

IN VITRO EXPLORING HORMONAL EFFECTS ON THE REGENERATION POTENTIAL OF INDIAN PENNYWORT (*CENTELLA ASIATICA* L.)

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(Received 28th Jun 2023; accepted 1st Sep 2023)

Abstract. This article strives to provide an improved and standardized *in vitro* regeneration protocol for Indian pennywort (*Centella asiatica* L.) utilizing nodal explants, specifically focuses on the cultivation of multiple shoots and the successful formation of roots for subsequent field transfer. The study utilized Murashige and Skoog (MS) medium supplemented with different concentrations of Benzyl Amino Purine (BAP), Kinetin (KIN), and α -naphthaleneacetic acid (NAA) to induce shoot formation from the nodal explants. Notably, multiple shoots were generated successfully across all concentrations of BAP, KIN, and NAA. The highest number of multiple shoots (8.20) was observed in MS medium supplemented with 3 mg L⁻¹ BAP. Furthermore, the addition of low levels of NAA (0.1 and 0.2 mg L⁻¹) alongside different concentrations of BAP did not significantly impact the formation of multiple shoots from the nodal explants. Comparing the two cytokinins tested, BAP was found to be more effective than KIN in terms of both shoot initiation and multiplication. Moreover, the highest number of roots (6.36) was obtained in MS medium supplemented with 3 mg L⁻¹ Indole Butyric Acid (IBA). Subsequently, the rooted plantlets were successfully transferred to the field, demonstrating an impressive 90% survival rate. This research article presents a comprehensive and scientifically rigorous approach to the development of an *in vitro* regeneration protocol for Indian pennywort. The successful cultivation of multiple shoots and the establishment of rooted plantlets lay the foundation for further exploration and application of this valuable medicinal plant.

Keywords: *hardening, medicinal plants, multiple shoots, nodal explants, rooting*

Introduction

Centella asiatica, commonly known as Indian pennywort, is a perennial medicinal herb with leaves and stems that can be consumed as a nutritious leafy vegetable (Shukurova et al., 2021). This plant has a rich historical background, being utilized for thousands of years in countries such as India, China, Sri Lanka, Nepal, and Madagascar for its medicinal properties (Chandrika and Kumara, 2015). It holds a significant position in ancient Ayurvedic remedies, where it has been employed to treat various mild and chronic ailments (Shukla et al., 1999).

The pharmacological value of *Centella asiatica* stems from its abundant flavonoids and terpenoid compounds, such as asiatic acid, asiaticoside, and madecassoside, which have been extensively studied (Gray et al., 2018; Rashid et al., 2023). These compounds contribute to its healing properties and make it a valuable herb in the treatment of skin problems and wound healing (Shukla et al., 1999; Diniz et al., 2023). Additionally, *Centella asiatica* holds significance as a psychoactive medicinal plant, deeply rooted in

Ayurvedic medicine as a Medhya Rasayana, known for its cognitive enhancement effects (Sarokte and Rao, 2013).

Various parts of the Indian pennywort plant are utilized as raw materials in the preparation of numerous pharmaceutical products (Sing et al., 2022). However, the popularity and usage of *Centella asiatica* have also led to concerns about its conservation status. It has been listed as a threatened plant species by the International Union for Conservation of Nature and Natural Resources (IUCN) and classified as an endangered species (Uddin et al., 2017). The recognition of its ecological significance emphasizes the need for conservation efforts to protect this valuable medicinal plant for future generations.

Indian pennywort (*Centella asiatica* L.) is traditionally propagated through cuttings and seeds. However, seed propagation is limited due to low viability (Martin, 2004). Conventional propagation methods are insufficient to meet the increasing demands of farmers and industries. To address this, large-scale multiplication of *C. asiatica* plants is essential. In vitro regeneration techniques offer a viable solution for producing a large number of high-quality plantlets in a short period.

Previous studies have reported on the *in vitro* regeneration of Indian pennywort using various explants, such as nodes (Nath Tiwari et al., 2000; Shashikala et al., 2005; Mohapatra et al., 2008; Karthikeyan et al., 2009; Jaheduzzaman et al., 2012; Panathula et al., 2014; Arpita and Navneeta, 2017; Siddiqui et al., 2019; Heidargholinezhad et al., 2023), leaves (Martin, 2004; Mohapatra et al., 2008; Joshi et al., 2013; Kumar, 2017; Gururajan et al., 2021), stems (Joshi et al., 2013; Kumari and Trivedi, 2020), and shoot tips (Nath and Buragohain, 2003; Sivakumar et al., 2006; Jaheduzzaman et al., 2012; Sing et al., 2014; Kundu et al., 2015).

In the present study, we investigate the influence of hormonal treatments on the *in vitro* regeneration of Indian pennywort using nodal explants.

Materials and methods

Explants collection and sterilization of explants

From March 2022 to May 2023, an investigation unfolded at the Plant Tissue Culture Laboratory of the Agricultural College and Research Institute, located at Eachangkottai, Thanjavur, Tamil Nadu. This captivating study aimed to unravel the secrets of *Centella asiatica*, a remarkable plant species with immense potential. Healthy plants of *C. asiatica* were carefully selected from the Medicinal Plant Garden maintained by the department of Horticulture at the Agricultural College and Research Institute in Eachangkottai, Thanjavur.

For micropropagation, nodal explants of *Centella asiatica* were chosen. To ensure their cleanliness, the explants underwent a series of preparation steps. Initially, they were thoroughly rinsed with running tap water to remove any dust and impurities. Next, the explants were subjected to surface sterilization by immersing them in 70% ethanol for 1 min, followed by a treatment with 0.1% mercuric chloride for 3 min. Finally, the explants were washed three times with sterile distilled water under a laminar airflow chamber to ensure complete removal of any sterilization agents.

Culture initiation and multiplication

Surface sterilized nodal explants were inoculated in MS (Murashige and Skoog, 1962) medium supplemented with BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L⁻¹),

KIN (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg L⁻¹) either alone and or in combination of BAP (3.0, 3.5 and 4.0 mg L⁻¹) with KIN (1.0 mg L⁻¹) and BAP (0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5) and in combination with NAA (0.1 and 0.2 mg L⁻¹) with 3% sucrose and 4 g L⁻¹ phytagel at 25°C under a 16 h photoperiod. The media were autoclaved at 12°C for 20 min at a pressure of 105 kPa after adjusting the pH to 5.8. The inoculated culture materials were kept in a culture room at a relative humidity of 80%. The shoots were initiated within 21 days of after inoculation which shows the good response of explants to the medium. Multiple shoots were observed 6 weeks after inoculation. Sub cultures were carried out every 2 weeks interval. The experiment for each treatment was replicated three times.

Root induction and hardening

The multiple shoots were carefully separated from the cluster and subsequently placed in MS medium supplemented with various concentrations of IBA ranging from 0.5 to 3.5 mg L⁻¹. This culture was maintained for a duration of three weeks to promote optimal growth. Following the growth period, rooted plantlets measuring approximately 6 to 8 cm in length were delicately removed from the culture bottles. To eliminate any residual media adhering to the root system, the plantlets underwent a thorough washing under gently running tap water for a duration of 10 min. To facilitate primary hardening, the rooted plantlets were successfully transplanted into plastic cups filled with a mixture of sterilized garden soil, farmyard manure, and sand in a ratio of 2:1:1. This primary hardening stage was maintained for a period of 14 days, allowing the plantlets to acclimatize to their new environment. Following the successful completion of the primary hardening phase, well-developed plantlets were transferred to the field for further growth and development.

Data collection and analysis

Throughout the experiment, various data points were carefully recorded, including the number of days required for shoot emergence, the number of shoots produced per explant, the number of roots formed per shoot, and the survival rate of the plantlets. These measurements were taken at specific time intervals to monitor the progress of the regeneration process. To assess the rate of multiplication, the ratio of the final shoot count after subculture to the initial number of shoots was calculated. This provided valuable insights into the efficiency of shoot production and multiplication. In order to derive meaningful conclusions from the collected data, a comprehensive statistical analysis was performed. The data collected from these experiments were analyzed through Analysis of variance (ANOVA) with the aid of SPSS Version 10 (SPSS, Chicago, IL) software. The significance differences within the treatment means were compared with the aid of Duncan's Multiple Range test (DMRT) at a 5% probability level. The resulted data were represented as mean $\pm$ Standard Error (SE). This robust statistical analysis helped to validate the findings and draw reliable conclusions from the experimental results.

Results

The purpose of this study was to investigate the influence of various plant growth regulators on the *in vitro* regeneration of Indian Pennywort (*Centella asiatica*). Nodal

explants were carefully selected as the primary plant material and were inoculated onto MS medium supplemented with different concentrations of BAP, NAA, and KIN.

As a result, the nodal explants exhibited successful induction of multiple shoots. The percentage of response varied depending on the specific type and concentration of the plant growth regulators used. Notably, BAP demonstrated higher efficacy compared to NAA and KIN for the induction of multiple shoots. The responses observed were diverse, highlighting the importance of selecting the appropriate growth regulators and their concentrations for optimizing shoot induction in Indian Pennywort.

Effect of cytokinins for shoot initiation and multiplication

Nodal explants of Indian Pennywort (*Centella asiatica*) were subjected to inoculation on MS medium supplemented with varying concentrations of BAP ranging from 0.5 to 4.0 mg L⁻¹ to induce multiple shoot formation (Fig. 1a). The results revealed that at lower concentrations of BAP, such as 0.5 mg L⁻¹, each explant produced an average of 2 shoots (Table 1). However, as the concentration of BAP increased gradually, the number of shoots per explant also increased. The maximum number of shoots (8.2 shoots) was observed when the MS medium contained 3 mg L⁻¹ of BAP (Fig. 1b, c). Subsequently increasing the BAP concentration to 3.5 mg L⁻¹ and 4.0 mg L⁻¹ did not significantly enhance shoot growth.

Table 1. Effect of BAP, Kinetin and NAA for shoot formation in *Centella asiatica* L.

Sl. No	Hormones (mg L ⁻¹)		Mean number of shoots/explants
	BAP	KIN	
1	0	0	0
2	0.5	0	2.60 ± 0.22g
3	1.0	0	2.40 ± 0.35g
4	1.5	0	3.80 ± 0.31f
5	2.0	0	5.20 ± 0.45d
6	2.5	0	6.10 ± 0.35bc
7	3.0	0	8.20 ± 0.40a
8	3.5	0	7.60 ± 0.15ab
9	4.0	0	6.20 ± 0.40bc
10	0	0.5	4.00 ± 0.30e
11	0	1.0	4.20 ± 0.31e
12	0	1.5	3.80 ± 0.22f
13	0	2.0	3.10 ± 0.36f
14	0	2.5	3.00 ± 0.31f
15	0	3.0	2.10 ± 0.23g
16	3.0	1.0	6.40 ± 0.35bc
17	3.5	1.0	5.60 ± 0.26d
18	4.0	1.0	4.40 ± 0.23e

Means in the same columns followed by different letters are significantly different ($P \leq 0.05$) with DMRT

Similarly, KIN alone was used at various concentrations (0.5, 1.0, 2.0, 2.5 and 3.0 mg L⁻¹) to promote shoot multiplication in Indian Pennywort (Table 1). The response of explants in terms of the frequency and number of shoots varied depending on the individual and combined concentrations of BAP and KIN. Notably, at 1 mg L⁻¹ of Kinetin, the explants exhibited a maximum of 4 shoots. However, excessive application

of Kinetin resulted in decreased shoot growth. Moreover, the combination of BAP and KIN did not favor the induction of a higher number of multiple shoots in Indian Pennywort (*Table 1*).

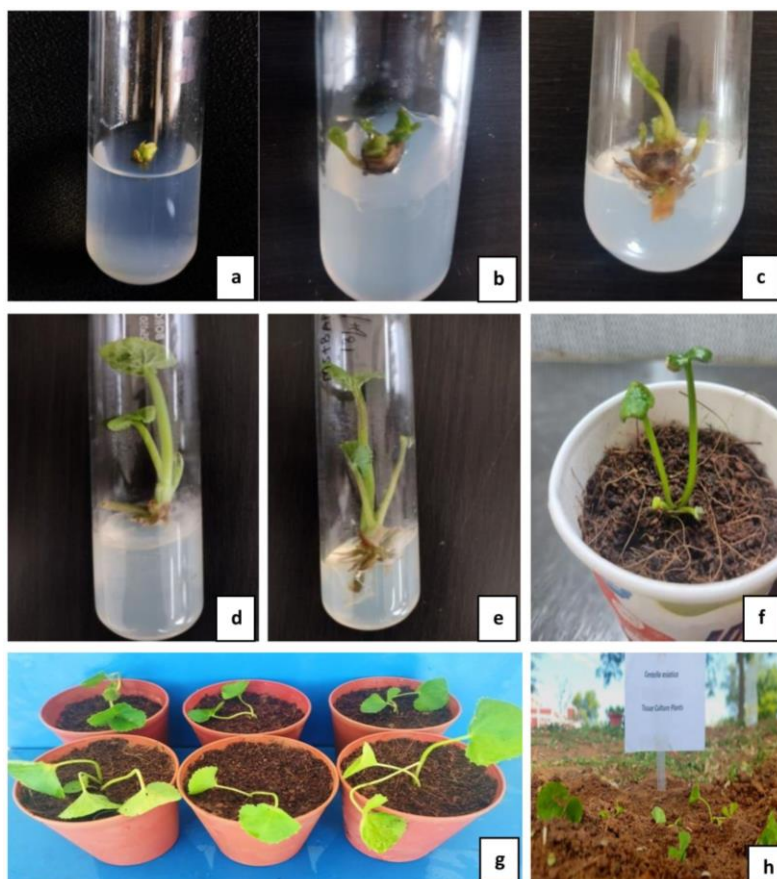


Figure 1. In vitro regeneration of Indian pennywort (*Centella asiatica* L.). (a) Explant. (b) Shoot initiation in MS + 3 mg L⁻¹ BAP. (c) Shoot multiplication in MS + 3 mg L⁻¹ BAP. (d) Root induction in MS + 3 mg L⁻¹ IBA. (e) Rooted plant. (f) Primary hardening of plants. (g) Primary hardening of plants. (h) TC plants grown in field

Effect of BAP and NAA for shoot initiation and multiplication

In the present study, we explored different combinations of BAP and NAA at varying concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg L⁻¹ for BAP; 0.1 and 0.2 mg L⁻¹ for NAA) to investigate their effects on shoot initiation and multiplication in Indian pennywort (*Centella asiatica*) (*Table 2*). The maximum number of multiple shoots (6.20) was observed in the MS medium supplemented with 3.0 mg L⁻¹ BAP and 0.1 mg L⁻¹ NAA.

Effect of auxin for root growth

In this study, we transferred explants with multiple shoots to a rooting medium supplemented with varying concentrations of IBA (*Table 3*). Adventitious root formation was observed in all shoots cultured on MS medium supplemented with different concentrations of IBA. The highest number of roots (6.36) was obtained in the MS medium containing 3 mg L⁻¹ IBA (*Fig. 1d, e*).

Table 2. Effect of BAP and NAA for shoot formation in *Centella asiatica* L.

Sl. No	Hormones (mg L ⁻¹)		Number of shoots/culture
	BAP	NAA	
1	0	0	0
2	0.5	0.1	2.20 ± 0.19e
3	1.0	0.1	3.00 ± 0.27d
4	1.5	0.1	4.10 ± 0.26c
5	2.0	0.1	4.60 ± 0.35c
6	2.5	0.1	5.00 ± 0.17b
7	3.0	0.1	6.20 ± 0.31a
8	3.5	0.1	5.80 ± 0.42b
9	4.0	0.1	4.20 ± 0.31c
10	0.5	0.2	2.00 ± 0.14e
11	1.0	0.2	2.40 ± 0.20e
12	1.5	0.2	2.40 ± 0.35e
13	2.0	0.2	3.00 ± 0.42d
14	2.5	0.2	3.10 ± 0.31d
15	3.0	0.2	3.58 ± 0.42d
16	3.5	0.2	2.82 ± 0.20e
17	4.0	0.2	2.60 ± 0.16e

Means in the same columns followed by different letters are significantly different ($P \leq 0.05$) with DMRT

Table 3. Effect of IBA for rooting in *Centella asiatica* L.

Sl. No	IBA (mg L ⁻¹)	Number of roots/shoot
1	0	0
2	0.5	1.82 ± 0.62f
3	1.0	2.10 ± 0.93e
4	1.5	3.80 ± 0.74d
5	2.0	4.32 ± 0.92c
6	2.5	5.83 ± 0.97b
7	3.0	6.36 ± 0.79a
8	3.5	5.10 ± 0.87b

Means value within same column bearing different letters a, b, c, d are significantly different ($P < 0.05$) on application of Duncan's multiple range test (DMRT)

Hardening and field transfer

After elongated and rooted plantlets were carefully removed from culture tubes, the roots were meticulously washed with running tap water to ensure the complete removal of agar. Subsequently, these rooted plantlets were successfully transplanted into plastic cups filled with autoclaved garden soil, farmyard manure, and sand in a ratio of 2:1:1 (Fig. 1f, g). This specific composition was chosen for the purpose of hardening the plantlets.

Upon successful establishment in the field (Fig. 1h), the regenerated plantlets exhibited morphological characteristics that were identical to the mother plants. The success rate of this transplantation and establishment process reached an impressive 90%.

Discussion

The results of this study demonstrate the influence of varying concentrations of BAP and KIN on the induction of multiple shoots in Indian Pennywort. The findings indicate that increasing the concentration of BAP resulted in an increased number of shoots per

explant up to a concentration of 3 mg L⁻¹, beyond which further increase did not have a significant effect on shoot growth. These results are consistent with previous studies that have reported the effectiveness of BAP in promoting shoot formation in Indian Pennywort. Previous studies have reported the effectiveness of BAP over other cytokinins in inducing a greater number of shoots in Indian Pennywort (Karthikeyan et al., 2009; Arpita and Navneeta, 2017). In contrast, our findings suggest that Kinetin favored shoot multiplication in Indian Pennywort. It is worth noting that Hossain et al. (2005) employed 1.5 mg L⁻¹ of BAP and 0.2 mg L⁻¹ of KIN for shoot regeneration in *Centella asiatica*, demonstrating the influence of different hormonal combinations in the regeneration process.

In contrast, the results showed that Kinetin had a favorable effect on shoot multiplication at lower concentrations (1 mg L⁻¹), but excessive application led to decreased shoot growth. This finding contradicts previous studies that have favored the use of BAP over other cytokinins for inducing a greater number of shoots in Indian Pennywort. The discrepancy in results could be attributed to variations in experimental conditions, such as plant genotype, culture medium composition, and hormone concentrations. The combination of BAP and KIN did not enhance the induction of multiple shoots compared to their individual application. This suggests that the interaction between these two growth regulators in Indian Pennywort may not have a synergistic effect on shoot multiplication.

Effect of BAP and NAA for shoot initiation and multiplication

The findings of this study support previous research that suggests the effectiveness of combinations of BAP and NAA in promoting shoot development in *Centella asiatica*. Nath and Buragohan (2003) reported that a combination of 1.5 mg L⁻¹ BAP and 0.5 mg L⁻¹ IAA was the most favorable concentration for the development of multiple shoots from nodal explants of *Centella asiatica*. Similarly, other studies by Shasikala (2005), Mohapatra et al. (2008), and Jaheduzzaman et al. (2012) have also reported that combinations of BAP and NAA were more effective in producing a larger number of plants compared to the use of BAP and KIN alone or in combination.

Interestingly, our findings differ from the results reported by Rao et al. (1999) and Singh et al. (2015), who found that the MS medium containing 0.5 mg L⁻¹ KIN and 2 mg L⁻¹ NAA promoted shoot growth in Indian pennywort. These differences could be attributed to variations in experimental conditions, including genotypic variations, culture medium composition, and hormonal concentrations.

Furthermore, Rozita et al. (2004) reported that the combination of BAP and IAA was particularly favorable for promoting shoot initiation and multiplication in *Centella asiatica*. Although the present study did not investigate the use of IAA, it suggests the potential benefits of exploring different combinations of BAP with other auxins for optimizing shoot development in Indian pennywort.

The results of this study contribute to the understanding of the effects of different combinations and concentrations of growth regulators on shoot initiation and multiplication in Indian pennywort. The findings indicate that the combination of 3.0 mg L⁻¹ BAP and 0.1 mg L⁻¹ NAA resulted in the highest number of multiple shoots. Further studies are needed to explore the underlying physiological and molecular mechanisms behind these observations and to optimize the hormonal combinations for efficient shoot multiplication in *Centella asiatica*.

Effect of auxin for root growth

The findings of this study are consistent with previous research conducted on Indian pennywort, which also reported similar results regarding the effect of IBA on root induction. Shasikala (2005), Mohapatra et al. (2008), Jaheduzzaman et al. (2012), Singh et al. (2015) and Siddiqui et al. (2019) have all reported successful adventitious root formation in Indian pennywort using IBA supplementation. Singh et al. (2015) specifically found that a combined concentration of 1.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ IBA resulted in the ideal treatment for root induction in Indian pennywort. This discrepancy in optimal hormonal concentrations for root induction could be attributed to variations in experimental conditions, including genotypic variations, culture medium composition, and hormonal concentrations.

The results of this study confirm the potential of IBA for promoting adventitious root formation in Indian pennywort. The highest number of roots was observed in the MS medium supplemented with 3 mg L⁻¹ IBA. Further investigations could explore additional hormonal combinations and concentrations to optimize root induction in this species.

Conclusion

In this current study, our objective was to optimize the medium composition that would be most effective for inducing shoot and root formation in Indian pennywort (*Centella asiatica*). Through our experiments, we successfully identified the ideal conditions for both shoot and root induction. The maximum number of multiple shoots (8.20) was achieved when using MS medium supplemented with 3 mg L⁻¹ BAP. This specific combination proved to be highly conducive to the proliferation of multiple shoots in the plant tissue. Furthermore, when focusing on root induction, the highest numbers of roots (6.36) were observed when utilizing MS medium supplemented with 3 mg L⁻¹ IBA. This concentration of IBA was found to be most effective in promoting the development and growth of roots in Indian pennywort.

By establishing a standardized *in vitro* regeneration protocol for Indian pennywort, this research provides valuable insights and practical applications for researchers. The protocol allows for the efficient multiplication of disease-free and high-quality planting materials. Additionally, it contributes to the conservation efforts of this significant medicinal plant, ensuring its preservation and availability for future use.

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