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Research Article

Induction, Proliferation and Differentiation of Callus in *Paris polyphylla* Sm. through Leaf Culture

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Abstract

Paris polyphylla Sm. is a vulnerable medicinal plant employed in the treatment of various ailments. This study seeks to establish a protocol for callus induction, proliferation, and differentiation of *P. polyphylla*. Immature leaf explants were cultured on MS medium with varying concentrations of plant growth regulators (PGRs), including 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn), 6-benzylaminopurine (BAP), Thidiazuron (TDZ), α-Naphthalene acetic acid (NAA), and Gibberellic acid (GA_3), along with 10% coconut water. After 12 weeks of primary culture, the optimal callus induction was observed in MS medium supplemented with 0.25 mg/l 2,4-D + 0.5 mg/l Kn. In the secondary culture at 8 weeks, the best callus proliferation, as determined by callus weight or growth index, occurred in MS medium supplemented with 2.0 mg/l BAP alone, 2.0 mg/l Kn alone, 1.0 mg/l TDZ alone, combinations of 2.0 mg/l Kn + 1.0 mg/l BAP + 2.0 mg/l GA_3, and combinations of 0.5 mg/l NAA + 2.0 mg/l BAP + 2.0 mg/l GA_3, as well as 10% coconut water. Furthermore, callus differentiation into mini rhizomes with root primordia was successfully achieved in MS media containing 2.5 mg/l Kn and 10% coconut water. This study reports, for the first time, the formation and differentiation of callus from leaf explants in *P. polyphylla*. Large-scale callus generation from leaf explants has the potential to enhance the production of bioactive secondary metabolites for therapeutic purposes and facilitate the development of plantlets through organogenesis.

Keywords: 2,4-D, Callus, GA_3, Growth index, Primary culture

Introduction

Paris polyphylla Sm., commonly known as Paris root in English, Rhizoma Paridis in Pharmacopoeia, and Satuwa in Nepali, is a vulnerable medicinal plant found in the subtropical to subalpine forests of South Asian countries at altitudes ranging from 1800 to 3500 meters above sea level (IUCN, 2004). This plant is extensively utilized in traditional ethnomedicine, traditional Chinese medicine (TCM), Ayurveda, and Homeopathy for treating a wide array of conditions such as cuts, wounds, burns, fever, anthelmintic, scabies, diarrhoea, dysentery, liver cancer, antipyretic, pain relief, anti-inflammatory, coughing and purgative, breast cancer, fractures, convulsions, and strains, acting as

an antidote, detoxicant, and soothing agent (Long et al., 2003; IUCN, 2004; Li et al., 2012). Steroidal saponin is the primary constituent of *P. polyphylla*, consisting of polyphyllin D, diosgenin, pennogenin, dioscin, and Paris saponin I, II, VI, VII, H, and polyphyllin VII (Lee et al., 2005; Zhang et al., 2014; Chen et al., 2019; Wang et al., 2019; Thapa et al., 2022).

Unfortunately, due to overutilization, habitat loss, and illegal trading for pharmaceutical and conventional medical purposes, the population of *P. polyphylla* is diminishing in its natural habitats. The process of developing callus tissue from plant cells in a nutrient medium with the use of plant growth regulators (PGRs) is known as in-vitro callus induction. The concentration of PGRs in the medium can be altered to guide the callus progression towards root formation, shoot growth, or somatic embryogenesis. Organ primordia corresponding to the callus tissues are then formed after additional cell proliferation and differentiation, contributing to the regeneration of complete plantlets and the production of bioactive compounds using precursors and elicitors. Callus culture is widely employed in both basic research and industrial applications, aiding in the establishment of cell suspensions, protoplast separation, extraction of bioactive compounds, and synthesis of significant pharmaceuticals in sizable quantities (Jhang et al., 1974; Furuya et al., 1983; Pant, 2014).

The induction of callus using various explants in culture is valuable for regenerating complete plants through organogenesis or embryogenesis, as well as for producing essential bioactive compounds. Additionally, the use of precursors and elicitors, along with chemical analysis of callus, contributes to laying the foundation for the production of natural drugs from in vitro-raised callus.

While existing literature discusses plant regeneration from rhizomes and other parts of *P. polyphylla* (Teerawatsakul et al., 2014; Raomai et al., 2014; Raomai et al., 2015; Devi et al., 2017), there is no information regarding callus induction and plant regeneration from the leaf. This study aims to fill this gap by investigating the process of callus induction, proliferation, and differentiation in MS media supplemented with various PGRs at different concentrations and combinations, utilizing leaf explants.

Materials and Methods

Plant material

P. polyphylla plants sourced from the Baglung district in western Nepal were collected in April and May of 2021 and potted. The herbarium specimen was documented, identified, and stored in the Tribhuvan University Central Herbarium (TUCH).

Preparation of explants

Immature leaves were carefully detached from the mother plants and thoroughly cleaned under running tap water for one and a half hours with the addition of a few drops of Tween-20 (Qualigens). This cleaning process was repeated three times using distilled water. The explants underwent surface sterilization with 70% ethanol for 30 seconds, followed by immersion in a solution containing 0.1% mercuric chloride ($HgCl_2$) for 3–4 minutes. To eliminate any $HgCl_2$ residue, the explants were washed three times with sterile distilled water. Using a sterile cork borer on the sterilized leaves, leaf discs (0.8–1.0 cm diameter) were created through gentle pressing.

Preparation of culture medium

Stock solutions were utilized to formulate Murashige and Skoog's (MS) medium (1962) for all leaf explant tissue cultures. The MS media were enriched with varying concentrations of plant growth regulators (PGRs), such as 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25, 0.5, 1.0, 1.5, and 2.0 mg/l) and kinetin (Kn) (0.25, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) individually and in combinations. The primary culture included 3% (w/v) sucrose, 10% coconut water (v/v), and 0.8% (w/v) agar. The pH was adjusted to 5.6 before autoclaving. For the secondary culture, Kn, BAP, and TDZ (0.5, 1.0, 1.5, and 2.0 mg/l) were added to MS medium separately, in combination with BAP, NAA, and GA3 (0.5, 1.0, 1.5, and 2.0 mg/l), and in combination with BAP, Kn, and GA_3 (0.5, 1.0, 1.5, and 2.0 mg/l). GA_3 was added at a constant concentration (2.0 mg/l) in MS media. Controls included full, 1/2, and 1/4 strengths of MS media without added PGR supplements and 10% coconut water. Approximately 25 mL of medium were dispensed into 300 mL sterile autoclaved culture jars (78 mm×122 mm) or 15 ml of medium in sterile culture tubes (25 mm×150 mm) for culture

initiation, covered with aluminum foil, and autoclaved at 121°C for 30 minutes under a pressure of 15 lb per square inch.

Culture establishment

Aseptically, small leaf discs (0.8–1.0 cm in diameter) were inoculated onto the MS medium. Following a 12-week primary culture, the regenerated callus was subcultured on MS medium. Calli for subculture were weighed in the airflow chamber to prevent contamination. The culture tubes and dishes were then transferred to the culture room and maintained at 25±2°C with 16-hour photoperiods. The experiment was conducted three times, with each treatment having three replicates. Eight-week-old calli were collected, and their growth index (%) and moisture contents (%) were determined according to Adhikari & Pant (2013) for further analysis.

"The moisture content of callus (%) = (Fresh callus wt. - Dry callus wt.) / Fresh callus wt. × 100"

"(Growth index) Increase in callus wt. (%) = (Fresh callus wt. - Fresh callus explant wt.) / Fresh callus explant wt. × 100"

Statistical analysis

Microsoft Excel 2010 was employed to calculate the percentages of moisture content and callus growth index, along with their average values and standard deviation. Additionally, the two-way ANOVA test was conducted in Microsoft Excel 2010.

Results and Discussion

Callus induction in MS medium

Inoculation of leaf discs onto MS media containing varying concentrations of 2,4-D and Kn (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/l) along with 10% coconut water resulted in the induction of callus (Figure 1). As stated by Trigiano & Gray (2000), many tissues in plant tissue culture require a specific combination of plant growth regulators (PGRs) to initiate the appropriate growth response. Notably, MS media at full, half, and quarter strength without PGR supplements and 10% coconut water failed to induce callus formation. Similarly, employing different concentrations of Kn alone, 2,4-D alone, and a combination of NAA+Kn in the MS medium did not induce callus in *P. polyphylla*. Soniya and Das (2002) found that kinetin alone in MS medium did not induce callus in *Piper longum* leaf explants, but when enriched with 2,4-D+Kn, callus formation from leaf explants was successful. While kinetin is not always essential, exogenous application of auxin is crucial for initiating callus development (Okazawa et al., 1967). In tissue culture, kinetin is often used to induce callus production (in combination with auxin) and to develop shoots from the callus (with reduced auxin concentrations) (Duszka et al., 2009).

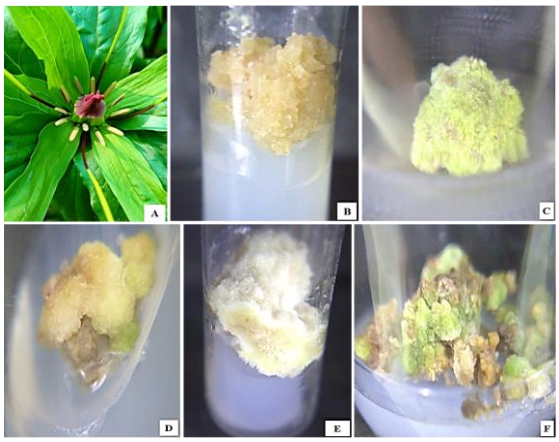

Figure 1: Induction of callus from leaf explant. Plant: *Paris polyphylla* (A), Callus: MS+0.25 mg/l 2,4-D+0.5 mg/l Kn (B), MS +2.0 mg/l Kn (C), MS+1.0 mg/l TDZ (D), MS+2.0 mg/l BAP+0.5 mg/l NAA (E), MS+1.0 mg/l BAP+2.0 mg/l Kn (F).

The most effective callus-inducing media in this study were MS media fortified with 0.25 mg/l 2,4-D+0.5 mg/l Kn (85%), followed by 0.25 mg/l 2,4-D+0.25 mg/l Kn (70%), 0.25 mg/l 2,4-D+1.0 mg/l Kn (60%), 0.25 mg/l 2,4-D+1.5 mg/l Kn (55%), and 0.5 mg/l 2,4-D+0.26 mg/l Kn (50%) after 12 weeks of initial culture (primary culture) (Table 1). The induction of callus was favoured when the concentrations of 2,4-D and Kn were nearly equal or when the Kn concentration exceeded the 2,4-D concentration. In nature, calli form on plants due to wounds, tumour-inducing bacteria (the Ti gene), and genetic tumours (Bhatiya, 2015). However, callus can also be artificially induced using a leaf or another explant and the appropriate nutritive medium. Generally, auxin alone or in combination with cytokinin induces callus formation in various plant species. The ratio of auxin to cytokinin determines callus induction (at an intermediate ratio), root development (at a high auxin to cytokinin ratio), and shoot regeneration (at a high cytokinin to

auxin ratio). In some species, callus induction may occur without auxin or cytokinin, as abscisic acid and brassinosteroid hormones can induce it (Goren et al., 1979; Hu et al., 2000).

This study also revealed that callus induction and growth initially increased, then decreased, as Kn concentration was raised from 0.25 mg/l to 1.0 mg/l in MS media with a constant 0.25 mg/l of 2,4-D (Table 1). Similarly, callus induction and growth decreased as 2,4-D concentrations in MS media increased from 0.25 mg/l to 1.5 mg/l, while Kn concentration remained constant. All calli were compact and light white in the primary culture. The morphology of the callus changed from compact to friable when subcultured in the same concentrations of 2,4-D and Kn. However, when subcultured in cytokinins alone, such as BAP, Kn, and TDZ, the callus morphology shifted from friable to more compact and green. This change may indicate the initiation of organogenesis in the callus. Previous researchers successfully induced callus from leaf segments in MS media fortified with 2,4-D alone, NAA alone, indole-3-butyric acid (IBA) alone, or a combination of 2,4-D+Kn in *Melaleuca alternifolia* (Kiong et al., 2007), a combination of IAA+BAP in *Piper longum* (Sathelly et al., 2016), a combination of 2,4-D+KN in *Piper auritum* (Domínguez et al., 2006), and a combination of NAA+BAP in *Bergenia ciliata* (Shrestha & Pant, 2011). The cumulative data indicate that callus induction from leaf explants in MS media enriched with auxins, either alone or in combination with cytokinins, is genotype-dependent. Additionally, callus induction within a plant species is influenced by factors such as explant type, orientation, plant growth regulators, plant and explant age, medium composition, plant metabolic state, temperature, growing conditions, and source plant type (Klimek-Chodacka et al., 2020).

Table 1: Induction of callus in different concentrations of Kinetin and 2,4-Dichlorophenoxy acetic acid.

Concentration of 2,4-D (mg/l)	Concentration of Kinetin (mg/l)					
	0.25	0.5	1.0	1.5	2.0	3.0
0.25	L	L	L	M	M	S
0.5	M	M	M	M	M	-
1.0	M	M	S	S	S	-
1.5	S	S	S	S	-	-
2.0	-	-	-	-	-	-

S=Small quantity (fresh wt. <0.5 g), M=Moderate quantity (fresh wt. 0.5-2.0 g), L=Large quantity (fresh wt. >2.0 g)

Callus proliferation in MS medium

The calli derived from MS medium, containing a combination of 2,4-D+Kn and 10% coconut water, were maintained by subculturing in the same concentration of 2,4-D and Kn. Subsequently, the calli were further subcultured in MS medium supplemented with different concentrations of BAP, Kn and TDZ alone, and in combinations such as NAA+BAP+GA_3, BAP+Kn+GA_3, and 10% coconut water. The proliferation of callus increased with the elevated concentrations of all PGRs, including BAP, Kn and TDZ alone, in the MS medium. Although callus proliferation occurred in all concentrations and combinations of PGRs, the most effective proliferation was observed in MS medium enriched with 2.0 mg/l Kn alone, as evidenced by the calli's fresh weight and dry weight after 8 weeks of subculture (Table 2). At 2.0 mg/l of Kn, there was a remarkable 647.33% increase in callus growth (growth index), with the initial fresh weight of the callus explant at 0.33 g yielding 6.52 g of callus. Similarly, it was observed that callus developed in MS medium in combination with BAP+Kn+GA_3 exhibited a higher growth index compared to callus grown in MS medium in combination with NAA+BAP+GA_3 (Tables 3 and 4). The maximum callus growth index (589.03%) was observed in 1.0 mg/l BAP + 2.0 mg/l Kn + 2.0 mg/l GA_3 from an initial callus explant weight of 0.35 g (Table 4), while the maximum callus growth index (570.58%) was observed in 2.0 mg/l BAP + 0.5 mg/l NAA + 2.0 mg/l GA_3 from an initial callus explant weight of 0.37 (Table 3). This suggests that the synergistic action of two cytokinins, BAP and Kn, played a role in promoting callus proliferation and development.

Furthermore, callus proliferation and development decreased when NAA (auxin) concentrations were increased from 0.5 mg/l to 2.0 mg/l while BAP (cytokinin) concentration remained constant (Table 3). This indicates that a higher concentration of auxins combined with cytokinins is not favourable for callus proliferation. Similarly, when the concentrations of BAP and Kn were increased, the proliferation of calli grown in MS medium supplemented with BAP+Kn+GA_3 increased in terms of fresh weight or growth index but slightly decreased after reaching a concentration of 1.0 mg/l Kn (Table 4). This may be attributed to the saturation concentration of BAP+Kn for callus proliferation.

The callus morphology underwent changes after subcultures, transitioning from slightly compact light white to compact white, compact yellowish, and compact green. Calli produced in combination with cytokinin and auxin (BAP+NAA) exhibited compact light yellow and compact white morphologies, whereas calli in MS media treated with cytokinins, either alone or in combinations, displayed compact light green and compact white morphologies. Genetic variability in the callus, metabolite release, and PGR content in the media could be contributing factors to the alteration of callus morphology following sub-culture. In tissue culture, explants with different genotypes respond diversely to the same growth conditions (Nehara et al., 1989, 1990), and PGRs influence the synthesis of metabolites, organogenesis, and cell development (Liang et al., 1991). The results of the two-way ANOVA test, examining the p-values of plant growth regulators such as BAP, Kn and TDZ alone at different concentrations (0.5, 1.0, 1.5, and 2.0 mg/l) versus callus growth (g) or growth index, demonstrated a significant difference at the <0.005 level ($p = 7.91\times e\text{-}26$ and $6.55\times e\text{-}16$). This indicates that callus proliferation, in terms of fresh weight (g) or growth index, was dependent on the types of plant growth regulators used and their various concentrations added to the MS medium. The successful induction of callus in leaf explants was achieved with the addition of an organic supplement (10% coconut water) in the MS medium. In MS media treated with 2,4-D+Kn and 10% coconut water, callus induction occurred, while media without 10% coconut water did not induce callus. According to Gnasekaran et al. (2009), coconut water stimulates cell division and growth in culture by acting as a cytokinin-like plant growth regulator and containing various nutritional and hormonal components.

Callus differentiation in MS medium

The callus, initially induced in MS medium enriched with 2,4-D and Kn, underwent subculturing in MS medium containing varying concentrations of Kn (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) along with 10% coconut water. In the presence of 2.5 mg/l Kn+10% coconut water, mini rhizomes with root primordia were successfully formed, and subsequent adventitious roots developed from these mini rhizomes (Figure 2). Kinetin, as a synthetic plant growth regulator, primarily facilitates shoot initiation and development. While rooting in the callus may have been initiated by the application of exogenous auxins (2,4-D) in combination with kinetin and 10% coconut water during the initial callus induction from the leaf explant. In tissue culture, the synthetic auxin 2,4-D is commonly used to stimulate somatic embryogenesis, but it is rarely employed commercially for root induction. Kinetin's role in supporting callus development suggests that the exogenous auxin-induced activation of callus growth is likely assisted by the inclusion of kinetin in the medium. Cytokinins like kinetin can induce the differentiation and regeneration of axillary buds (Le Bris, 2017). For roots to form in culture, the medium must contain a relatively low amount of auxin (Okazawa et al., 1967).

Table 2: Independent impacts of BAP, Kn, & TDZ on the proliferation and growth of callus following secondary culture.

BAP (mg/l)	Kn (mg/l)	TDZ (mg/l)	Callus explant fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5			0.33±0.03	1.19±0.14 CW	0.38±0.04	91.19
1.0			0.30±0.07	2.42±0.23 CW	0.88±0.12	229.65
1.5			0.30±0.01	3.71±0.13 CY	1.54±0.06	362.67
2.0			0.33±0.04	4.57±0.26 CG	2.10±0.16	449.95
	0.5		0.32±0.04	2.56±0.38 CY	0.79±0.09	243.98
	1.0		0.35±0.03	3.76±0.17 CW	1.30±0.06	366.98
	1.5		0.31±0.03	5.24±0.28 CG	2.15±0.08	518.42
	2.0		0.33±0.04	6.52±0.35 CG	2.96±0.15	647.33
		0.5	0.34±0.06	3.55±0.17 CW	1.12±0.04	345.22
		1.0	0.31±0.06	5.88±0.01 CG	2.83±0.09	582.30
		1.5	0.32±0.04	4.33±0.38 CG	1.04±0.16	425.77
		2.0	0.37±0.05	3.44±0.18 CY	0.83±0.07	334.77

CW: compact light white, CG: compact light green, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Table 3: Cumulative impacts of BAP+NAA+GA_3 on the proliferation and growth of callus following secondary culture (2.0 mg/l GA_3).

BAP (mg/l)	NAA (mg/l)	Callus explants fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5	0.5	0.33±0.07	1.96±0.67 CY	0.71±0.26	179.68
1.0	0.5	0.37±0.08	2.53±0.11 CW	0.98±0.01	238.59
1.5	0.5	0.36±0.06	2.85±0.07 CW	1.31±0.01	273.08
2.0	0.5	0.36±0.06	5.76±0.18 CW	2.74±0.05	570.58
0.5	1.0	0.34±0.06	2.69±0.22 CW	0.99±0.10	256.41
1.0	1.0	0.35±0.05	3.42±0.29 CY	1.47±0.02	332.54
1.5	1.0	0.37±0.04	3.73±0.10 CY	1.71±0.01	363.87
2.0	1.0	0.35±0.07	4.66±0.15 CW	2.33±0.10	458.68
0.5	1.5	0.39±0.03	1.30±0.06 CY	0.46±0.03	100.32
1.0	1.5	0.36±0.07	2.54±0.09 CW	1.01±0.01	240.66
1.5	1.5	0.37±0.03	2.79±0.02 CY	1.28±0.03	266.36
2.0	1.5	0.33±0.03	3.28±0.15 CY	1.38±0.27	318.41
0.5	2.0	0.35±0.03	1.01±0.11 CY	0.37±0.03	67.15
1.0	2.0	0.37±0.04	1.44±0.10 CY	0.56±0.05	118.55
1.5	2.0	0.35±0.03	2.43±0.10 CW	1.12±0.06	229.52
2.0	2.0	0.36±0.06	2.74±0.11 CW	1.30±0.08	261.13

CW: compact light white, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Table 4: Cumulative impacts of BAP+Kn+GA_3 on the proliferation and growth of callus following secondary culture (2.0 mg/l GA_3).

BAP (mg/l)	Kn (mg/l)	Callus explants fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5	0.5	0.37±0.04	2.09±0.13 CW	0.75±0.05	191.27
1.0	0.5	0.39±0.04	2.33±0.05 CW	0.93±0.03	216.46
1.5	0.5	0.33±0.05	2.40±0.02 CG	0.98±0.01	226.65
2.0	0.5	0.39±0.03	2.52±0.04 CG	1.26±0.02	236.33
0.5	1.0	0.37±0.08	2.62±0.04 CW	0.94±0.01	247.59
1.0	1.0	0.33±0.04	2.74±0.05 CY	1.13±0.02	262.73
1.5	1.0	0.35±0.05	2.82±0.05 CG	1.21±0.02	270.31
2.0	1.0	0.32±0.07	2.89±0.01 CG	1.33±0.02	278.59
0.5	1.5	0.35±0.07	3.21±0.03 CW	1.14±0.02	310.98
1.0	1.5	0.36±0.06	3.34±0.04 CY	1.35±0.04	323.26
1.5	1.5	0.36±0.06	3.59±0.01 CG	1.67±0.05	348.95
2.0	1.5	0.34±0.03	3.67±0.03 CY	1.81±0.02	358.19
0.5	2.0	0.45±0.20	5.18±0.06 CG	1.79±0.02	509.48
1.0	2.0	0.35±0.08	5.94±0.04 CG	2.84±0.13	589.03
1.5	2.0	0.36±0.05	5.80±0.01 CG	2.61±0.01	573.96
2.0	2.0	0.34±0.05	5.74±0.09 CY	2.74±0.17	568.63

CW: compact light white, CG: compact light green, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Figure 2: Various stages of mini rhizomes and root initiation in MS media supplemented with 2.5 mg/l Kn from callus.

Conclusion

In MS medium supplemented with 2,4-D+Kn and 10% coconut water, callus formation can be initiated from leaf segments. The most effective plant growth regulators for callus proliferation in MS medium are kinetin and the combination of BAP+Kn+GA_3, surpassing BAP and TDZ alone, and the combination of BAP+NAA+GA_3. Kinetin alone also supports the differentiation of callus into mini rhizomes with adventitious roots. While increased concentrations of BAP, TDZ and Kn alone, as well as the combination of BAP+Kn+GA_3 in MS medium, elevate callus proliferation, no distinct pattern of proliferation is observed following subculture in MS medium with NAA+BAP+GA_3. The protocol devised for in-vitro callus induction and growth from leaf segments, utilizing elicitors and precursors, holds promise for the synthesis and enhancement of crucial bioactive compounds. Furthermore, the callus can serve as a source for organogenesis and somatic embryogenesis, facilitating plant regeneration.

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