators. A) 0.5 mg/L Kinetin, B) 1 mg/L Kinetin, C) 1.5 mg/L Kinetin, D) 2 mg/L Kinetin, E) 0.5 mg/L BA, F) 1 mg/L BA, G) 1.5 mg/L BA, H) 2 mg/L BA, I) 0.5 mg/L IAA, J) 1 mg/L IAA, K) 1.5 mg/L IAA, L)0.5 mg/L NAA, M) 1 mg/L NAA, N) 1.5 mg/L NAA, O) 0.5 mg/L IBA, P) 1 mg/L IBA, Q) 1.5 mg/L IBA, R) 0.5 mg/L Kinetin+1 mg/L BA, S) 0.5 mg/L BA+1 mg/L Kinetin, T) MS basal (control).

Acclimatization

Plantlets grown *in vitro* were successfully transferred to *ex-vitro* conditions, where they thrived and remained healthy (**Figures 2**). Their shoots flourished in the field, and their leaves became more vibrant. The plants produced through micropropagation closely resembled the mother plants in both structure and function.

Figure 2: *In vitro*, culture establishment of *R. cordifolia.* (A) Habit; (B) Culture established in medium containing 0.5 mg/L Kinetin; (C) Acclimatized plantlets in sterile soil at lab conditions and (D) well established plantlet in Garden pot.

Table 1: Effect of PGRs on shoot multiplication from nodal segments of *R. cordifolia* in MS medium

KIN (mg/L)	IAA (mg/L)	BA (mg/L)	NAA (mg/L)	IBA (mg/L)	Length of shoot (cm)	Length of root (cm)	No. of shoots	Fresh weight (gms)	Dry weight (gms)
-	-	-	-		8.22±0.75	4.84±0.75	7.428±0.07	3.17±0.25	0.24±0.04
0.5	-	-	-		9.7±0.34	8.24±0.36	8.692±0.03	3.64±0.14	0.26±0.02
1	-	-	-		5.08±0.49	4.04±0.35	4.16±0.05	0.053±0.02	0.06±0.04
1.5	-	-	-		3.24±0.28	3.5±0.78	3.098±0.04	0.05±0.01	0.01±0.00
2	-	-	-		4.42±0.52	4.2±0.81	4.178±0.03	0.11±0.02	0.02±0.00
-	0.5	-	-		6.78±0.39	3.24±0.46	3.262±0.11	0.29±0.04	0.04±0.01
-	1	-	-		3.1±0.37	2.74±0.41	4.072±0.01	0.27±0.05	0.04±0.01
-	1.5	-	-		0.82±0.50	1.64±0.58	3.01±0.0	0.08±0.03	0.02±0.01
-	-	0.5	-		2.04±0.22	1.48±0.50	3.064±0.01	0.03±0.00	0.01±0.00
-	-	1	-		4.24±0.33	1.7±0.32	6.29±0.06	0.09±0.01	0.02±0.00
-	-	1.5	-		4.02±0.48	2.88±0.54	1.144±0.04	0.14±0.03	0.02±0.00
-	-	2	-		4.28±0.32	1.58±0.62	2.588±0.47	0.14±0.02d	0.06±0.04
-	-	-	0.5		0.06±0.04	0.7±0.18	0±0.0	0.18±0.01	0.03±0.01
-	-	-	1		0±0.0	1.38±0.25	0±0.0	0.30±0.06	0.05±0.02
-	-	-	1.5		0±0.0	2.8±0.42	0±0.0	0.47±0.05	0.14±0.03
				0.5	1.2±1.54	1.6±0.86	1.087±0.03	0.03±0.00	0.01±0.00
				1	0.8±1.37	2.5±1.06	1.060±0.11	0.09±0.01	0.02±0.01
				1.5	0.4±1.05	1.5±0.57	0.30±0.02	0.04±0.03	0.02±0.00
0.5	-	1	-		1±0.42	0.56±0.56	2.07±0.0	0.06±0.01	0.01±0.02
1	-	0.5	-		0.88±0.51	1.02±0.44	1.044±0.03	0.06±0.01	0.03±0.02

Data in each column represents mean ± standard deviation. According to Duncan's multiple range test, significant differences are denoted as $p< 0.05$ (Duncan, 1955).

Conclusion

The developed protocol for *in vitro* axillary bud multiplication of *R. cordifolia* is an efficient method for rapid propagation of this important medicinal plant. Using MS medium with 0.5 mg/L of Kinetin yielded the best results for shoot production. This method enables the propagation of contamination-free plants in a limited time, allowing for further studies on enhancing and assessing secondary metabolite concentrations for pharmaceutical purposes.

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Conflict of Interest

No potential conflict of interest was reported by the authors.

Ethics Approval

The matter given in this manuscript is in compliance with ethical standards. The research work given in this paper does not involve human participants or animals.

Authors' contribution

Satheesh George had the idea of the article and suggested the topic. Aparna Prasad performed the literature search and wrote the first draft. Satheesh George revised and formatted the manuscript.

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