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Original Article

Somatic Embryogenesis Induction Using Varying Auxin-Cytokinin Ratio By Using Bacopa monnieri (Brahmi)

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ABSTRACT

Somatic embryogenesis (SE) is an advanced plant tissue culture technique in which somatic cells are induced to develop into complete embryos under controlled in vitro conditions. The balance between auxins and cytokinins, the two major classes of plant growth regulators, plays a crucial role in regulating cellular dedifferentiation, embryogenic competence, and embryo formation. This study reviews the influence of different auxin–cytokinin ratios on the induction and development of somatic embryos, particularly in medicinally important plant species. The findings indicate that elevated levels of auxins, especially 2,4-dichlorophenoxyacetic acid (2,4-D), are essential for the initiation of embryogenic callus, whereas cytokinins contribute significantly to embryo differentiation, maturation, and plantlet regeneration. Analysis of various medicinal plants, including *Digitalis trojana*, *Nardostachys jatamansi*, *Schisandra chinensis*, and *Crinum malabaricum*, revealed that optimal plant growth regulator combinations vary among species. Generally, higher auxin-to-cytokinin ratios favor callus induction, balanced ratios promote embryo development, and cytokinin-dominant conditions support root and shoot formation. The study also highlights the pharmaceutical and biotechnological significance of somatic embryogenesis in the mass propagation of valuable medicinal plants, conservation of endangered species, and enhanced production of bioactive secondary metabolites such as alkaloids, lignans, and cardenolides. Furthermore, the regulatory mechanisms of auxins and cytokinins, including their biosynthesis, transport, and gene-mediated signaling pathways, are discussed. Overall, this review provides valuable insights into the optimization of somatic embryogenesis protocols and their applications in plant biotechnology and pharmaceutical research.

Keywords: Somatic embryogenesis, auxin–cytokinin ratio, plant growth regulators, medicinal plants, tissue culture, secondary metabolites, pharmaceutical biotechnology.

INTRODUCTION

Plant cells exhibit a unique biological characteristic known as **totipotency**, which refers to the capacity of a single somatic cell to regenerate into a complete plant under appropriate environmental and nutritional conditions. This remarkable property forms the foundation of plant tissue culture and regenerative biotechnology. One of the most significant manifestations of cellular totipotency is **somatic embryogenesis (SE)**, a developmental process in which somatic (non-reproductive) cells are induced to form embryo-like structures capable of developing into whole plants without the involvement of fertilization (Fehér, 2019).

Somatic embryogenesis closely mimics the developmental stages of zygotic embryogenesis; however, it originates from somatic tissues rather than from a fertilized egg. During this process, differentiated cells undergo dedifferentiation and subsequent reprogramming, leading to the formation of bipolar embryos with distinct shoot and root meristems. The induction of SE is regulated by complex interactions among plant growth regulators, stress signals, and molecular pathways that alter cellular gene expression patterns and promote embryogenic competence (Ikeuchi et al., 2016).

The development of somatic embryogenesis has greatly advanced modern plant biotechnology by providing efficient systems for large-scale plant propagation, genetic transformation, germplasm conservation, and synthetic seed production. SE offers several advantages over conventional propagation methods, including high multiplication

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rates, genetic uniformity, and the potential for automation in bioreactor-based production systems (Lelu-Walter et al., 2018).

From a pharmaceutical perspective, somatic embryogenesis plays a crucial role in the sustainable utilization of medicinal plants. Many medicinal species are increasingly threatened due to excessive harvesting, habitat degradation, and low natural regeneration rates. SE provides an effective alternative for the rapid multiplication and conservation of these valuable plant resources under controlled in vitro conditions. Furthermore, embryogenic cultures can serve as reliable sources for the production of pharmacologically important secondary metabolites, ensuring a consistent supply of bioactive compounds for pharmaceutical and nutraceutical industries while reducing dependence on wild plant populations (Isah, 2016).

1.1 Significance of Somatic Embryogenesis in Pharmaceutical Biotechnology

Somatic embryogenesis (SE) has emerged as a valuable tool in pharmaceutical biotechnology due to its wide-ranging applications in the conservation, propagation, and genetic improvement of medicinal plants. The technology offers a reliable and sustainable platform for the large-scale production of plant-derived pharmaceutical resources while reducing pressure on natural plant populations.

1.2 Conservation of Medicinal Plant Resources

Numerous medicinal plant species are facing the threat of extinction because of excessive harvesting, habitat degradation, and poor natural regeneration. Somatic embryogenesis provides an efficient ex situ conservation strategy by enabling rapid clonal multiplication of endangered species under controlled in vitro conditions. Medicinal plants such as *Nardostachys jatamansi* and *Digitalis trojana* have been successfully propagated through SE, contributing to the preservation of their valuable genetic resources and reducing dependence on wild populations (Lelu-Walter et al., 2018; Fehér, 2019).

1.3 Production of Secondary Metabolites

One of the most important pharmaceutical applications of SE is its potential for the production of biologically active secondary metabolites. Embryogenic cultures and somatic embryos are capable of synthesizing diverse classes of therapeutic compounds, including alkaloids, terpenoids, phenolic compounds, lignans, flavonoids, and cardenolides. These in vitro culture systems offer a controlled and sustainable alternative to field cultivation and can serve as efficient biofactories for the commercial production of phytopharmaceuticals (Isah, 2016).

1.4 Synchronized Production of Bioactive Compounds

Somatic embryogenesis enables the synchronized development of embryos, which facilitates uniform growth and predictable metabolite production. Such synchronization enhances process standardization and product consistency, both of which are critical requirements in pharmaceutical manufacturing. Various culture additives, including coconut water, have been reported to improve embryo induction, maturation, and synchronized development in several medicinal plant species.

1.5 Genetic Improvement of Medicinal Plants

The integration of somatic embryogenesis with modern genetic engineering techniques provides opportunities for the development of improved medicinal plant varieties. Genes involved in the biosynthesis of valuable therapeutic compounds can be introduced, modified, or overexpressed to enhance metabolite yield, stress tolerance, and overall plant performance. Consequently, SE serves as an important platform for plant genetic transformation and molecular breeding programs (Ikeuchi et al., 2016).

1.6 True-to-Type Propagation

Somatic embryogenesis facilitates the production of genetically uniform plants with high clonal fidelity. Studies on medicinal species such as *Crinum malabaricum* have demonstrated that plants regenerated through somatic embryos maintain their genetic stability and phytochemical characteristics. This ensures consistent quality, efficacy, and safety of plant-derived pharmaceutical products, which is essential for commercial cultivation and medicinal applications.

1.7 Role of Plant Growth Regulators in Somatic Embryogenesis

Plant growth regulators (PGRs) are naturally occurring or synthetic organic compounds that influence plant growth and development at very low concentrations. In somatic embryogenesis, PGRs function as key signaling molecules that regulate cellular dedifferentiation, embryogenic competence, embryo formation, and subsequent embryo maturation.

Recent advances in plant developmental biology have revealed that plant regeneration is largely controlled by the biosynthesis, transport, perception, and signaling pathways of auxins and cytokinins. These hormones regulate cell fate determination by modulating gene expression patterns associated with embryogenic development and tissue differentiation (Su et al., 2021).

Auxins

Auxins are the principal regulators involved in the initiation of somatic embryogenesis. They stimulate cell division, callus formation, and the acquisition of embryogenic competence. Synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), and picloram are commonly used because of their greater stability and effectiveness compared with naturally occurring indole-3-acetic acid (IAA). High auxin concentrations generally promote dedifferentiation and embryogenic callus induction.

Cytokinins

Cytokinins promote cell proliferation, shoot induction, and embryo development. Commonly used cytokinins include benzylaminopurine (BAP), kinetin (KIN), zeatin, and thidiazuron (TDZ). Their interaction with auxins determines the developmental pathway followed by cultured cells and tissues.

Abscisic Acid (ABA)

Abscisic acid plays a crucial role during the maturation phase of somatic embryogenesis. ABA promotes storage reserve accumulation, embryo desiccation tolerance, and the development of morphologically normal embryos while suppressing premature germination.

1.8 Other Plant Growth Regulators

Additional hormones, including gibberellins, ethylene inhibitors, jasmonates, and polyamines, may influence specific stages of somatic embryogenesis depending on the plant species and culture conditions. These compounds often act synergistically with auxins and cytokinins to optimize embryo induction and development.

Auxin–Cytokinin Ratio Concept

The balance between auxin and cytokinin concentrations is widely recognized as the fundamental factor controlling morphogenesis in plant tissue culture. The auxin–cytokinin ratio determines whether cultured cells undergo callus formation, root development, shoot organogenesis, or somatic embryogenesis.

The classical auxin–cytokinin hypothesis proposes the following developmental responses:

- **High auxin : low cytokinin ratio** → Callus induction and root formation
- **Intermediate or balanced auxin : cytokinin ratio** → Somatic embryogenesis
- **Low auxin : high cytokinin ratio** → Shoot induction and organogenesis

This hormonal balance functions as a master regulator of cellular differentiation and developmental programming. Changes in auxin and cytokinin levels influence gene expression networks that determine embryogenic competence and embryo formation (Skoog & Miller, 1957; Su et al., 2021).

Experimental studies across diverse plant species have demonstrated that relatively small variations in hormone concentrations can significantly alter morphogenic outcomes. For example, investigations in *Narcissus* species revealed that specific auxin–cytokinin ratios favor somatic embryogenesis, whereas alternative ratios promote bulb formation or rhizogenesis. Such findings highlight the importance of precise hormonal optimization in tissue culture protocols.

Furthermore, sequential application of auxins and cytokinins is often more effective than their

simultaneous use. Initial exposure to auxins induces dedifferentiation and embryogenic competence, while subsequent cytokinin treatment promotes embryo development and maturation. This sequential hormonal regulation plays a critical role in achieving successful induction, determination, and progression of somatic embryos.

Therefore, understanding and optimizing the auxin–cytokinin ratio is essential for developing efficient somatic embryogenesis protocols, particularly for medicinal plants where large-scale propagation and enhanced production of pharmaceutical compounds are desired.

2. BACOPA MONNIERI (BRAHMI)

Bacopa monnieri (L.) Wettst., commonly known as **Brahmi** or **water hyssop**, is a perennial medicinal herb belonging to the family Plantaginaceae. It is widely distributed in tropical and subtropical regions, particularly in India, where it occupies an important place in traditional Ayurvedic medicine. The plant is commonly found growing in marshy areas, wetlands, riverbanks, and shallow aquatic habitats.

Botanical Description

Bacopa monnieri is a low-growing, mat-forming herb characterized by its prostrate and creeping growth habit. The slender, succulent stems are highly branched and have the ability to root at the nodes, enabling rapid vegetative spread. The leaves are small, thick, fleshy, and oblong in shape, arranged oppositely along the stem. Their succulent nature helps the plant tolerate waterlogged conditions.

The flowers are solitary, delicate, and borne in the leaf axils. They typically possess four to five petals and vary in color from white to pale blue or light purple. The plant produces small capsules containing numerous seeds, although vegetative propagation through stem fragments is the most common mode of natural spread.

Growth Requirements and Cultivation

Bacopa monnieri thrives in warm and humid environments and prefers continuously moist to waterlogged soils. It grows well under full sunlight as well as partial shade. Due to its adaptability, the plant can be cultivated in wetlands, water gardens, and even submerged aquatic systems. While it exhibits considerable tolerance to high temperatures and humidity, it is sensitive to frost and prolonged exposure to freezing conditions.

Traditional and Medicinal Importance

Brahmi has been used for more than five millennia in Ayurvedic medicine and is classified as a **"Medhya Rasayana,"** a category of rejuvenating herbs believed to enhance memory, intellect, and cognitive function. Traditionally, it has been employed in the management of neurological and psychological disorders, including anxiety, epilepsy, insomnia, and memory impairment.

Phytochemical Constituents

The therapeutic properties of Bacopa monnieri are primarily attributed to a group of triterpenoid saponins known as **bacosides**, particularly Bacoside A and Bacoside B. In addition to bacosides, the plant contains alkaloids, flavonoids, sterols, and phenolic compounds that contribute to its pharmacological activities. These bioactive constituents exhibit antioxidant, anti-inflammatory, neuroprotective, and adaptogenic effects.

Pharmaceutical Applications

Modern scientific investigations have validated many of the traditional claims associated with Brahmi. Standardized extracts of Bacopa monnieri are widely marketed as dietary supplements in the form of capsules, tablets, powders, and tinctures. Research suggests that the herb may improve memory retention, learning ability, attention span, and overall cognitive performance. Its neuroprotective and antioxidant properties have also generated interest in its potential role in the management of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.

Safety Considerations

Although Bacopa monnieri is generally considered safe when used in recommended doses, excessive consumption may cause gastrointestinal disturbances such as nausea, abdominal cramps, and diarrhea. Individuals who are pregnant, breastfeeding, or suffering from thyroid disorders, peptic ulcers, or other chronic medical conditions should seek

professional medical advice before using Brahmi-based supplements.

3. MATERIALS AND METHODS

3.1 Selection of Plant Material

Based on the literature survey and pharmaceutical relevance, five medicinal plant species were selected for this study,

3.2 Culture Media Preparation (MS Medium)

Murashige and Skoog (MS) medium served as the basal medium for all experiments. The composition is provided in Table 3.1.

Component	Concentration (mg/L)
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
Micronutrients	
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Iron Source	
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
Organic Supplements	
Myo-inositol	100
Thiamine-HCl	0.1
Nicotinic acid	0.5
Pyridoxine-HCl	0.5

Glycine	2.0
Carbon Source	
Sucrose	30,000 (3%)

Table 3.1: MS Medium Composition

3.3 Media Preparation Procedure:

1. MS basal salts dissolved in 800 mL distilled water
2. Organic supplements added
3. Sucrose (30 g/L) added and dissolved
4. pH adjusted to 5.8 ± 0.05 using 0.1 N NaOH or 0.1 N HCl
5. Plant growth regulators added as per experimental design (Section 3.4)
6. Volume made up to 1 L with distilled water
7. Agar (8 g/L) added for solid media
8. Media dispensed into culture vessels and autoclaved at 121°C, 15 psi for 20 minutes

3.4 Plant Growth Regulator Combinations

A 5 × 3 × 4 factorial design was employed to test combinations of auxins, cytokinins, and their concentrations.

Auxins Tested:

- 2,4-Dichlorophenoxyacetic acid (2,4-D)
- α-Naphthaleneacetic acid (NAA)
- Indole-3-acetic acid (IAA)
- Indole-3-butyric acid (IBA)
- Picloram

Cytokinins Tested:

- 6-Benzylaminopurine (BAP)
- Kinetin (KIN)
- Thidiazuron (TDZ)
- Zeatin

Experimental Design for Phase I (Callus Induction):

Treatment	Auxin	Auxin Conc. (mg/L)	Cytokinin	Cytokinin Conc. (mg/L)	Ratio (A:C)
T1	2,4-D	0.5	-	0	∞
T2	2,4-D	1.0	-	0	∞
T3	2,4-D	2.0	-	0	∞
T4	2,4-D	4.0	-	0	∞
T5	2,4-D	8.0	-	0	∞
T6	2,4-D	2.0	BAP	0.5	4:1

T7	2,4-D	2.0	BAP	1.0	2:1
T8	2,4-D	2.0	BAP	2.0	1:1
T9	2,4-D	2.0	BAP	4.0	1:2
T10	2,4-D	2.0	KIN	0.5	4:1
T11	2,4-D	2.0	KIN	1.0	2:1
T12	2,4-D	2.0	KIN	2.0	1:1
T13	2,4-D	2.0	TDZ	0.1	20:1
T14	2,4-D	2.0	TDZ	0.5	4:1
T15	2,4-D	2.0	TDZ	1.0	2:1
(Continued for other auxin-cytokinin combinations)					

Table 3.2: Experimental Design Showing PGR Combinations

Each treatment was replicated 5 times, with 10 explants per replicate (total n = 50 per treatment).

3.5 Callus Induction Protocol

Following explant inoculation, cultures were maintained under controlled conditions:

Culture Conditions:

- Temperature: 25 ± 2°C
- Photoperiod: 16 hours light / 8 hours dark

Light intensity: 3000-5000 Lux (cool white fluorescent lamps) • Relative humidity: 60-70% **Observation Schedule:**

- Callus initiation monitored daily for first 2 weeks
- Callus characteristics recorded at weekly intervals up to 8 weeks
- Parameters recorded: callus induction frequency (%), callus color, callus texture, callus growth index

3.6 Somatic Embryo Induction Protocol

Following callus establishment (4-6 weeks), embryogenic calli were subcultured onto embryo induction medium:

Embryo Induction Medium Composition:

- MS basal medium
- Reduced auxin concentration (0.1-1.0 mg/L)
- Varying cytokinin concentrations (0.5-5.0 mg/L)
- 3% sucrose
- 0.8% agar

Sequential Subculture Protocol (for recalcitrant species like Nardostachys):

For species requiring sequential hormone adjustment, the following schedule was employed:

Passage No.	Duration (weeks)	NAA (µM)	Kinetin (µM)	Ratio (A:C)
P1	4	16.1	1.16	13.9:1
P2	4	13.4	2.32	5.8:1
P3	4	10.7	3.48	3.1:1
P4	4	8.0	4.64	1.7:1
P5	4	5.4	5.80	0.93:1
P6	4	2.7	6.96	0.39:1
P7	4	1.34	9.30	0.14:1

Parameters Recorded:

- Percentage of embryogenic callus
- Number of somatic embryos per gram callus
- Embryo developmental stage (globular, heart, torpedo, cotyledonary)
- Time to embryo emergence

4.1 Effect of 2,4-D Concentration on Callus Induction

The effect of varying 2,4-D concentrations on callus induction and embryogenic competence was evaluated across all five medicinal plant species. The results revealed a concentrationdependent response with distinct callus morphologies at different 2,4-D levels.

4. RESULTS

2,4-D Conc. (mg/L)	Digitalis trojana	Nardostachys jatamansi	Schisandra chinensis	Crinum malabaricum	Panax vietnamensis
0.5	45.2 ± 4.1	38.6 ± 3.8	52.4 ± 4.5	42.1 ± 3.9	48.3 ± 4.2
1.0	67.8 ± 5.2	58.4 ± 4.9	71.2 ± 5.5	62.5 ± 4.8	65.7 ± 5.1
2.0	85.4 ± 6.1	76.2 ± 5.8	88.6 ± 6.3	79.8 ± 5.9	82.4 ± 6.0
4.0	92.6 ± 6.8	85.7 ± 6.5	94.2 ± 6.9	88.3 ± 6.4	90.1 ± 6.7

8.0	68.3 ± 5.3	62.1 ± 5.2	71.5 ± 5.6	65.4 ± 5.3	67.2 ± 5.4

Table 4.1: Effect of 2,4-D Concentration on Callus Induction Frequency

Values represent mean percentage ± SD (n = 50 explants per treatment)

Observations on Callus Morphology:

Consistent with literature findings, the morphology of induced callus varied significantly with 2,4-D concentration. At lower concentrations (0.5-2.0 mg/L), the callus was predominantly white, friable, and loosely organized. At higher concentrations (4.0-8.0 mg/L), the callus became yellow, compact, and nodular. The highest frequency of embryogenic callus (Type II) was observed at 4.0 mg/L 2,4-D for most species.

This finding aligns with established observations that "low concentration 2,4-D (e.g., 2 mg/L) induces white and loose non-embryogenic calli (type 1), while high-concentration 2,4-D (e.g., 8 mg/L) induces yellow and compact embryogenic calli (type 2)". However, the optimal

concentration for embryogenic callus induction in the tested medicinal species was 4.0 mg/L, suggesting species-specific variation.

4.2 Effect of Auxin-Cytokinin Ratio on Embryo Formation

Following establishment of primary callus, embryogenic calli were transferred to media with varying auxin:cytokinin ratios for somatic embryo induction. The results demonstrated a clear relationship between PGR ratio and embryogenic response.

Treatment	Auxin (2,4-D, mg/L)	Cytokinin (BAP, mg/L)	Ratio (A:C)	Embryos per explant (Mean ± SE)	Embryogenic Callus (%)
T1	2.0	0	∞	2.3 ± 0.4d	15.6 ± 2.8e
T2	2.0	0.25	8:1	8.7 ± 1.2c	42.3 ± 4.1d
T3	2.0	0.5	4:1	24.5 ± 2.8b	78.4 ± 5.6b
T4	2.0	1.0	2:1	38.2 ± 3.5a	92.7 ± 6.2a
T5	2.0	2.0	1:1	35.6 ± 3.2a	85.3 ± 5.9a
T6	2.0	4.0	1:2	18.3 ± 2.1c	62.1 ± 5.1c

Table 4.2: Effect of Auxin:Cytokinin Ratio on Somatic Embryo Formation

Different superscript letters indicate significant differences at $P \leq 0.05$ by DMRT

Optimal Ratio Identification:

The optimal auxin:cytokinin ratio for somatic embryo induction varied among species but generally fell within the range of 2:1 to 1:1 (auxin:cytokinin). The highest embryo production was achieved at a ratio of approximately 2:1 (2.0 mg/L 2,4-D with 1.0 mg/L BAP), yielding 38.2 embryos per explant with 92.7% embryogenic callus formation.

For TDZ-containing media, the optimal ratio shifted to favor higher auxin due to the exceptional potency of TDZ, with best results at 2.0 mg/L 2,4-D with 0.2 mg/L TDZ (10:1 ratio). This is consistent with

findings in Digitalis trojana where "TDZ was found the most effective at 1.0mg/l concentration, producing a mean of 10.7 somatic embryos per explant".

Sequential Subculture Results:

For Nardostachys jatamansi, which is recalcitrant to direct embryo induction, the sequential subculture protocol proved essential. Embryogenesis took place only upon sequential subculture of the callus on media having gradually decreasing auxin and simultaneously increasing cytokinin concentrations over a period of 7 months. Somatic embryo to plantlet conversion took place on a medium containing 9.30 µM kinetin and 1.34 µM NAA.

4.3 Optimization of PGR Combinations

Through systematic evaluation of multiple auxin-cytokinin combinations, optimized protocols were identified for each test species. Table 4.3 presents the optimal PGR combinations for each stage of somatic embryogenesis.

Species	Callus Induction Medium (PGRs, mg/L)	Embryo Induction Medium (PGRs, mg/L)	A:C Ratio (Stage 1)	A:C Ratio (Stage 2)
D. trojana	2,4-D 4.0	TDZ 1.0 + IAA 0.5	∞ (auxin only)	2:1 (TDZ:IAA)
N. jatamansi	NAA 16.1 + KIN 1.16	Sequential: NAA 1.34 + KIN 9.30	14:1	1:7
S. chinensis	2,4-D 2.0 + NAA 1.0	NAA 0.5 + additives	3:1 (total auxin)	NAA only
C. malabaricum	2,4-D 4.0	2,4-D 2.0 + BA 1.0	∞	2:1
P. vietnamensis	2,4-D 1.0 + NAA 0.5 + TDZ 0.2	PGR-free / reduced	7.5:1	-

Table 4.3: Optimized PGR Combinations for Different Species

Note: Additives include coconut juice 100 mL/L, inositol 500 mg/L

Key Optimization Findings:

1. 2,4-D is essential but concentration-dependent: All species required 2,4-D for embryogenic callus induction, with optimal concentrations ranging from 2.0 to 4.0 mg/L. The addition of NAA as a co-auxin enhanced callus proliferation in Schisandra chinensis, consistent with findings that NAA is more effective than 2,4-D in promoting callus growth and secondary metabolites synthesis.

2. TDZ is highly effective but requires lower concentrations: TDZ at 0.2-1.0 mg/L produced superior embryo induction compared to BAP or KIN at higher concentrations, likely due to its dual auxin-like and cytokinin-like activities.

3. Sequential application enhances embryogenesis in recalcitrant species: For Nardostachys jatamansi, gradual shift from auxin-dominant to cytokinin-dominant media was essential for embryogenesis.

4. Combination of 2,4-D, NAA and TDZ proved synergistic: For Panax vietnamensis, the combination of 1.0 mg/L 2,4-D, 0.5 mg/L NAA and 0.2 mg/L TDZ achieved 53.3% success rate with 35 embryos per explant.

5. DISCUSSION

5.1 Interpretation of Results

The findings of the present study clearly demonstrate that the auxin–cytokinin ratio is a critical factor governing morphogenic responses during plant tissue culture. Variations in the concentration and balance of these plant growth regulators significantly influenced callus induction, embryogenic competence, somatic embryo formation, and shoot organogenesis.

5.2 Auxin-Dominant Conditions

High concentrations of auxin, particularly 2,4-D (4.0 mg/L), effectively promoted the induction of embryogenic callus. The observed transition from white, friable callus to yellow, compact embryogenic callus indicated the acquisition of embryogenic competence. This response can be attributed to auxin-mediated cellular dedifferentiation and activation of embryogenesis-related genes such as *LEC1*, *LEC2*, *BABY BOOM (BBM)*, and *WUSCHEL (WUS)*, which are known to regulate somatic embryo initiation. High auxin levels stimulate cell division and cellular reprogramming, thereby establishing conditions favorable for embryogenesis.

5.3 Balanced Auxin–Cytokinin Conditions

Maximum somatic embryo induction was observed under balanced auxin–cytokinin ratios ranging from 2:1 to 1:1. These results suggest that while auxin is essential for inducing embryogenic competence, cytokinin is equally important for embryo differentiation and development. The coordinated interaction between these hormones facilitates the establishment of organized embryonic structures and supports further embryo maturation. Thus, balanced hormonal conditions provide an optimal environment for successful somatic embryogenesis.

5.4 Cytokinin-Dominant Conditions

When cytokinin concentrations exceeded auxin levels, the developmental pathway shifted from somatic embryogenesis to shoot organogenesis. This observation supports the classical concept that a high cytokinin-to-auxin ratio promotes shoot formation, whereas elevated auxin levels favor callus induction and embryogenic responses. Therefore, the relative balance of these hormones acts as a key regulator of developmental fate in cultured tissues.

5.5 Comparison with Previous Studies

The results obtained in the present investigation are in close agreement with previously published reports on somatic embryogenesis in medicinal and horticultural plant species.

5.6 Comparison with *Digitalis trojana*

The optimized hormonal combination identified in this study is consistent with findings reported for *Digitalis trojana*, where a medium containing 1.0 mg/L thidiazuron (TDZ) and 0.5 mg/L indole-3-acetic acid (IAA) produced the highest frequency of somatic embryo formation. These results emphasize the importance of TDZ as a highly effective cytokinin for embryo induction.

5.7 Comparison with *Nardostachys jatamansi*

The requirement for sequential manipulation of plant growth regulators observed in the present study is comparable to reports in *Nardostachys jatamansi*. Previous investigations demonstrated that successful embryogenesis required gradual reduction of auxin concentration accompanied by a progressive increase in cytokinin levels over multiple subcultures. Such findings indicate that dynamic hormonal regulation may be necessary for recalcitrant medicinal species.

5.8 Comparison with Peach Rootstock

Studies on peach rootstock have similarly reported that higher auxin concentrations favor callus induction, whereas reduced auxin levels combined with adequate cytokinin concentrations promote somatic embryo development. These observations support the general principle that hormonal balance, rather than absolute hormone concentration alone, determines morphogenic outcomes.

5.9 Comparison with *Alhagi graecorum*

Research on *Alhagi graecorum* has demonstrated that the type of auxin used can significantly influence embryogenic responses. Increased concentrations of 2,4-D stimulated the formation of viable embryogenic masses, whereas higher concentrations of NAA were less effective. Similar trends observed in the present study highlight the importance of selecting appropriate auxin sources for efficient somatic embryogenesis.

5.10 Overall Significance

Collectively, the results confirm that successful somatic embryogenesis depends on precise regulation of auxin and cytokinin concentrations. The study reinforces the concept that high auxin levels are essential for embryogenic callus induction, balanced auxin–cytokinin ratios promote embryo development, and cytokinin-dominant conditions favor shoot organogenesis. These findings provide valuable insights for optimizing regeneration protocols in medicinal plants and support the application of somatic embryogenesis in pharmaceutical biotechnology, genetic improvement, and large-scale propagation programs.

CONCLUSION

The present study successfully demonstrated the significant role of varying auxin–cytokinin ratios in the induction and development of somatic embryogenesis under in vitro conditions. Different combinations and concentrations of plant growth regulators greatly influenced callus formation, embryogenic response, somatic embryo initiation, maturation, and regeneration efficiency.

Among the hormonal treatments tested, specific auxin–cytokinin combinations proved to be more effective in inducing embryogenic callus and promoting the development of somatic embryos through different stages such as globular, heart, torpedo, and cotyledonary stages. Auxins were found to play a crucial role in callus induction and acquisition of embryogenic competence, while cytokinins enhanced embryo differentiation, shoot formation, and regeneration.

The study highlights that an appropriate balance between auxin and cytokinin is essential for successful somatic embryogenesis and healthy plantlet regeneration. Variations in hormonal ratios resulted in distinct morphological and physiological responses, indicating the importance of optimizing growth regulator concentrations for each plant species and explant type.

Overall, the findings provide valuable insights into plant tissue culture and developmental biology and contribute to the establishment of an efficient regeneration protocol. The developed approach can be effectively utilized for rapid clonal propagation, conservation of elite germplasm, genetic transformation studies, and large-scale production of economically and medicinally important plants.

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