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Callogenesis in *Hemidesmus indicus* (L.): Optimization of *In vitro* culture conditions for medicinal plant propagation

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Abstract
Hemidesmus indicus (L.), an important medicinal plant widely used in traditional systems of medicine, faces challenges in large-scale propagation and conservation. The present study focuses on the induction and optimization of callus formation (callogenesis) using different explants (leaf, nodal, and petiole) under *in vitro* conditions. Murashige and Skoog (MS) medium supplemented with varying concentrations and combinations of plant growth regulators (2,4-D, NAA, BAP, and IBA) was employed. Among all explants, leaf segments exhibited the highest callogenic response (100%) on MS medium supplemented with 2,4-D (1.5 mg/L) and NAA (1.5 mg/L). Callus initiation was observed within 9–12 days of inoculation. The study demonstrates that optimized hormonal combinations significantly influence callus induction efficiency. The developed protocol can be utilized for large-scale propagation, conservation, and secondary metabolite production in *H. indicus*.

Keywords: Callogenesis, *Hemidesmus indicus*, MS medium, plant tissue culture, medicinal plant, auxins, cytokinins

Introduction

Plant tissue culture is used widely in plant science, it also has a number of commercial applications.
That include:

- A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance (Gill *et. al*., 2008).
- Production of identical sterile hybrid species can be obtained (Srivastava *et al*., 2006).
- Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals (Georgiev *et al.,* 2009) [14].
- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid (Manstein *et al.,* 1880).
- To cross-pollinate distantly related species and then tissue culture the resulting embryo which would otherwise normally die (Embryo Rescue) (George *et al*., 2008) [12, 13].
- For Production of doubled monoploid (dihaploid) plants from haploid cultures to achieve homozygous lines more rapidly in breeding programmes, usually by treatment with colchicine which causes doubling of the chromosome number (Edwin *et al.,* 2009).
- As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.
- Certain techniques such as meristem tip culture can be used to produce clean plant material from virus free stock, such as potatoes and many species of soft fruit.
- Micropropagation using meristem and shoot culture to produce large numbers of identical individuals.

Importance and scope of Medicinal Plant

Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or other were used for medicinal purposes (Kumar 1998) [20].
Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds (Chand *et al.,* 1997) [6].
The Ayurveda system of medicine uses about 70 species, Unani 700, Siddha 600 and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower seed etc. Scientific study of traditional medicines, derivation of drugs through bioprospecting and systematic conservation of the concerned medicinal plants are thus of great importance (Joy *et al.,* 1998).

Botanical description of plant

Hemidesmus *indicus* (Anantmool) is a perennial, slender, lactiferous and twinning climbing vine shrub. It has long, cylindrical, slightly twisted aromatic roots and brown-coloured bark. Stems are slender and having thickened nodes.
The leaves are simple, shortly petioled, exstipulate, opposite, entire, smooth, acute and striated down the middle with white colour. The mature leaves are generally broad lanceolate, sometimes ovate or oval. It is 5-10 cm long dark green with reticulate veins.
Flowers are greenish-purple in colour. It is crowded in sub-sessile cymes with opposite axils. Fruits are cylindrical and long up to 10 cm. Seeds are flat, oblong with a long tuft of white silky hair. (Shifali Thakur *et al*, 2021)

Geographical Distribution

H. Indicus is widely distributed in India, Pakistan, Sri Lanka, Bangladesh, Iran, Iraq and Indonesia. It is cultivated at an altitude of 600 m. In India, it is distributed through the Gangetic Plain, the arid region of the Chota Nagpur and the southern dry regions. It is commonly growing in deciduous forests, uncultivated lands and moist hedges. (Shifali Thakur *et al*, 2021)

Medicinal plants have been an integral part of traditional healthcare systems worldwide and continue to serve as a major source of therapeutic agents. It is estimated that a significant proportion of the global population relies on plant-derived medicines for primary healthcare due to their accessibility, affordability, and minimal side effects (Kumar, 1998; World Health Organization, 2013) [20]. The therapeutic potential of medicinal plants is largely attributed to the presence of diverse bioactive compounds such as alkaloids, flavonoids, glycosides, tannins, and terpenoids (Chand *et al*., 1997; George *et al*., 2008) [6, 12, 13].

Hemidesmus indicus (L.) R. Br., commonly known as Indian Sarsaparilla or Anantmool, is an important medicinal plant belonging to the family Apocynaceae. It is widely distributed in tropical and subtropical regions and is extensively used in Ayurvedic and Unani systems of medicine for the treatment of various ailments including inflammation, skin diseases, diabetes, and cancer (Banerjee and Ganguly, 2014; Austin, 2008) [1, 3, 4].

The pharmacological importance of *H. indicus* is primarily due to its rich phytochemical composition. Different parts of the plant contain distinct classes of secondary metabolites. The roots are particularly rich in essential oils, mainly 2-hydroxy-4-methoxy benzaldehyde, along with saponins, tannins, sterols such as β-sitosterol and stigmasterol, and triterpenoids including α-amyrin, β-amyrin, and lupeol (Saraswathy *et al*., 2010; Banerjee and Ganguly, 2014) [3, 4, 29]. Additionally, glycosides such as hemidesmin I and II and phenolic compounds have also been reported in root extracts.

The stem of *H. indicus* contains several bioactive constituents including terpene lactones such as 3-keto-lup-12-ene-21→28-olide, along with lupanone derivatives, aromatic aldehydes, and pregnane glycosides (Gupta *et al*., 2008) [17].

Leaves are reported to contain cardiac glycosides, tannins, saponins, and flavonoids such as rutin and hyperoside, whereas flowers are rich in flavonoid glycosides including rutin, hyperoside, and isoquercetin (Banerjee and Ganguly, 2014) [3, 4].

Due to this diverse phytochemical profile, *H. indicus* exhibits a wide range of pharmacological activities. Several studies have demonstrated its anticancer potential against breast (MCF-7), colon (HT-29), and Ehrlich Ascites tumor cell lines, primarily through induction of apoptosis via mitochondrial dysfunction and modulation of intracellular signaling pathways (Patil *et al*., 2011; Banerjee and Ganguly, 2014) [3, 4, 21]. Furthermore, the plant has been shown to enhance the efficacy of conventional chemotherapeutic agents such as methotrexate and cytarabine.

In addition to anticancer activity, *H. indicus* exhibits significant chemopreventive and antioxidant properties, protecting cells against oxidative stress and lipid peroxidation (Shetty *et al*., 2005) [32].

Its extracts have been reported to possess immunomodulatory effects, enhancing IgG secretion and adenosine deaminase activity in lymphocytes (Das *et al*., 2010) [7]. The plant also demonstrates wound healing properties, accelerating epithelialization and wound contraction, particularly in chronic conditions (Kumar *et al*., 2007) [19].

Moreover, *H. indicus* shows antiulcer activity through mucosal protection and inhibition of prostaglandins, with effects comparable or superior to standard drugs such as omeprazole (Austin, 2008) [2].

It also exhibits nootropic effects, improving memory and learning ability, indicating its potential use in neurodegenerative disorders (Rao *et al*., 2012) [27].

The antioxidant potential of *H. indicus* is well documented, particularly in reducing doxorubicin-induced cardiotoxicity, where it restores antioxidant enzyme levels such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Naidu *et al*., 2006) [23].

Materials and Methods

1. Collection of Plant Material

In the present investigation the plants of *Hemidesmus indicus* was collected from Sant Gadge Baba Amravati University, forest area and was established in the garden of Department of Botany, Sant Gadge Baba Amravati University, Amravati and different explants such as nodal explants, shoot tips and leaves from plantlets of *Hemidesmus indicus* were used.

The complete experiment was performed under aseptic and controlled conditions in plant tissue culture laboratory of Department of Botany, Sant Gadge Baba Amravati University, Amravati.

2. Preparation of Stock Solutions of Plant Growth Regulators

Stock solutions of plant growth regulators (PGRs), including auxins and cytokinins, were prepared according to standard protocols used in plant tissue culture. Appropriate solvents such as ethanol, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were used to facilitate dissolution of growth regulators prior to dilution with sterile double distilled water (Murashige and Skoog, 1962; George *et al*., 2008) [12, 13, 22].

2.1. Preparation of Auxin Stock Solutions

a. 2,4-Dichlorophenoxyacetic Acid (2,4-D)

A stock solution of 2,4-D was prepared by dissolving 20 mg of the compound in 3 mL of ethanol to ensure complete solubility. The volume was then adjusted to 20 mL using sterile double distilled water under aseptic conditions (George *et al*., 2008) [12, 13].

b. α-Naphthalene Acetic Acid (NAA)

For NAA, 20 mg of the compound was dissolved in 3 mL of 1N NaOH, as auxins are more soluble in alkaline solutions. The final volume was adjusted to 20 mL with sterile double distilled water (Bhojwani and Razdan, 1996) [5].

c. Indole-3-Acetic Acid (IAA)

Similarly, 20 mg of IAA was dissolved in 3 mL of 1N NaOH, followed by volume adjustment to 20 mL with sterile double distilled water. The alkaline medium aids in complete dissolution of IAA (George *et al*., 2008) [12, 13].

2.2. Preparation of Cytokinin Stock Solutions

a. 6-Benzylaminopurine (BAP)

A stock solution of BAP was prepared by dissolving 20 mg in 1 mL of 1N NaOH, followed by dilution up to 20 mL with sterile double distilled water. Cytokinins like BAP require slight alkalinity for proper solubilization (Murashige and Skoog, 1962) [22].

b. Kinetin

For kinetin, 20 mg of the compound was dissolved in 1 mL of 1N HCl to enhance solubility, and the volume was made

up to 20 mL using sterile double distilled water (Bhojwani and Razdan, 1996) [5].

2.3. Storage of Stock Solutions

All prepared stock solutions were stored in sterile, labeled containers and kept at 4°C until further use. Light-sensitive compounds such as IAA were stored in amber-colored bottles to prevent degradation (George *et al*., 2008) [12, 13]

2.4. Excision and surface sterilization of explant:

In vitro plant tissue culture techniques require strict aseptic conditions to ensure successful establishment and growth of explants. Surface sterilization of explants is a critical step to eliminate microbial contamination without affecting tissue viability (Murashige and Skoog, 1962; George *et al*., 2008) [12, 13, 22]. Proper sterilization protocols involving detergents, alcohol, and chemical sterilants such as mercuric chloride ($HgCl_2$) are widely used in plant tissue culture studies (Bhojwani and Razdan, 1996) [5].

The selection of healthy and disease-free explants, followed by sterilization and inoculation on nutrient media supplemented with plant growth regulators, plays a vital role in successful callus induction and plant regeneration (George *et al*., 2008) [12, 13]. Controlled environmental conditions such as temperature, photoperiod, and humidity further influence *in vitro* growth.

Selection of healthy plant material

↓

Excision of explants (shoot tip / nodal / leaf)

↓

Washing with running tap water

↓

Rinsing with sterile distilled water

↓

Treatment with soap solution (Labolin)

↓

Rinsing with sterile distilled water

↓

Surface sterilization with 0.1% $HgCl_2$ (2–3 min)

↓

Rinsing with sterile distilled water (3–4 times)

↓

Treatment with 70% ethanol (2–3 min)

↓

Final washing with sterile distilled water

↓

Drying on sterile tissue paper

↓

Trimming edges using sterile scalpel

↓

Inoculation on MS medium + PGRs

↓

Incubation under controlled conditions

Results and Discussion

3.1. Callus Initiation and Growth Response

In the present investigation, different explants (leaf, nodal, and petiole) of *Hemidesmus indicus* were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of plant growth regulators (PGRs).Callus initiation was observed within 9–12 days of inoculation across all treatments. The initiation response varied significantly depending on:.

- Type of explant

- Concentration of growth regulators
- Combination of auxins and cytokinins

Among the explants tested, nodal explants showed the earliest callus initiation, whereas leaf explants exhibited the highest overall callogenic response

In the present investigation which was carried out in the well equipped plant tissue culture laboratory. Murashige and Skoog's media supplemented with combination of different growth regulators including auxins and cytokinins i.e. 2,4-D. IAA, NAA, and BAP, kinetin respectively. Leaf, Petiole, Node and Internodes were used as explants.

Different concentrations of plant growth regulators had a significant effect on callus regeneration. During the present investigation Leaf, nodal and internodal explants of *Hemidesmus indicus* were cultured on MS media fortified with 2, 4-D, 2,4- D-NAA, NAA-BAP, and BAP +IBA in different concentrations to test the efficiency of sprouting of various explants. It was observed that after 9-12 days of inoculation, the explants showed callus initiation in all the concentrations.

The nodal explants were the first to show callus initiation. The callogenic response showed variations among the hormonal combinations/concentrations and types of explants used. The callus response varied from 50 to 80% in nodal explants, 60 to 100% in leaf explants and 50 to 90% in Petiole explants. The study confirms that plant growth regulators play a critical role in callogenesis. Auxins, particularly 2,4-D in combination with NAA, significantly enhanced callus formation. Similar findings have been reported in other medicinal plants.

Among different types of explants used, leaf segments proved to be best for callus induction. Highest callogenic response, i.e. 100%, was noticed with leaf explants at 1.5 mg/L. 2, 4-D+1.5 mg/L NAA and lowest callogenic response i.e. 50% was observed in nodal explants at 2, 4-D 3.0 mg/l. A total of 14 combinations of auxins and cytokinins were tried for callus formation. The degree of callus formation varies from very little in nodal explants at 2,4-D (1.5mg/l) and large amount of callus in leaf explants at 2,4-D +NAA(1.5+1.5mg/l) denoted by + and +++ respectively. (Table 1, plate 2, graph 1).

Table 1: Callus induction from Leaf, Nodal and Petiole explants at different concentrations and combinations of plant growth regulators

		Nodal explant	Degree of callus formations	Leaf explants	Degree of callus formations	Petiole explants	Degree of callus formations
PGR	Conc.(mg/l)	RP (%)		RP (%)		RP (%)	
2, 4 D	1.5	50	+	90	+++	70	++
	2.0	65	++	80	++	80	++
	2.5	40	+	70	++	60	+
	3.0	45	+	70	++	60	+
2,4 D + NAA	1.0 + 1.0	60	+	80	++	60	+
	1.5 + 1.5	50	++	100	+++	90	+++
	1.5 + 1.0	70	++	90	+++	80	++
NAA + BAP	1.5 + 2.5	60	+	90	+++	70	++
	2.0 + 2.0	70	++	100	+++	70	++
	2.5 + 1.5	75	++	90	+++	80	++
	2.5 + 2.0	60	+	90	+++	70	++
BAP + IBA	1.0 + 0.5	50	+	60	+	70	++
	1.0 + 2.0	65	++	80	++	80	++
	2.0 + 1.0	50	+	60	+	50	+

+ = Little Callus,
++ = Average Callus,
+++ = Large amount of callus.

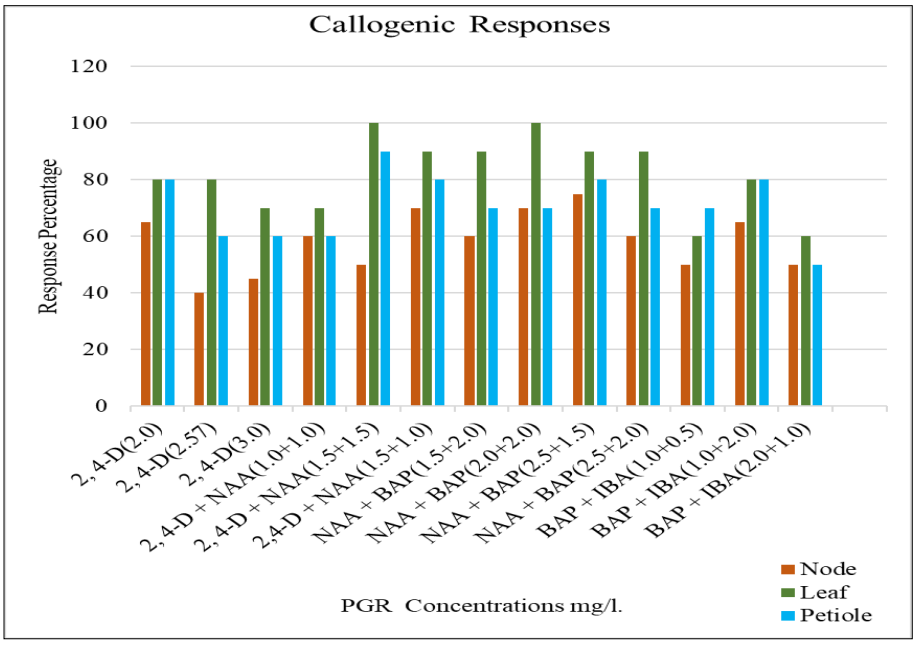

Fig 2: Callogenic response from Leaf, Nodal and Internodal Explants

Table 2: Response of the explants for callusing to different combinations of growth regulators used at different concentrations

Sr. No.	Explant used	Growth Regulators	Concentrations mg/lit	Time taken by explants to form callus (days)	Result	Colour & Morphology
1.	Leaf	2,4-D	1.5	14	+	Yellowish brown & Compact
2.	Leaf	2,4-D + NAA	1.5 +1.5	13	++	Yellowish brown & Compact
3.	Leaf	NAA+BAP	2.0 + 2.0	14	++	Yellowish brown & Compact
4.	Petiole	2,4-D + NAA	1.5 + 1.5	11	+	Whitish & Friable

Callus induction in leaf explants was started earlier in MS medium containing 2, 4 D (1.5 ~mg/lit), 2, 4-D + NAA (1.5 + 1.5 ~mg/lit), (1.5 + 1.0 ~mg/lit) and NAA + BAP (2.0 + 2.0~mg/lit) (Table-2). During after inoculation of 1 week leaf explants start initiation for callus induction. After the second week the inoculated tissue showed the sign of callusing in MS medium containing 2, 4 D (1.5 ~mg/lit), 2, 4-D + NAA (1.5 + 1.5 ~mg/lit), (1.5 + 1.0 ~mg/lit) and NAA + BAP (2.0 + 2.0~mg/lit) showed good amount of callus. However, the present investigation proved that leaf explants responded better at a higher level of 2, 4-D + NAA (1.5 + 1.5 ~mg/lit), NAA + BAP (2.0 + 2.0~mg/lit) and NAA + BAP (2.0 + 2.0~mg/lit) However, leaf explants show response to any concentrations. It was also observed Petiole explants too showed response to concentrations of 2, 4-D + NAA (1.5 + 1.5 ~mg/lit), Morphologically the callus showed yellowish Brown and compact & Yellow and Friable in appearance in 07-15 days from leaf explants etc. (Table-2).

Discussion

The study confirms that plant growth regulators play a critical role in callogenesis. Auxins, particularly 2,4-D in combination with NAA, significantly enhanced callus formation. Similar findings have been reported in other medicinal plants.

Leaf explants exhibited superior responsiveness, likely due to higher metabolic activity and better hormonal sensitivity. The variation in callus morphology indicates differential cellular responses influenced by hormonal balance.

Callus culture provides a platform for:

- Secondary metabolite production
- Genetic improvement
- Mass propagation

Figures

Fig 1: Habit

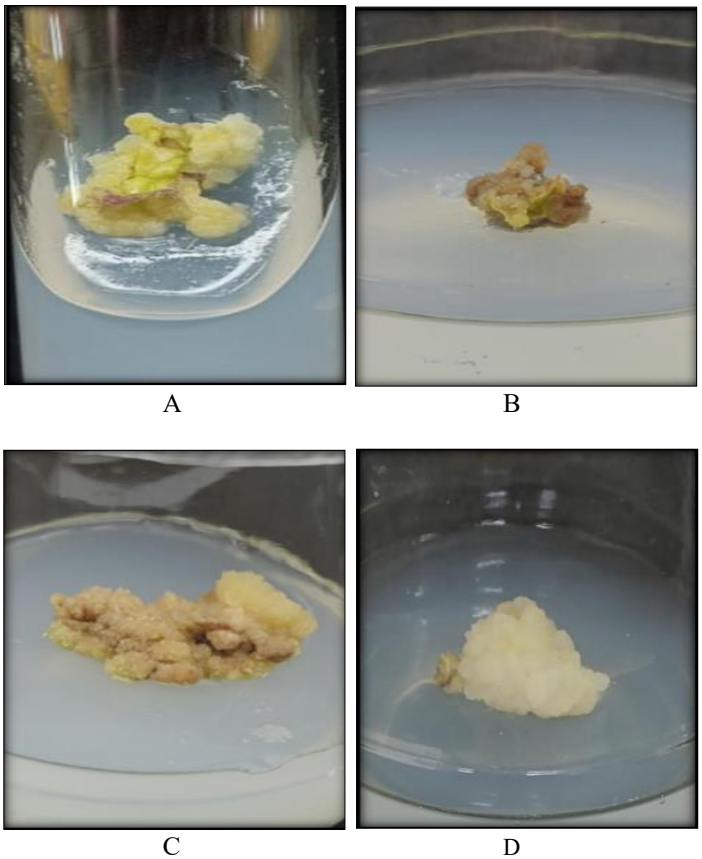

Fig 2: Callus induction from Leaf, Nodal and Petiole explants at different concentrations and combinations of plant growth regulators

A = Callogenic response from leaf explants at 2, 4 – D 1.5 mg/l.
B = Callogenic response from leaf explants at 2, 4 - D + NAA (1.5 + 1.5) mg /l
C = Callogenic response from leaf explants at NAA + BAP (2.0 + 2.0) mg /l.
D = Callogenic response from Petiole explants at 2, 4 - D + NAA (1.5 + 1.5) mg /l, respectively.

Conclusion

The present study successfully established an efficient and reproducible protocol for callogenesis and organogenesis in *Hemidesmus indicus* (L.) using different explants, including leaf, petiole, nodal, and internodal segments. Among the explants tested, leaf explants exhibited the highest callogenic response, achieving up to 100% callus induction on Murashige and Skoog (MS) medium supplemented with 2,4-D (1.5 mg/L) and NAA (1.5 mg/L).

The study clearly demonstrates that callus induction is significantly influenced by the type of explant and the concentration and combination of plant growth regulators. The combination of auxins, particularly 2,4-D and NAA, proved to be most effective for inducing compact, yellow-colored callus, indicating its suitability for further applications.

The established callus culture system provides a promising platform for:

- Large-scale propagation of *Hemidesmus indicus*
- Sustainable production of bioactive secondary metabolites.
- Genetic improvement and stress resistance studies.
- Cytological and biochemical investigations.

Furthermore, the ability to regenerate whole plants from callus through manipulation of culture conditions highlights the potential of this protocol for mass multiplication and conservation of this medicinally important species.

Overall, the present study contributes significantly toward the conservation, biotechnological advancement, and commercial utilization of *Hemidesmus indicus*, particularly for enhanced biomass production and secondary metabolite synthesis.

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