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Research article

In vitro morphogenesis and callus induction from nodal explants of *Rubia manjith* Roxb. ex Fleming

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Abstract

Rubia manjith, a perennial herbaceous climber, is one of the important medicinal and dye plants prioritized for research and development in Nepal. It is categorized as a vulnerable species in Nepal. Locally, this plant is used by various communities to treat different ailments. However, unsustainable exploitation and large-scale export have led to a decline in its wild populations. The present study aims to develop a standardized protocol for *in vitro* propagation of the species through organogenesis. Nodal explants of *R. manjith* were cultured in Murashige and Skoog (MS) medium supplemented with various combinations of plant growth regulators: BAP and NAA. Cultures were incubated at 25 ± 2°C with a 16/8-hour light/dark cycle and a light intensity of ± 3000 lux. Callus induction was highest in MS medium supplemented with either 2.0 mg/L NAA or 0.1 mg/L BAP + 1.0 mg/L NAA. The highest mean shoot number of 8.33 ± 0.33 and root number of 8.00 ± 1.00 were observed on MS medium without any growth regulators. The greatest shoot length (14.33 ± 1.54 cm) was observed on MS medium supplemented with 0.1 mg/L BAP, while the highest root length (6.80 ± 1.27 cm) was observed with 2.0 mg/L BAP. The *in vitro*-grown plants propagated from nodal segments can be transplanted as needed to support wild populations and promote large-scale cultivation.

Keywords: BAP, MS media, NAA, node culture, tissue culture.

Introduction

Rubia manjith Roxb. ex Fleming, a climbing herb that can grow up to 3 meters tall, is listed as vulnerable in Nepal according to the Conservation Assessment and Management Plan (CAMP) 2001 (Bhattarai *et al.* 2002). The species is one of the 30 non-timber forest products that the Government of Nepal has identified as particularly important for the nation's economic development and thus prioritized for conservation and research purposes (DPR 2006). Native to the Himalayan regions, South-Central China, and the Qinghai-Tibet Plateau (POWO 2023), it is found in the temperate and subtropical parts of Nepal, typically between 1200 and 3000 m asl (Ghimire *et al.* 2008).

R. manjith is known for its astringent, antidysenteric, antiseptic, and deobstruent properties and is used to treat rheumatism, ulcers, inflammations, and skin disorders (IUCN Nepal 2000). The root, stem, leaf, and fruit of this species are used in various traditional medical systems, including Ayurveda and folk healing practices; of these, dried root and stem are the most traded parts (Pradhan *et al.* 2021). The Newar community of Pharping, Nepal, prescribes it as an antiseptic and a remedy to treat rheumatism (Balami 2004). The Chepang community in Nepal uses its root and whole plant to treat fever, diarrhea, and burns (Tamang *et al.* 2017). In Sikkim, it is valued as a tonic and blood purifier, and used to treat conditions such as jaundice, urinary tract infections, liver complaints, menstrual irregularities, joint discomfort, and leucoderma (Maity *et al.* 2004). Other medicinal uses of the plant include the treatment of stomachaches, headaches, liver disorders, jaundice, urinary tract infections, and as an antidote for snake and scorpion bites (Pradhan *et al.* 2021). The plant is also used to treat menstrual disorders, including menorrhagia (Bhatia *et al.* 2015).

Internationally, *R. manjith* is in high demand for its application in cosmetics, dyeing, and traditional medicines (DPR 2006). In Asia, Europe, and Africa, *Rubia* species are key sources of red pigment (St Clair 2016). These plants contain anthraquinones, particularly alizarin and purpurin, which are responsible for the red color used as natural dyes in food, cosmetics, textiles, and pharmaceuticals (Mori *et al.* 1990).

In Nepal, *R. manjith* plays an important economic role. Nepal exported 61.567 tons of *R. manjith* between 2011 and 2015, with a total value of NRs. 9.235 million (Bhujel and Pokharel 2018). In the fiscal year 2018/2019, the county exported 219.4 metric tons of *R. manjith*, valued at NRs. 52.442 million, mainly to India and Bangladesh (Plant Quarantine and Pesticide Management Center 2019). Nepal ranks as the third-largest exporter of *R. manjith* globally, following India and Afghanistan (Volza Grow Global 2023). In addition, the roots and stems of 2–3-year-old *R. manjith* plants are used for dyeing textiles,

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particularly in clothing and carpet industries in Nepal (Manandhar 2002; DPR 2006; Gurung and Pyakurel 2017).

The plant is often collected haphazardly, using destructive practices that involve cutting stems to the ground with no scope for regeneration (Pandit and Thapa 2003). These unsustainable harvesting practices have led to the depletion of natural populations of *R. manjith*. Furthermore, over-harvesting and habitat degradation have led to substantial threats to its survival, emphasizing the urgent need for immediate conservation efforts to protect this species from further decline.

One promising approach for conserving the *R. manjith* populations is the development of efficient propagation methods, particularly micropropagation. With the help of this method, one can produce a large number of genetically uniform adventitious shoots within a short period and in limited space, without harming the parent stock. Production of callus from plant tissues is another aspect by which a large number of plantlets may be produced through organogenesis. Finally, the plants produced by either method can be rooted *in vitro* or *in vivo* and acclimatized before transplanting them into their natural habitats (Gaspar *et al.* 2002; Doran 2009).

Until now, no established protocols exist for the *in vitro* propagation of *R. manjith*, particularly through micropropagation, and hence, it is essential to find out the possibility of this method in its propagation. Therefore, the present study aims to address this gap by developing standardized protocols for rapid multiplication and callogenesis of *R. manjith*.

Materials and methods

COLLECTION OF MATERIALS

Stems of *R. manjith* (Figure 1) were collected from Godawari Municipality-6 (27.5607° N, 85.3191° E) at an elevation of 1650 m in Lalitpur District, Central Nepal. The plant materials were collected periodically, without destroying the natural habitat, and stored at 4°C until further use. Species identification was carried out using relevant floristic literature and verified against specimens housed at the Tribhuvan University Central Herbarium (TUCH), Kirtipur, Kathmandu. A voucher specimen (No. 101-2023) has been deposited at TUCH.

SURFACE STERILIZATION OF PLANT SAMPLES

Techniques given by Torres (1988) were followed with slight modifications for sterilization in tissue culture. Single nodes were gently cleaned with tap water, soaked in Tween-20 for 5 minutes, and then rinsed for 20 minutes under running tap water. The explants were then surface sterilized with 70% ethanol for 1 minute and washed thoroughly three times with distilled water to eliminate residual alcohol. Further surface sterilization was performed inside a laminar airflow chamber using 0.1% mercuric chloride ($HgCl_2$) solution for 5 minutes, followed by three rinses with sterile distilled water to remove any trace of $HgCl_2$.

SHOOT AND ROOT INDUCTION

Sterilized nodal sections (1.5 ± 2 cm) were inoculated, under aseptic conditions, onto Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing different concentrations and combinations of NAA (naphthaleneacetic acid) and BAP (6-benzylaminopurine), either separately or in combination, to induce shoot multiplication and elongation. The culture tubes were incubated at 25 ± 2°C with a relative humidity of 60–70%, under a 16/8-hour light/dark photoperiod with approximately 3000 lux light intensity under aseptic conditions. The *in vitro*-grown plants were used as explants again after neutralizing the hormonal effects by subculturing them in MS medium for 8 weeks (Figure 2.C).

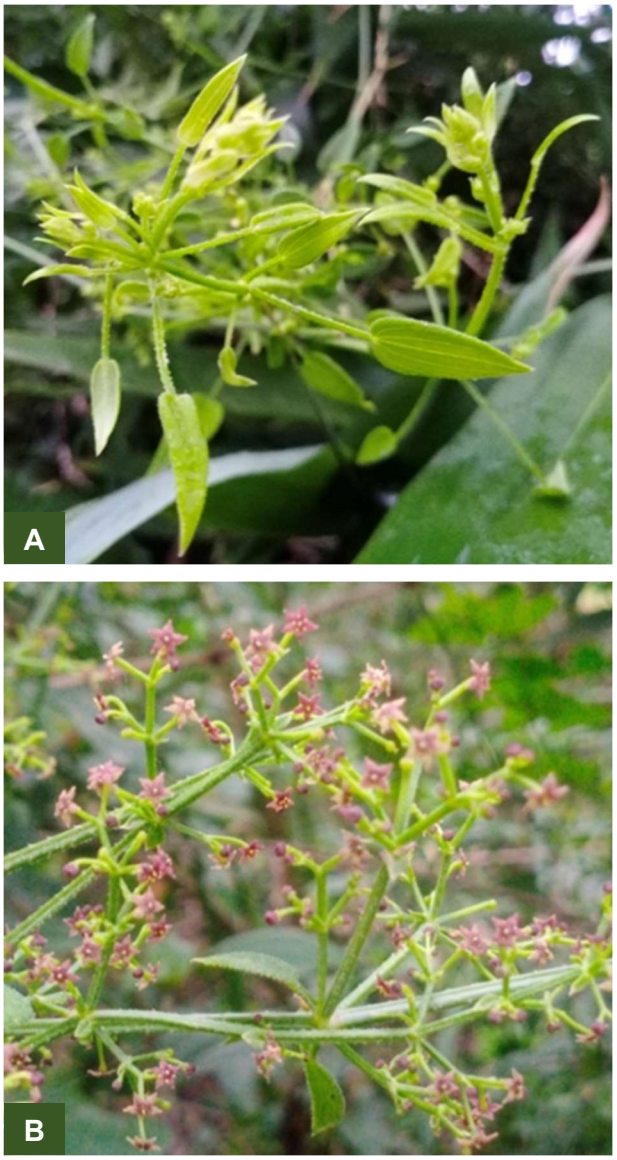

Figure 1. *Rubia manjith* from Godawori-6, Lalitpur, Nepal. A: Whole plant in natural habitat. B: Inflorescence.

CALLUS INDUCTION

In vitro-grown nodal segments (1.5 ± 2 cm) with leaves were used as explants for callus induction. Before use, these explants were cultured on hormone-free MS media for 8 weeks to neutralize residual hormonal effects. Subsequently, the explants were transferred to MS media supplemented with different

concentrations (0.1–2.0 mg/L) of growth regulators, NAA and BAP.

DATA COLLECTION AND STATISTICAL ANALYSIS

Data related to shoot, root, and callus induction were collected in triplicate after 8 weeks of culture and organized in a Microsoft Excel spreadsheet. Descriptive statistical analysis was performed, and the results were expressed as mean ± standard error (SE). Statistical significance was assessed using one-way ANOVA, followed by Tukey's post hoc test. Finally, charts were generated to represent the findings.

Results

Single nodes from wild *R. manjith* and single nodes from their cultured progeny (Figure 2.C) were used as explants for *in vitro* culture on MS medium supplemented with various concentrations of BAP and NAA (0.1–2.0 mg/L). Younger nodal segments were excluded from the culture process, as they were more likely to be damaged during the harsh sterilization process. Results were significantly different ($p<0.05$) in all treatments.

SHOOT INDUCTION

In the first week of culture, two shoot tips were often observed; however, typically only one continued to elongate, producing shoots with mostly 5 nodes (with a maximum of 7). Although the number of individual shoots per explant was generally low (2.33), long lateral shoots (5 ± 2 cm) readily developed from the second and third nodes of plants grown on MS medium (control) and MS supplemented with BAP. These lateral shoot nodes were counted individually due to their potential to develop into independent plants upon rooting.

The highest mean shoot number (8.33 ± 0.33) was observed in MS medium alone (control), followed by MS + 0.1 mg/L BAP and MS + 1.0 mg/L BAP, both yielding a mean shoot number of 5.33 ± 0.33 (Table 1). For shoot elongation, the longest average shoot length (14.33 ± 1.54 cm) was recorded in MS + 0.1 mg/L BAP, closely followed by the control MS medium (14.13 ± 1.13 cm). No shoot development was observed in the MS media containing 0.1 mg/L BAP + 0.1 mg/L NAA and 0.5 mg/L BAP + 0.1 mg/L NAA. In addition, long lateral shoots measuring approximately 5 ± 2 cm were obtained in the second and third nodes in every plant in single BAP treatments and MS media (control) (Table 2; Figure 2.B).

ROOT INDUCTION

Explants that gave successful shoots without callus formation also showed spontaneous root development (Figure 2.E). Therefore, there was no need to subculture the *in vitro*-grown shoots specifically for root induction. The MS medium (control) and MS supplemented with a single hormone were particularly effective in inducing roots in *R. manjith*. Roots varied in both number and length. Long roots (4–5 cm) developed at the second and third nodes in MS medium, in all MS + BAP treatments, and in the MS + 2 mg/L BAP + 1 mg/L NAA combination. Only these long roots had secondary roots and root hairs.

The highest mean root number (8.0 ± 1.0) was recorded in MS medium, followed closely by 7.67 ± 0.33 roots in MS + 2 mg/L NAA (Table 3). The highest mean root length (6.08 ± 1.27 cm) was observed in MS + 2.0 mg/L BAP, followed by 4.27 ± 1.07 cm in MS + 0.1 mg/L BAP. Likewise, MS medium alone, all the MS + BAP treatments, and the MS + 2 mg/L BAP + 1 mg/L NAA combination produced long roots (4–5 cm) at the second and third nodes.

Short roots appeared from the basal ends of explants along with small amounts of callus in MS medium containing 2 mg/L NAA. Similarly, short roots were also observed on the upper portions of nodal explants in the MS + 1 mg/L BAP + 2 mg/L NAA and MS + 2.0 mg/L BAP + 2.0 mg/L NAA treatments without the formation of adventitious shoots or callus (Figure 2.D). This phenomenon was also seen in the MS + 0.1 mg/L BAP + 2.0 mg/L NAA and MS + 0.5 mg/L BAP + 1.0 mg/L NAA combinations (Table 4).

Table 1. Responses in the shoot number induction of *R. manjith* in different hormonal treatments.

		BAP mg/L				
		0	0.1	0.5	1	2
NAA mg/L	0	8.33 ± 0.33^{a}	5.33 ± 0.33^{b}	4.66 ± 0.33^{bc}	5.33 ± 0.33^{b}	2.66 ± 0.33^{cd}
	0.1	0.67 ± 0.33^{de}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	1.00 ± 0.00^{de}	1.50 ± 0.50^{de}
	0.5	1.33 ± 0.33^{de}	1.00 ± 0.58^{de}	0.33 ± 0.33^{e}	1.50 ± 0.41^{de}	1.33 ± 0.33^{de}
	1	1.67 ± 0.33^{de}	1.00 ± 0.00^{de}	1.00 ± 0.00^{de}	1.00 ± 0.58^{de}	2.00 ± 0.00^{de}
	2	0.67 ± 0.67^{de}	0.67 ± 0.67^{de}	2.00 ± 0.00^{cd}	0.33 ± 0.33^{e}	1.00 ± 0.00^{de}

Table 2. Responses in shoot length induction of *R. manjith* in different hormonal treatments.

		BAP mg/L				
		0	0.1	0.5	1	2
NAA mg/L	0	14.13 ± 1.12^{a}	14.33 ± 1.54^{ab}	8.00 ± 1.00^{bcd}	7.35 ± 3.15^{cde}	7.30 ± 1.43^{cde}
	0.1	3.10 ± 2.95^{def}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	2.23 ± 1.08^{ef}	0.20 ± 0.10^{f}
	0.5	0.10 ± 0.00^{f}	0.63 ± 0.58^{f}	0.03 ± 0.03^{f}	0.10 ± 0.00^{f}	1.60 ± 0.67^{f}
	1	0.93 ± 0.19^{f}	0.10 ± 0.00^{f}	0.15 ± 0.05^{f}	0.37 ± 0.32^{f}	11.0 ± 0.50^{abc}
	2	0.03 ± 0.03^{f}	0.10 ± 0.10^{f}	0.40 ± 0.00^{f}	0.03 ± 0.03^{f}	0.30 ± 0.20^{f}

Table 3. Responses in root number induction of *R. manjith* in different hormonal treatments.

		BAP mg/L				
		0	0.1	0.5	1	2
NAA mg/L	0	8.00 ± 1.00^{a}	6.00 ± 1.15^{abc}	3.5 ± 0.50^{abcd}	4.00 ± 2.00^{abcd}	3.67 ± 1.76^{abcd}
	0.1	4.00 ± 0.58^{abcd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.67 ± 0.67^{d}	0.00 ± 0.00^{d}
	0.5	0.00 ± 0.00^{d}	2.33 ± 0.67^{cd}	0.00 ± 0.00^{d}	2.00 ± 0.00^{cd}	4.67 ± 0.88^{abcd}
	1	2.67 ± 1.76^{bcd}	0.00 ± 0.00^{d}	4.50 ± 1.50^{abcd}	0.00 ± 0.00^{d}	3.50 ± 0.50^{abcd}
	2	7.67 ± 0.33^{ab}	2.00 ± 1.15^{cd}	4.00 ± 0.00^{abcd}	4.33 ± 1.86^{abcd}	3.33 ± 2.03^{abcd}

Table 4. Responses in root length induction of *R. manjith* in different hormonal treatments.

		BAP mg/L				
		0	0.1	0.5	1	2
NAA mg/L	0	2.07 ± 0.07^{bcde}	4.27 ± 1.07^{ab}	3.35 ± 0.85^{bc}	3.50 ± 1.50^{bc}	6.80 ± 1.27^{a}
	0.1	2.07 ± 1.02^{bcde}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.73 ± 0.73^{cde}	0.00 ± 0.00^{e}
	0.5	0.00 ± 0.00^{e}	0.73 ± 0.28^{cde}	0.00 ± 0.00^{e}	0.25 ± 0.05^{de}	1.23 ± 0.32^{cde}
	1	0.40 ± 0.31^{cde}	0.00 ± 0.00^{e}	1.55 ± 1.25^{bcde}	0.00 ± 0.00^{e}	0.15 ± 0.05^{de}
	2	0.33 ± 0.03^{de}	0.33 ± 0.18^{de}	0.70 ± 0.00^{cde}	0.27 ± 0.03^{de}	0.27 ± 0.15^{de}

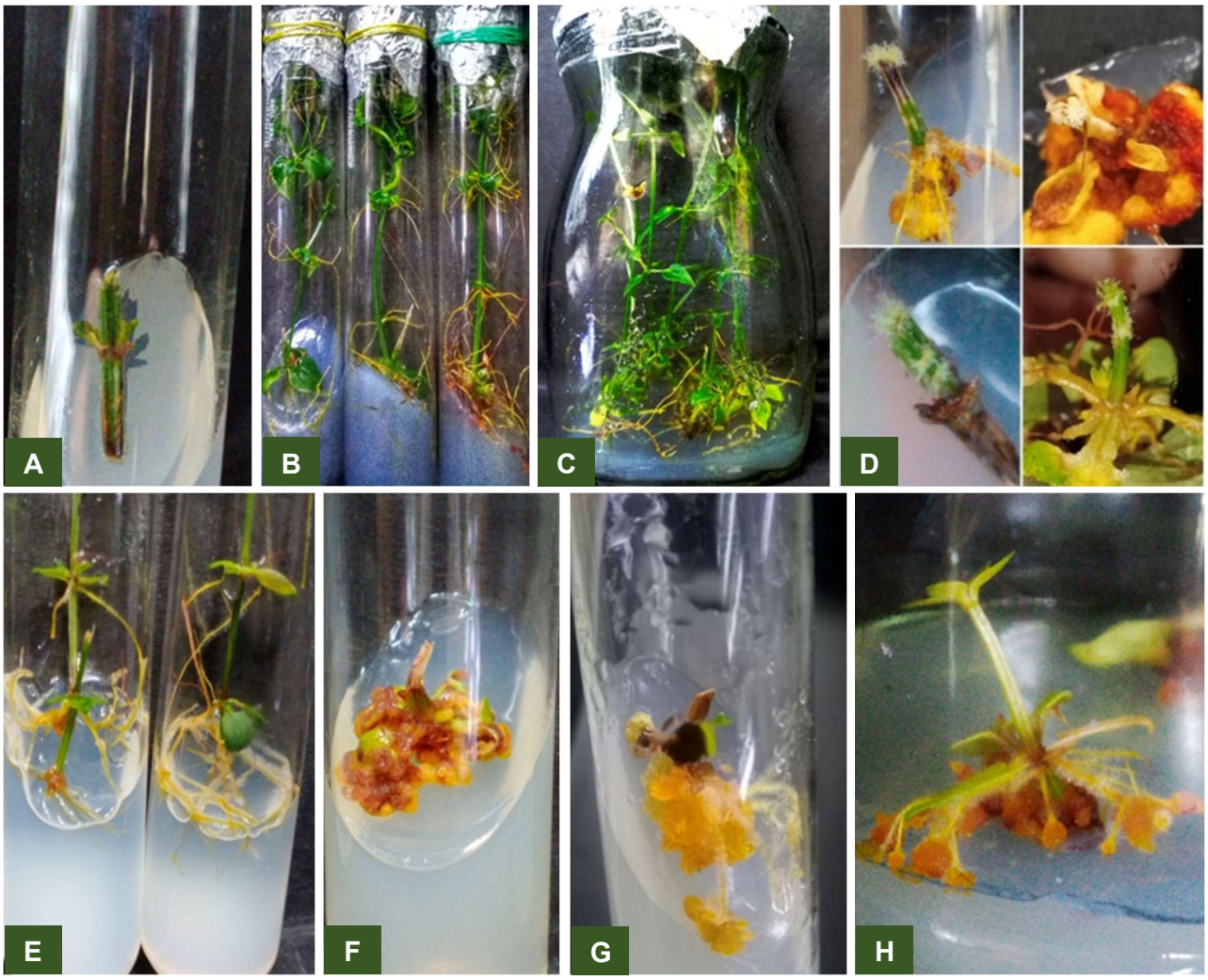

Figure 2. Different stages of shoot induction of *R. manjith*. A: Initial stage of the node after one week of inoculation. B: Elongated shoots, along with long roots. C: Sub-cultured plant on MS media. D: Fuzzy tufts, seen in the upper part of the node. E: Induction of roots in the shoot. F: Yellow to dark orange callus in cultured node and leaves. G: Yellow callus in the grown roots. H: Orange to dark orange callus in the grown roots.

Table 5. Responses in callus induction of *R. manjith* in different hormonal treatments.

		BAP mg/L				
		0	0.1	0.5	1	2
NAA mg/L	0	-	+ (R)	+ (R)	-	+ (R)
	0.1	+ (N-L)	+ (N-L)	+ (N-L)	+ (L-R)	+ (N)
	0.5	+ (N-L)	++ (N-L-R)	+ (N-L)	+ (N)	++ (N-R)
	1	+ (N-L-R)	+++ (N)	+ (N-L-R)	+++ (N-L)	-
	2	+++ (L-R)	+++ (N-L-R)	++ (L-R)	++ (N-L)	++ (N-L-R)

Callus size was categorized as -, +, ++, and +++ (no callus, ~0.1 × 0.5 cm, ~ 0.5 × 1.0 cm, and >1.0 × 1.5 cm, respectively). Callus observed in different parts.: 'no' callus; L: in leaves (residual); N: in node; R: in grown roots; N-L: in node along with leaves (residual); L-R: in leaves (residual) along with grown roots; N-R: in node with grown roots; N-L-R: in node along with leaves (residual) and grown roots.

CALLUS INDUCTION

The nodal segments, including leaves, were cultured. Calluses were observed on pre-existing leaves, nodes, and newly formed roots (Figure 2 F, G, H). These explants were cultured on MS medium supplemented with various concentrations and combinations of BAP and NAA, and observed for eight weeks. Nodal and leaf swellings occurred within one week, while root callus appeared 4–5 weeks after root development. Callus growth and coloration (dark orange to yellow) varied with hormone concentrations and combinations. The largest callus formation (>1.0 × 1.5 cm) was observed in MS + 2.0 mg/L NAA and MS + 0.1 mg/L BAP + 1.0 mg/L NAA. Moderate callus growth (~0.5 × 1.0 cm) was observed in treatments such as MS + 0.1 mg/L BAP + 0.5 mg/L NAA and MS + 0.5 mg/L BAP + 2.0 mg/L NAA. No callus formation was observed in control (MS medium only), MS + 1.0 mg/L BAP, and MS + 2.0 mg/L BAP + 1.0 mg/L NAA (Table 5).

Discussion

As there is no available work on *Rubia manjith in vitro* propagation, we present this study as the first of its kind on this species. The current results revealed that MS medium without any plant growth regulators gave better results in shoot (with 8.33 ± 0.33 shoots per explant) and root (with 8.00 ± 1.00 roots per explant) induction. Similar results were observed by Hapsari and Ermayanti (2020) in *Rubia akane*, where the highest number of shoots (4.5) and roots (9.14) per explant were produced within 8 weeks in the MS medium without cytokinins. Similar observations have been reported in other species, viz. *Stevia rebaudiana* (Galo 2019), *Colocasia esculenta* (Alam and Kadir 2022), and rice varieties (Puhan and Siddiq 2013), where optimum root and shoot induction was achieved in MS media alone rather than in media supplemented with growth regulators.

Since the MS medium is inherently nutrient-rich, comprising a combination of macronutrients, micronutrients, vitamins, and organic compounds, it is extremely suitable and supportive for a broad range of plant species (Aktar *et al*. 2008). Hence, it can be safely said that in plants like *R. manjith*, nutritional components play a more crucial role than hormones in shoot and root induction under *in vitro* conditions. This also indicates that for species like *R. manjith*, proper *in vitro* morphogenetic responses, especially the induction of adventitious roots and shoots, can occur without exogenous hormones. The limited or negative response of the species toward exogenous hormones might be related to the endogenous hormones already present in the explants, causing an overdose.

The highest induction of shoot length in the present study was observed in MS medium supplemented with BAP. Similar findings have been reported by Ghatge *et al*. (2011) and Radha *et al*. (2011) in *R. cordifolia*, Yuniastuti *et al*. (2018) in *Sterculia foetida*, and Durrani *et al*. (2010) in spinach. Yuniastuti *et al*. (2018) also mentioned that higher concentrations of BAP can enhance root length. BAP, a synthetic cytokinin, is known to have promotory effects on shoot and root length by stimulating cell division in meristematic tissue (Yuniastuti *et al*. 2018).

In this study, callogenesis and callus growth were enhanced by the combinations of MS + BAP + NAA, while they were poorly supported by MS + single hormone (BAP or NAA) treatments, and not supported at all by MS media only without exogenous supply of hormone (i.e., control). These findings are supported by Radha *et al*. (2011) in *Rubia cordifolia*, where similar hormone combinations were essential for callus formation. A compact red callus was observed at the cut ends of the nodal explants in MS + BAP + NAA media. Earlier studies also stated that the combination of cytokinin and auxin is most suitable for the initiation and proliferation of calli in *R. cordifolia* (Labade 2009; Khadke *et al*. 2013). These results indicate the necessity of an exogenous supply of hormones, along with endogenous hormone levels, for triggering the cells to divide rapidly, forming an undifferentiated mass of cells (callus).

Conclusions

This study demonstrates that *Rubia manjith* nodal explants have specific nutritional requirements rather than exogenously supplemented hormones for effective *in vitro* propagation. MS media lacking exogenous hormones are suitable for the rapid induction of shoots (8.33 ± 0.33 shoots) and roots (8.0 ± 1.0 roots). Similarly, the lowest (0.1 mg/L) concentration of BAP or BAP-free media is effective in inducing the shoot length (14.33 ± 1.54 and 14.13 ± 1.13 cm, respectively); but, comparatively higher concentration (2.0 mg/L) of BAP is required to achieve the highest mean root length (6.08 ± 1.27 cm). This suggests that different concentrations of BAP exert different effects on shoot and root development, with lower concentrations favoring shoot elongation and higher concentrations enhancing root growth.

However, cytokinin (BAP) and auxin (NAA) interaction is necessary to trigger callus formation, which suggests that hormone synergy is crucial for supporting callogenesis. Overall, the results of the present study provide a foundation for developing *in vitro*-grown plants that can be transplanted to support conservation and sustainable use of *R. manjith*.

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