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Preserving Nature's Treasure: A Journey into the In Vitro Conservation and Micropropagation of the Endangered Medicinal Marvel—*Podophyllum hexandrum* Royle

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Abstract: *Podophyllum hexandrum* Royle, also known as *Podophyllum emodi* Wall, holds significant ecological, ornamental, and medicinal values. However, it has become endangered due to overexploitation, prolonged seed dormancy, slow natural regeneration, and climate change. This study developed an efficient in vitro protocol for callogenesis and micropropagation of *P. hexandrum* to conserve germplasm in in vitro conditions. Callus formation from various plant parts, including the leaf, stem, rhizome, radicle, and cotyledon, was induced using Murashige and Skoog (MS) medium supplemented with different plant growth regulators. The combination of benzyladenine at 1 mg L^{-1} and 4-dichlorophenoxy acetic acid at 3 mg L^{-1} was optimal for biomass production, yielding 215.88 ± 0.31 mg, with growth per gram at 8.32 ± 0.32 and a growth rate of 13.62 ± 0.25 mg/day on MS medium. For shoot proliferation, benzyladenine (3.5 mg L^{-1}) and naphthalene acetic acid (0.5 mg L^{-1}) combined with activated charcoal showed the highest shoot induction percentage per explant. For shoot regeneration from calluses, 6-benzylaminopurine (0.5 mg L^{-1}) and thidiazuron (2 mg L^{-1}) were most effective, producing superior shoot length, number of regenerations, and regeneration percentage. Root induction was successful with α-naphthalene acetic acid supplementation (0.5 to 1.5 mg L^{-1}) in MS medium, resulting in the highest number per explant (4.08 ± 0.08), length (5.45 ± 0.15 cm), and rooting rate (87.00 ± 1.66%) of roots in plantlets. Subculturing for callus culture was performed every 28 days for up to four subcultures to prevent nutrient depletion and toxic metabolite accumulation, ensuring tissue health and viability. Continuous subculturing of callus on MS medium maintained healthy *P. hexandrum* germplasm in vitro. Overall, this micropropagation protocol provides a rapid system for conserving *P. hexandrum* germplasm.

Keywords: germplasm; podophyllotoxin; callogenesis; organogenesis; micropropagation

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1. Introduction

Podophyllum hexandrum Royle, synonym *P. emodi* Wall, is an important ornamental and medicinal plant. *P. hexandrum* is cherished for its large, deeply lobed leaves, that resemble elegant umbrellas. These leaves emerge in spring, adding a lush and exotic appearance to shaded garden areas. These leaves, with their waxy texture and rich green color, span up to 12 inches (30 cm) in diameter, creating a striking focal point. In late spring to early summer, the plant produces charming nodding flowers with six white to pink petals, adding an element of mystery and allure beneath the large foliage [1]. Following the flowers, decorative fruit resembling miniature apples adorn the plant, enhancing its ornamental value, especially in fall when they ripen and add vibrant color to the landscape [2,3]. *P. hexandrum*'s ability to thrive in shaded environments makes it a valuable addition to shade gardens, where it can fill in under trees or alongside other shade-loving plants. Its low maintenance requirements and historical significance in traditional medicine practices further elevate its appeal among gardeners interested in ethnobotany or cultural gardening. Overall, the combination of its attractive foliage, charming flowers and fruit, shade tolerance, and cultural significance solidifies *P. hexandrum*'s status as an esteemed ornamental plant in garden landscapes [4].

P. hexandrum belongs to the well-known plant family Berberidaceae, thriving in the northern Himalayan regions, including various parts of Pakistan. Its distribution spans altitudes ranging from 2300 m to 3700 m above mean sea level [5,6]. *P. hexandrum* has been an immensely significant source of nutrition, medicinal substances, and other healthcare products since ancient times. It has been a revered Aindri (a divine medicine), dating back to prehistoric eras [7]. It contains various bioactive compounds, such as lignans, proteins, glycosides, flavonoids, saponins, and terpenes [8].

One of the most notable natural secondary metabolites found in *P. hexandrum* is podophyllotoxin (PTOX), or podophyllin. It is used in the treatment of various cancers [9]. Additionally, PTOX and its derivatives exhibit antimicrobial, antiviral, antifungal, antineoplastic, insecticidal, antiparasitic, and radioprotective properties. They are used in the treatment of leukemia and rheumatoid arthritis [10]. Podophyllotoxin, or podophyllin, can also be used as a hepatic stimulant, purgative, emetic, antipyretic, antiseptic for wound healing, and a strong analgesic. Additionally, PTOX exhibits strong antioxidant properties, helping to neutralize free radicals and reduce oxidative stress. It can reduce inflammation, modulate the immune system, improve digestion, and alleviate gastrointestinal issues such as bloating and indigestion [11]. Podophyllotoxin can be incorporated into skincare products for its healing and protective effects. It is useful in treating respiratory conditions by reducing congestion and soothing the respiratory tract and can relieve muscle spasms and cramps [12]. *P. hexandrum* has several interesting cultivars and variants, including *P. hexandrum* var. axillare, *P. hexandrum* var. bhootanense, *P. hexandrum* var. emodi, and *P. hexandrum* var. Jaeschkei. Among these, *P. hexandrum* var. Emodi (the Emodi variety) is known for its relatively higher PTOX content compared to other cultivars [5–7].

The wild *P. hexandrum* population has drastically declined in recent years due to overexploitation, habitat destruction, prolonged winter conditions, slow regeneration, and limited fruit production. Deforestation and land use changes further contribute to habitat loss and fragmentation [13]. Climate change triggers phenological mismatch, altering the plant life-cycle events of *P. hexandrum*. Factors like temperature, precipitation, and day length impact flowering, fruiting, and leafing [14]. Limited awareness and incentives among local communities and stakeholders lead to unsustainable exploitation and neglect of the plant's conservation status [15].

Multiple factors collectively drive the *P. hexandrum* species to endangered status. Studies report a population reduction of 68% to 97% in the Swat region of Pakistan, categorizing it as critically endangered [5,10,16]. Despite being internationally recognized as a rare plant on the IUCN Red List, excessive harvesting persists, contributing to its vulnerability. The absence of organized conservation plans and limited efforts in in vitro conservation further exacerbate the species' precarious status [6,17]. In vitro conservation methods, being rapid,

cost-effective, and season-independent, are invaluable for preserving species diversity, especially for threatened species [18,19]. To enhance genetic diversity and PTOX content, favoring breeding, selection, and biotechnological approaches is crucial for both ex situ and in situ conservation measures of *P. hexandrum* [15,20].

The in vitro culture of *P. hexandrum*, an endangered medicinal plant from Pakistan, is a challenging task. The in vitro culture of *P. hexandrum* faces several challenges, such as establishing initial cultures from explants, contamination, low germination rates, and a low frequency of explant regeneration. Inducing roots in regenerated shoots and acclimatizing them to ex vitro conditions requires finely tuned protocols to ensure high survival rates [5]. The plant also experiences somatic mutations or chromosomal abnormalities during successive subcultures, which can negatively affect plant quality. Scaling up plantlet production to meet conservation or commercial demands is resource intensive and requires efficient multiplication protocols [11]. Cell necrosis, browning, and eventual death also occur due to the release of secondary metabolites from the explants of *P. hexandrum*. Establishing and maintaining a tissue culture facility is costly and demands specialized equipment and skilled personnel. Additionally, *P. hexandrum* seeds do not germinate easily in laboratory conditions, complicating tissue culture initiation [15]. Overcoming seed dormancy is critical for successful propagation, as many plants exhibit poor responses to tested medium formulations, making it essential to find the right combination of growth regulators and nutrients [17,19].

Only a few studies have reported tissue culture with limited success for *P. hexandrum* [6,7,15,19–22]. The issues and limitations identified in these studies included high contamination rates, which made it difficult to maintain sterile cultures [13,15]. Additionally, there were challenges with low regeneration efficiency and slow growth rates, resulting in the inconsistent development of explants. Ensuring genetic stability was another significant concern, as somaclonal variations could affect the medicinal properties of the plant [20]. Optimizing the culture medium composition, including the balance of hormones and nutrients, proved complex and critical for successful propagation. Furthermore, developing a robust rooting system and acclimatizing plantlets to external conditions posed significant difficulties, often leading to high mortality rates during the transition from in vitro to ex vitro environments [21]. To address the urgent need for conservation and propagation, efficient methods, both conventional and in vitro, such as rooting of rhizome segments, seed germination, somatic embryogenesis, and micropropagation, must be developed. Successful tissue culture studies for *P. hexandrum* are limited, and protocols for callus cultures from mature explants are particularly lacking. Overexploitation and climate change necessitate immediate action, making plant tissue culture a crucial tool for achieving conservation goals. In view of the preceding discussion, the current study was designed, aiming to develop a cost-effective and efficient protocol for in vitro conservation and micropropagation of *P. hexandrum*.

2. Materials and Methods

2.1. Plant Material

The plant materials of *P. hexandrum* were collected from Saifullah Lake and Koz Lalko in the Swat district of Pakistan, using a random sampling approach. A total of 15–20 *P. hexandrum* plants were collected as representative samples for in vitro culture between May and September of 2021 and 2022 (Figure 1). The rationale for collecting plant samples of *P. hexandrum* between May and September 2021 and 2022 likely stems from the plant's growth cycle and seasonal variations. During these months, the availability of all plant parts such as stems, roots, leaves, flowers, and seeds is at its peak. This period typically coincides with the active growing season for many plants in temperate regions. During this time, plants are more vigorous and have higher metabolic activity, making it easier to obtain healthy and viable explants for in vitro culture [5,6].



Figure 1. *P. hexandrum* at flowering and fruiting stage, and seeds (photo taken at Saif Ullah Lake, 2021).

2.2. Sterilization of Plant Materials

Surface sterilization of seeds, leaf, stem, rhizome, radicle, and cotyledon was carried out by washing with running tap water to remove any external contaminants. The explants were then submerged in a 20% Tween-20 solution for 5 min, effectively eliminating the surface contaminants present. Following this, aqueous solutions of mercuric chloride ($HgCl_2$) at concentrations of 0.05%, 0.1%, and 0.2% were used, with each concentration applied for 5 min to the explants. After each sterilization treatment, the explants were rinsed three times with double-distilled water (ddH_2O) and gently dried by blotting before inoculation in an airflow chamber. These concentrations of mercuric chloride were tested to evaluate their effectiveness in preventing fungal and bacterial contamination, following a partially modified protocol based on Boruah et al. [22]. Mercuric chloride was utilized for seed germination and contamination percentage of *P. hexandrum*.

2.3. Isolation and Aseptic Transfer of Explants

Explants were inoculated inside a laminar flow chamber that was thoroughly cleaned with 70% ethanol. All the tissue culture tools and the prepared MS medium in flasks were autoclaved and were UV sterilized for 30 min. Following sterilization, explants were prepared and inoculated on the growth medium in 250–500 mL flasks and Petri dishes, following a partially modified protocol based on the work of Nadeem et al. [13].

2.4. In Vitro Conservation and Propagation of P. hexandrum

MS medium was used for in vitro propagation and conservation of *P. hexandrum* explants. Callus induction, indirect organogenesis (shooting and rooting) and direct organogenesis (shooting) were carried out using the respective growth substances as described below. Three replications were used for each combination with four explants per Petri dish and flask.

2.5. Callus Induction in Explants of P. hexandrum

Leaf, stem, rhizome, radicle, and cotyledon explants were used for callus induction with various growth hormones. The explants were cultured on MS media supplemented with different hormone combinations:

1. BA (0.5, 1.5, and 2.0 mg L^{-1}) + 2,4-D (1.0, 2.5, and 3.0 mg L^{-1})
2. KIN (0.2, 0.5, and 1.0 mg L^{-1}) + 2,4-D (1.0, 2.5, and 3.0 mg L^{-1})
3. BA (1.0, 1.5, and 2.5 mg L^{-1}) + NAA (0.5 and 1.0 mg L^{-1})
4. TDZ (1.0, 1.5, and 2.0 mg L^{-1}) + NAA (0.5 and 1.0 mg L^{-1})

The synergistic and antagonistic effects of these combinations on callus induction were examined. Ascorbic acid (50, 100, and 150 mg L^{-1}) and activated charcoal (3 mg L^{-1}) were added to the MS media to prevent callus necrosis and browning. Cultured explants were incubated in a growth room at 26 ± 2 °C under dark conditions for 45 days. After callus initiation, the cultures were maintained at the same temperature and relative humidity (54–65%) but exposed to a 16 h photoperiod using cool fluorescent tubes. Callus induction percentage was recorded for each combination, and to prevent browning and necrosis, explants were subcultured on fresh media every 28 days following a partially modified protocol based on Sharma et al. [6] and Nadeem et al. [13].

2.6. Indirect Shoots and Root Induction

The embryogenic calluses at the maturity stage were placed in culture flasks containing shoot-inducing media supplemented with various combinations and concentrations of auxins and cytokinins. Plant growth regulators (PGRs) used included the following:

1. BA (0.1–0.5 mg L^{-1})
2. KIN (0.1–1.0 mg L^{-1})
3. TDZ (1.0–2.0 mg L^{-1})
4. BA (0.1–0.5 mg L^{-1}) + IAA (0.1–0.5 mg L^{-1})

The percentage of shoot induction, the number of shoots per explant, and the shoot length per callus clump were recorded. For rooting, the shoots, approximately 1–2 cm in length, were excised from callus clusters and transferred to MS media supplemented with different auxins:

1. NAA (0.5–1.5 mg L^{-1})
2. IAA (0.5–1.5 mg L^{-1})
3. 2,4-D (1.0–2.0 mg L^{-1})

The cultures were incubated at 26 ± 2 °C with 54–65% relative humidity and a 16 h photoperiod for four weeks. The explants on MS media were monitored daily, and data on root induction, root number per explant, and root length were noted.

2.7. Direct Shoot Induction

Healthy rhizome explants were utilized for direct organogenesis and cultured on a medium supplemented with activated charcoal, cytokinins, and auxins, either individually or in combinations. The hormone combinations included the following:

1. BA (1.5–3.5 mg L^{-1})
2. TDZ (2.0–4.5 mg L^{-1})
3. KIN (2.5–4.5 mg L^{-1})
4. BA (3.5–4.5 mg L^{-1}) + IAA (0.1–0.5 mg L^{-1})
5. BA (2.0–4.5 mg L^{-1}) + NAA (0.1–0.5 mg L^{-1})

The inoculated cultures were maintained at 25 °C with 54–65% relative humidity and a 16 h photoperiod for four weeks. The explants were observed daily, and data regarding shoot induction, the number of shoots per explant, shoot length, the number of roots per explant, and root length were collected, following a partially modified protocol of Chakraborty et al. [23].

2.8. Hardening off and Acclimatization of In Vitro Plantlet

The plantlets developed in vitro were removed from flasks and rinsed with tap water to remove the nutrient medium and were planted in pots with a mixture of vermiculite and sterilized soil (1:1). To maintain high humidity, pots were covered with transparent polyethylene bags. The plantlets were placed in a growth chamber with a photoperiod of 16 h with a relative humidity of 65–70%. The temperature was set at 25 °C during the day and 18 °C during the night. The plantlets were watered every two days with half-strength Hoagland's solution for 3–4 weeks. The survival and growth of the plants were recorded every week. After a month, the successfully hardened off plantlets were transplanted to bigger pots with garden soil and kept in a glasshouse.

2.9. Statistical Analysis and Data Visualization

Parameters related to callus, shoot, and root development were evaluated approximately 2–8 weeks after culture initiation. Each parameter related to callus formation, shoot development, and root growth was measured in three repetitions per experimental condition, ensuring reliable results and robust statistical analysis. Four explants were placed in each flask and Petri dish to maintain uniformity and enable accurate comparisons. The data collected were analyzed statistically via MS Excel 2010, Statistix 8.1 and visualized via Sigma Plot 12.0. An analysis of variance (ANOVA) was performed to assess the overall significance of the data. The significance of differences among different treatments was assessed using the least significant difference (LSD) test at 1 and 5% levels of significance.

3. Results

3.1. Sterilization of Plant Materials

In this experiment, *P. hexandrum* seeds underwent sterilization using different concentrations of $HgCl_2$ for 5 min at a consistent temperature (25 ± 2 °C) to assess their impact on seed decontamination and germination. Results indicated that the 0.1% $HgCl_2$ treatment was the most successful, yielding significantly higher percentage germination (% germ) and lower percentage contamination (% cont) compared to other treatments. However, the 0.2% $HgCl_2$ treatment negatively affected germination. Thus, 0.1% $HgCl_2$ emerged as the optimal concentration for effective decontamination and germination of *P. hexandrum* seeds (Figure 2).

3.2. Callus Induction from the Explants of P. hexandrum

In this study, various combinations of plant growth regulators (PGRs) were assessed to induce callogenesis in *P. hexandrum* explants, aiming at germplasm conservation and micropropagation. Callogenesis was observed in stem, root, radicle, and cotyledon explants. These explants were successfully induced to form calluses on the MS medium supplemented with PGRs within 3 to 5 weeks. (Figure 3). The results indicated that

the maximum callus induction PGR combinations induced callogenesis in *P. hexandrum* explants for conservation and micropropagation. MS basal media and supplements optimized the callus-inducing medium. Stem, root, radicle, and cotyledon explants formed calluses in 3 to 5 weeks on MS media with PGRs. The results indicated that the highest callus induction occurred in leaf explants (95.67%), followed by radicle explants (85.67%). Stem and cotyledon explants showed moderate frequencies (78% and 63%, respectively) on MS media with BA (1 mg L^{-1}) and 2,4-D (3 mg L^{-1}). However, the callus induction frequency (53%) of root explants was relatively lower (Figure 3). Within 10 days, primary callus formation extended across all sides of the cuttings, swelling along the cut edges. The addition of BA (1 mg L^{-1}) and 2,4-D (3 mg L^{-1}) to MS media was most effective in callogenesis ($p \leq 0.05$), yielding the highest callus weight (215 mg), induction percentage (100%), and growth rate (13.620 mg/day) (Table 1; Figure 4A,B).

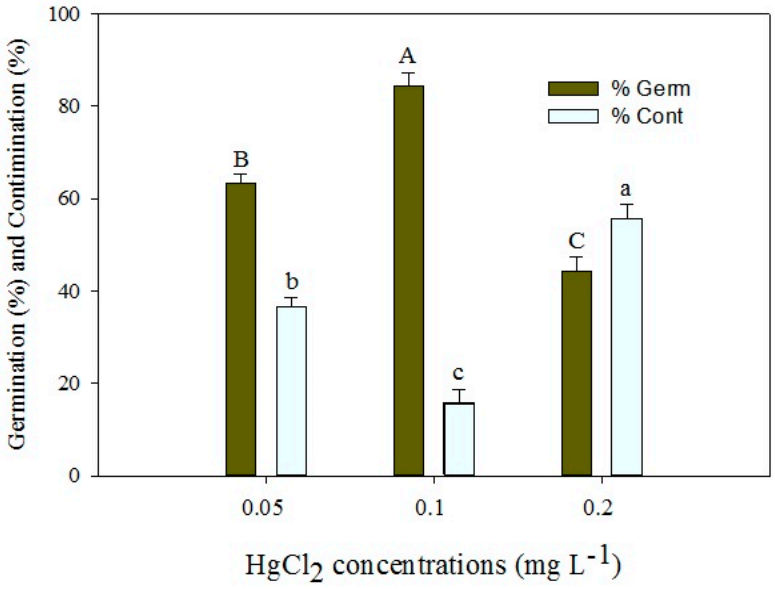

Figure 2. Effects of different concentrations of $HgCl_2$ on seed germination percentage (% germ) and contamination percentage (% cont) of *P. hexandrum*. Error bars represent the standard error of the respective means for three replicates. Capital and small letters represent mean comparisons at $p \leq 0.01\%$ and $p \leq 0.05\%$.

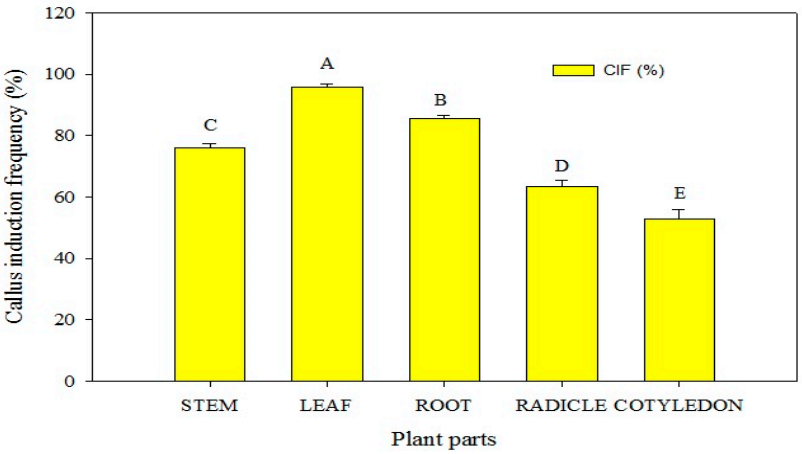

Figure 3. Callus induction frequency (CIF) of various explants of *P. hexandrum* under different combinations of cytokinins and auxins. Capital letters represent data mean comparisons at $p \leq 0.01\%$. Error bars show standard errors of respective means.

Table 1. Effects of different combinations and concentrations of plant growth regulators on callogenesis for in vitro conservation of *P. hexandrum*.

Cytokinin + Auxin	Concentration (mg L^{-1})	Weight (g)	Growth Rate mg/day	Growth (g)	Callus Induction %
MS0	0	0	0	0	0
TDZ + NAA	1 + 0.5	203.72 ± 0.43 (14.29) c	3.11 ± 0.52 (1.90) c	3.10 ± 0.69 (1.77) c	80.00 ± 0.00 (8.97) b
	1.5 + 0.5	207.07 ± 0.45 (14.41) b	5.55 ± 0.74 (2.46) b	5.55 ± 0.73 (2.37) b	80.00 ± 0.00 (8.97) b
	2 + 1.0	210.59 ± 0.57 (14.53) a	8.75 ± 0.41 (3.04) a	8.75 ± 0.23 (2.97) a	88.33 ± 1.66(9.43) a
LSD ($p \leq 0.05$)		3.03	2.39	0.98	2.89
BA + 2,4-D	0.5 + 1.0	205.82 ± 1.11 (14.36) c	7.41 ± 0.57 (2.81) c	4.47 ± 0.49 (2.23) c	100.00 ± 0.00 (10.02) a
	1.0 + 2.5	210.08 ± 1.17 (14.51) b	10.59 ± 0.59 (3.33) b	6.27 ± 0.19 (2.60) b	100.00 ± 0.00 (10.02) a
	1.0 + 3.0	215.88 ± 0.31 (14.71) a	13.62 ± 0.25 (3.76) a	8.32 ± 0.32 (2.97) a	100.00 ± 0.00 (10.02) a
LSD ($p \leq 0.05$)		4.26	1.28	0.26	-
BA + NAA	1.0 + 0.5	204.28 ± 0.34 (14.31) c	3.47 ± 0.39 (1.99) c	3.27 ± 0.35 (1.94) c	81.66 ± 1.66 (9.06) c
	1.5 + 0.5	208.91 ± 0.68 (14.47) b	7.26 ± 0.49 (2.79) b	5.89 ± 0.49 (2.53) b	91.6 ± 1.66 (9.60) b
	2.5 + 1	212.97 ± 1.30 (14.58) a	9.76 ± 0.80 (3.20) a	8.50 ± 0.66 (3.00) a	100.00 ± 0.00 (10.02) a
LSD ($p \leq 0.05$)		2.34	1.26	2.02	5.36
KIN + 2,4-D	0.2 + 1.0	207.59 ± 0.52 (14.43) c	7.04 ± 0.08 (2.75) c	3.26 ± 0.05 (1.82) c	86.78 ± 1.66 (9.34) b
	0.2 + 2.5	210.56 ± 0.91 (14.53) b	10.49 ± 0.42 (3.32) ab	5.51 ± 0.38 (2.36) b	100.00 ± 0.00 (10.02) a
	1.0 + 3.0	213.76 ± 0.33 (14.64) a	12.87 ± 0.26 (3.66) a	7.88 ± 0.42 (2.82) a	100.00 ± 0.00 (10.02) a
LSD ($p \leq 0.05$)		2.01	3.26	0.39	4.26
BA + IAA	1.0 + 0.5	202.28 ± 0.59 (14.24) c	2.66 ± 0.32 (1.78) c	2.57 ± 0.05 (1.75) c	41.66 ± 1.66 (6.49) c
	1.5 + 0.5	206.66 ± 0.22 (14.39) b	4.92 ± 0.11 (2.33) b	3.37 ± 0.26 (1.97) b	58.00 ± 1.66 (7.47) b
	3 + 1.0	209.37 ± 0.57 (14.49) a	8.08 ± 0.42 (2.93) a	5.40 ± 0.28 (2.43) a	73.33 ± 1.66 (8.59) a
LSD ($p \leq 0.05$)		2.98	0.98	0.27	5.68

Each treatment was conducted in triplicate with ten explants per replicate. Means with standard errors followed by the different letters differ significantly ($p < 0.05$, LSD test). The data in parentheses represent angle transformation values.

In contrast, the lowest callus weight (209.37 mg), callus induction percentage (69%), and growth rate (8.0833 mg/day) were observed with BA and IAA supplementation in MS culture media (Table 1) (Figure 4F). The addition of 100 mg L^{-1} ascorbic acid and 3 mg L^{-1} activated charcoal to the MS media prevented callus browning in in vitro cultures. Various PGRs resulted in calluses of different sizes, colors, and textures. Leaf explants produced compact, friable, and soft calluses in colors ranging from dark yellow to white and green (Table 1 and Figure 4). The induced callus was subcultured on MS media with various PGR concentrations and combinations, including BA + 2,4-D and TDZ + NAA, which led to the successful transformation of the proliferative callus into highly nodular callus formations in all tested combinations.

3.3. Shoot Induction of P. hexandrum from Calluses via Indirect Organogenesis

The present study successfully developed an in vitro protocol for *P. hexandrum* shoot multiplication from calluses, influenced by different PGRs and activated charcoal. The effects of various plant growth regulators (PGRs) on shoot frequency, the number of shoots, and shoot length per callus clump of *P. hexandrum* were evaluated to promote indirect organogenesis (Table 2 and Figure 5). Shoot regeneration from callus clumps was significantly induced by four different cytokinin PGRs, either alone or in combination with auxins. Statistical analysis of the data indicated that the highest shoot length (5.50 ± 0.10 cm), number of shoot regenerations (6.50 ± 0.10), and shoot regeneration percentage (85.00 ± 0.00%) were observed in calluses cultured on the MS growth medium fortified with BA (0.5 mg L^{-1}) (Table 2 and Figure 5G–I), indicating its effectiveness in promoting shoot growth and regeneration. The second-highest shoot regeneration percentage (78.00 ± 1.66%), number of shoots per callus clump (4.80 ± 0.08), and shoot length (4.00 ± 0.11 cm) were obtained on the MS growth medium containing TDZ (0.5 mg L^{-1}) (Table 1 and Figure 5C,D).

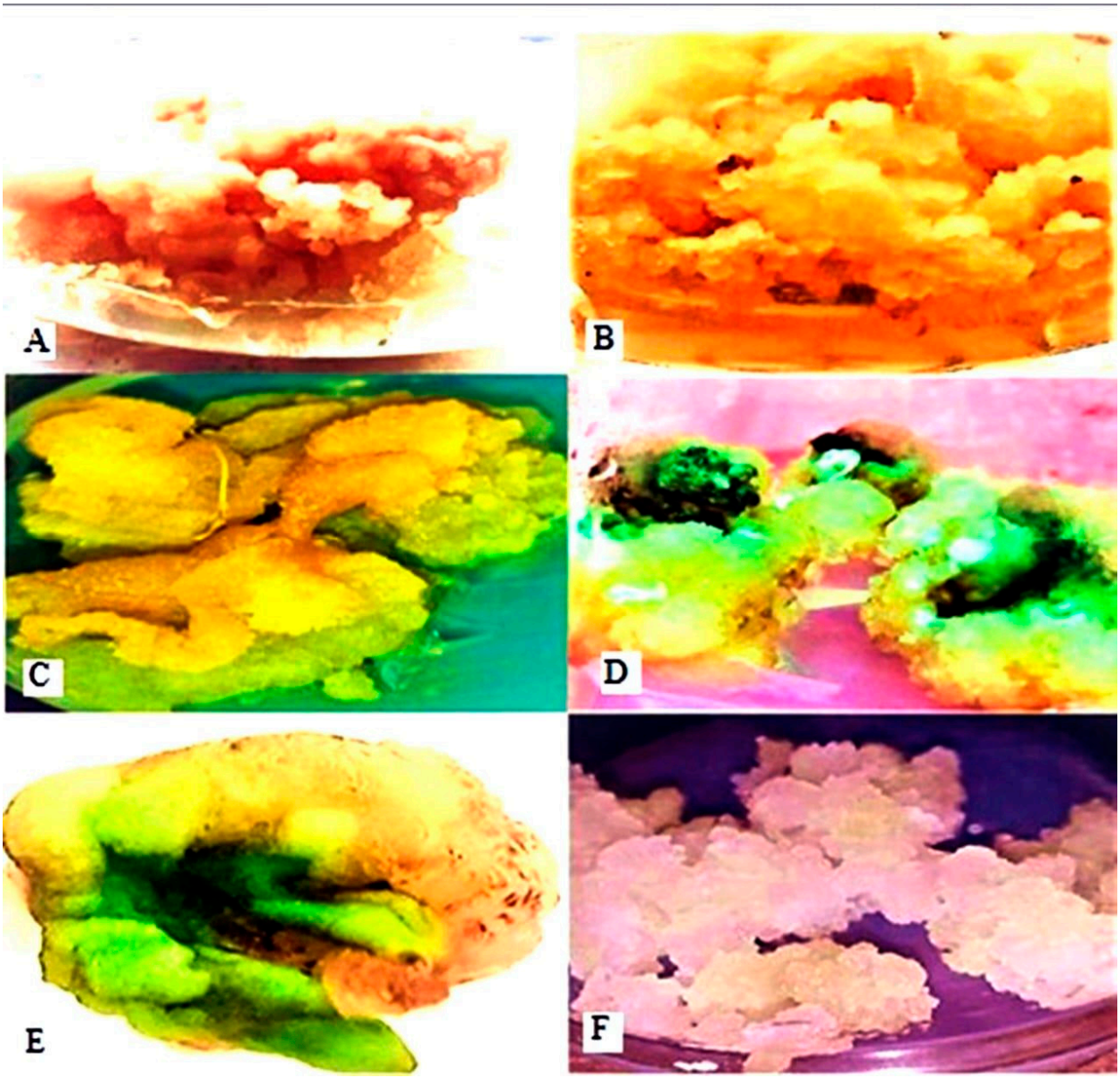

Figure 4. Callus induction on MS media supplemented with different PGRs. Callus culture induced from explants on MS medium supplemented with 1 mg L^{-1} BA and 3 mg L^{-1} 2,4-D (**A**,**B**). Callus cultured on MS medium supplemented with 1 mg L^{-1} KIN and 3 mg L^{-1} 2,4-D (**C**). Callus cultured on MS medium supplemented with 2.5 mg L^{-1} BA and 1 mg L^{-1} NAA (**D**). Callus cultured on MS medium supplemented with 2 mg L^{-1} TDZ and 1 mg L^{-1} NAA (**E**). Callus cultured on MS medium supplemented with 3 mg L^{-1} BA and 1.0 mg L^{-1} IAA (**F**).

Table 2. Effects of various plant growth hormones on shoot induction of *P. hexandrum* from calluses via indirect organogenesis.

Cytokinin + Auxin	Concentration (mg L^{-1})	Number of Shoots Per Callus Clump	Shoot Length (cm)	Shoot Induction %
MS0	0	0	0	0
BA + IAA	1.5 + 0.1	1.08 ± 0.08 (1.06) c	1.67 ± 0.08 (1.31) c	13.33 ± 1.66 (3.66) c
	2.0 + 0.1	1.66 ± 0.22 (1.31) b	2.35 ± 0.11 (1.55) b	21.66 ± 1.66 (4.66) b
	2.0 + 0.5	2.58 ± 0.08 (1.62) a	3.31 ± 0.08 (1.83) a	40.00 ± 2.88 (6.33) a
LSD ($p \leq 0.05$)		0.78	0.26	4.89

Table 2. *Cont.*

Cytokinin + Auxin	Concentration (mg L^{-1})	Number of Shoots Per Callus Clump	Shoot Length (cm)	Shoot Induction %
TDZ	1.0	3.75 ± 0.14 (2.06) c	2.97 ± 0.05 (1.86) c	41.66 ± 1.66 (6.49) c
	1.5	4.33 ± 0.08 (2.20) b	3.71 ± 0.06 (2.05) b	58.33 ± 3.33 (7.67) b
	2.0	4.83 ± 0.08 (2.31) a	4.85 ± 0.11 (2.31) a	78.58 ± 1.66 (8.88) a
LSD ($p \leq 0.05$)		0.26	0.63	5.98
BA	0.1	4.33 ± 0.08 (2.20) b	3.77 ± 0.07 (2.07) c	61.66 ± 3.33 (7.88) c
	0.2	5.41 ± 0.08 (2.43) b	4.71 ± 0.09 (2.28) b	73.33 ± 1.66 (8.59) b
	0.5	6.50 ± 0.14 (2.65) a	5.54 ± 0.18 (2.46) a	85.00 ± 0.00 (9.25) a
LSD ($p \leq 0.05$)		0.56	0.41	6.02
KIN	0.1	1.83 ± 0.08 (1.53) c	2.08 ± 0.14 (1.61) c	33.45 ± 1.66 (5.82) c
	0.5	2.66 ± 0.08 (1.78) b	2.75 ± 0.06 (1.80) b	53.333 ± 1.66 (7.34) b
	1.0	3.41 ± 0.08 (21.98) a	3.33 ± 0.10 (1.96) a	60.00 ± 0.00 (7.78) a
LSD ($p \leq 0.05$)		0.21	0.15	8.65

Each treatment was conducted in triplicate with ten explants per replicate. Means with standard errors followed by the different letters differ significantly ($p < 0.05$, LSD test). The data in parentheses represent angle transformation values.

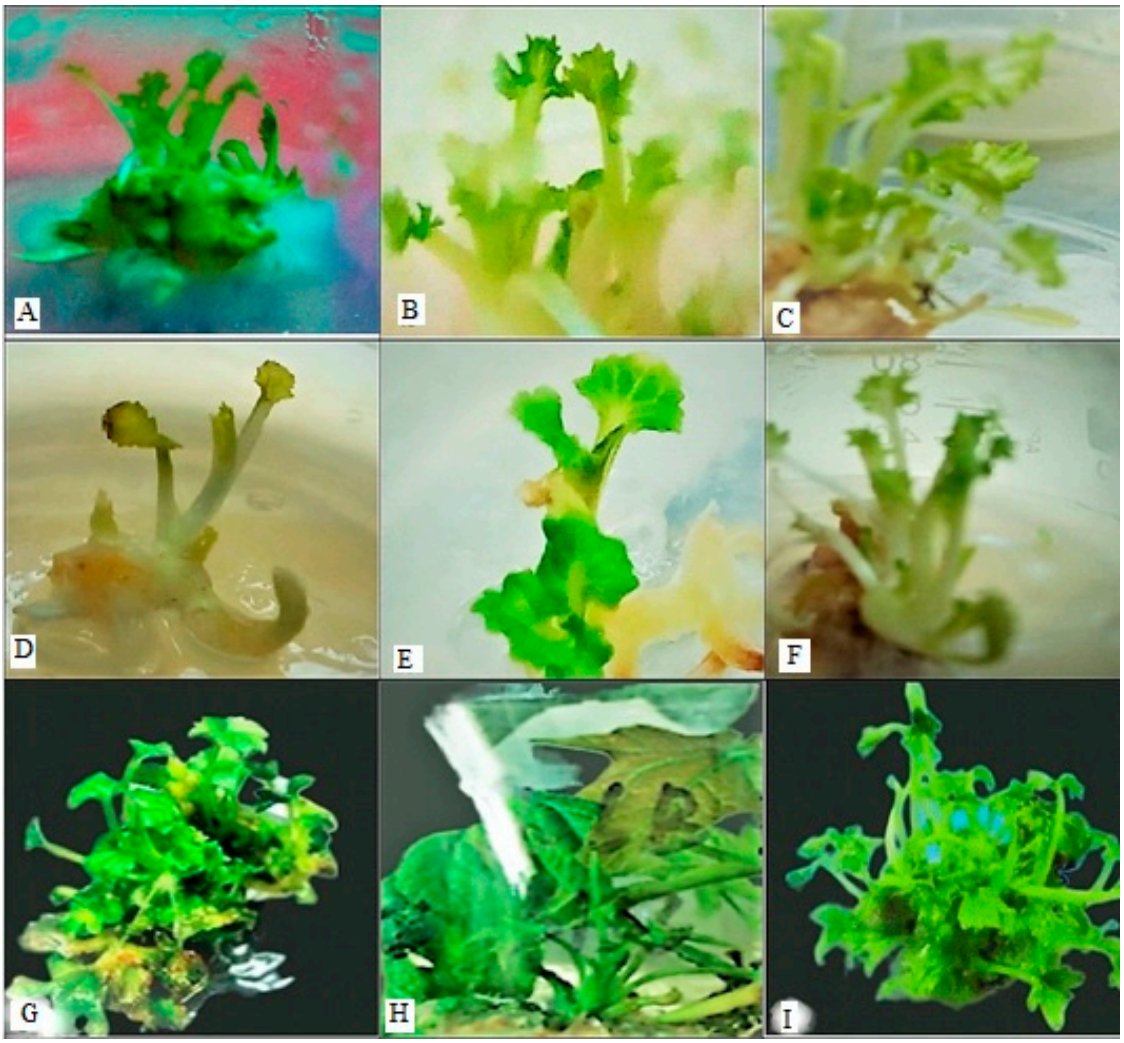

Figure 5. Indirect organogenesis on MS media. Shoot induction/growth from callus clump on MS medium supplemented with the following: 2.0 mg L^{-1} BA and 0.5 mg L^{-1} IAA (**A**,**B**); 2.0 mg L^{-1} TDZ (**C**,**D**); 1.05 mg L^{-1} KIN (**E**,**F**); and 0.5 mg L^{-1} BA (**G**–**I**).

Conversely, the lowest number of shoot regenerations was observed on the MS medium containing a combination of BA (2.0 mg L^{-1}) and indole-3-acetic acid (IAA) (0.5 mg L^{-1}) (Table 2) (Figure 5A,B). However, when IAA and BA were used in combination, it also resulted in a significant improvement in the number of shoots per callus clump, shoot length, and shoot regeneration percentage. The use of BA alone or in combination with other PGRs can significantly impact shoot regeneration percentage, the number of shoots, shoot length, and shoot induction (as shown in Table 2 and Figure 5).

3.4. Root Induction in Calluses of P. hexandrum

Root induction plays a crucial role in the in vitro reproduction of plants that are challenging to root. Auxins, crucial hormones for initiating and fostering root growth, necessitate specific types and concentrations tailored to individual plant species and the conditions of their cultivation. In the present study, the effect of three different auxins, namely 2,4-D, IAA, and NAA, on root induction in the calluses of *P. hexandrum* via indirect organogenesis was investigated. These results were based on different concentrations of auxins and their corresponding effects on the number and length of roots, as well as the rooting percentage through indirect organogenesis.

The maximum rooting, characterized by the number of roots per explant (4.08 ± 0.08), root length (5.40 ± 1.56 cm), and rooting percentage (87.00% ± 1.66), was observed in the MS medium supplemented with NAA (1.5 mg L^{-1}) (Table 3 and Figure 6C). However, the lowest response in terms of the number of roots per explant (2.33 ± 0.08) and root length (2.85 ± 0.06 cm) was observed in MS media IAA (Table 3) (Figure 6A). Among the various auxins tested, the response of the explants was more favorable when MS media were fortified with NAA. These results suggested that the supplementation of 1.5 mg L^{-1} of NAA, IAA, and 2,4-D to the MS medium rapidly promotes root induction in *P. hexandrum* calluses through indirect organogenesis (Table 3 and Figure 6).

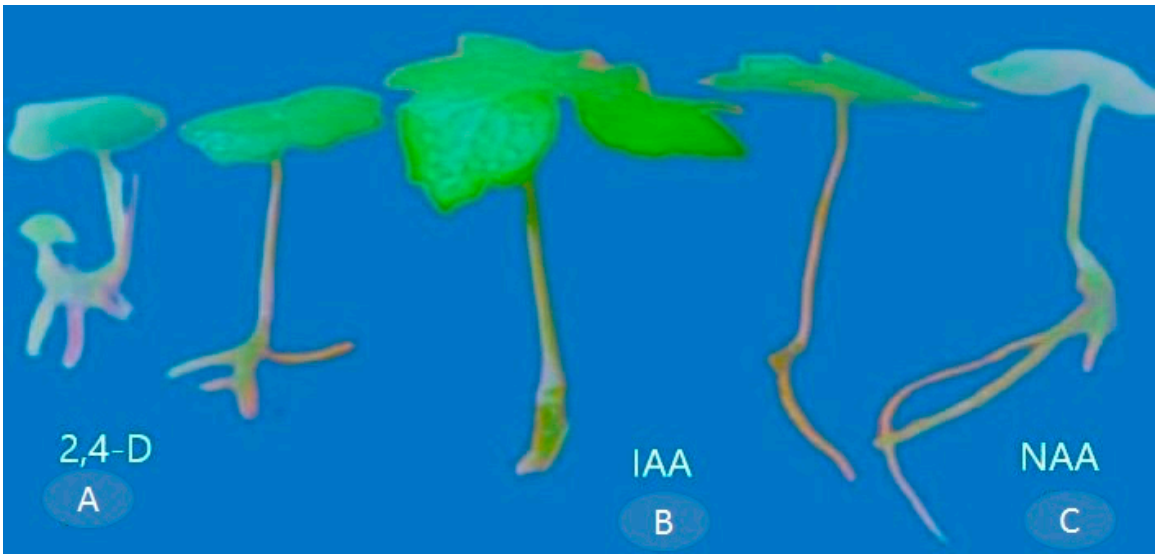

Figure 6. Root induction on MS medium supplemented with (**A**) 2,4-D (0.5 mg L^{-1}), (**B**) IAA (0.5 mg L^{-1}), and (**C**) NAA (1.5 mg L^{-1}).

Table 3. Effect of various auxins on root induction in calluses of *P. hexandrum* during organogenesis.

Auxin	Concentration (mg L^{-1})	No. of Root Explants	Root Length (cm)	Rooting %
MS0	0	0	0	0
IAA	0.5	1.08 ± 0.08 (1.26) c	1.64 ± 0.04 (1.46) c	48.00 ± 1.66 (6.99) c
	1.0	1.58 ± 0.08 (1.44) b	2.56 ± 0.19 (1.75) b	56.00 ± 1.66 (7.56) b
	1.5	2.33 ± 0.08 (1.68) a	2.85 ± 0.06 (1.83) a	66.00 ± 1.66 (8.20) a

Table 3. *Cont.*

Auxin	Concentration (mg L^{-1})	No. of Root Explants	Root Length (cm)	Rooting %
LSD ($p \leq 0.05$)		0.45	0.65	2.26
2,4-D	1	1.92 ± 0.08 (1.55) c	2.53 ± 0.04 (1.74) c	51.00 ± 1.66 (7.22) c
	1.5	2.67 ± 0.08 (1.78) b	3.42 ± 0.14 (1.98) b	65.00 ± 1.66 (8.20) b
	2.0	3.33 ± 0.08 (1.96) a	4.31 ± 0.08 (2.19) a	83.00 ± 1.66 (9.16) a
LSD ($p \leq 0.05$)		0.09	0.16	3.32
NAA	0.5	2.67 ± 0.08 (1.78) c	3.93 ± 0.11(2.10) a	53.00 ± 1.66 (7.31) c
	1	3.25 ± 0.08 (1.94) b	4.64 ± 0.06 (2.27) b	60.00 ± 1.66 (7.82) b
	1.5	4.08 ± 0.08 (2.14) a	5.45 ± 0.15 (2.44) a	87.00 ± 1.66 (9.35) a
LSD ($p \leq 0.05$)		0.19	0.14	4.26

Each treatment was conducted in triplicate with ten explants per replicate. Means with standard errors followed by the different letters differ significantly ($p < 0.05$, LSD test). The data in parentheses represent angle transformation values.

3.5. In Vitro Shoot Proliferation from the P. hexandrum Explants through Direct Organogenesis

In this study, an efficient method for the in vitro regeneration of *P. hexandrum* has been successfully developed, using rhizome explants freshly collected from the plant through direct organogenesis for the micropropagation of *P. hexandrum*. The statistical analysis showed that the highest response in terms of the number of shoots per explant (5.30 ± 0.08), shoot length (12.40 ± 0.61), and shooting percentage (86.67 ± 1.66%) was obtained when the MS medium was augmented with BA (4.5 mg L^{-1}) + NAA (0.5 mg L^{-1}) and 3 mg L^{-1} of activated charcoal (Figures 7A,B and 8A,F).

The second-highest number of shoots per explant (4.92 ± 0.08), shoot length (7.52 ± 0.14), and shooting percentage (76.67% ± 1.60) was achieved with a medium containing BA (2.5 mg L^{-1}) (Figures 7C,D and 8B,G). TDZ treatment resulted in an average of 4.50 ± 0.14 shoots per explant, with a shoot length of 5.90 ± 0.11 cm and a shooting percentage of 73.33 ± 1.66%. (Figures 7E,F and 8C,H). Similarly, KIN treatment also yielded 3.70 ± 0.08 shoots per explant, with a shoot length of 3.40 ± 0.07 cm and a shooting percentage of 70.00% ± 0.00 (Figures 7G,H and 8D,I).

Explants cultured on media augmented with cytokinins alone and in combination with auxins (TDZ, BA + NAA, BA, KIN, and BA + NAA) exhibited varying levels of organogenesis induction with distinct responses (Figures 7 and 8). In conclusion, the BA + NAA treatment demonstrated the most favorable outcomes in terms of shoot induction in this experiment, leading to high shoot multiplication and longer shoot lengths. The combination of auxin (NAA) and cytokinin (BA), along with activated charcoal in the growth medium, proved to be effective in promoting shoot formation from the explants through direct organogenesis.

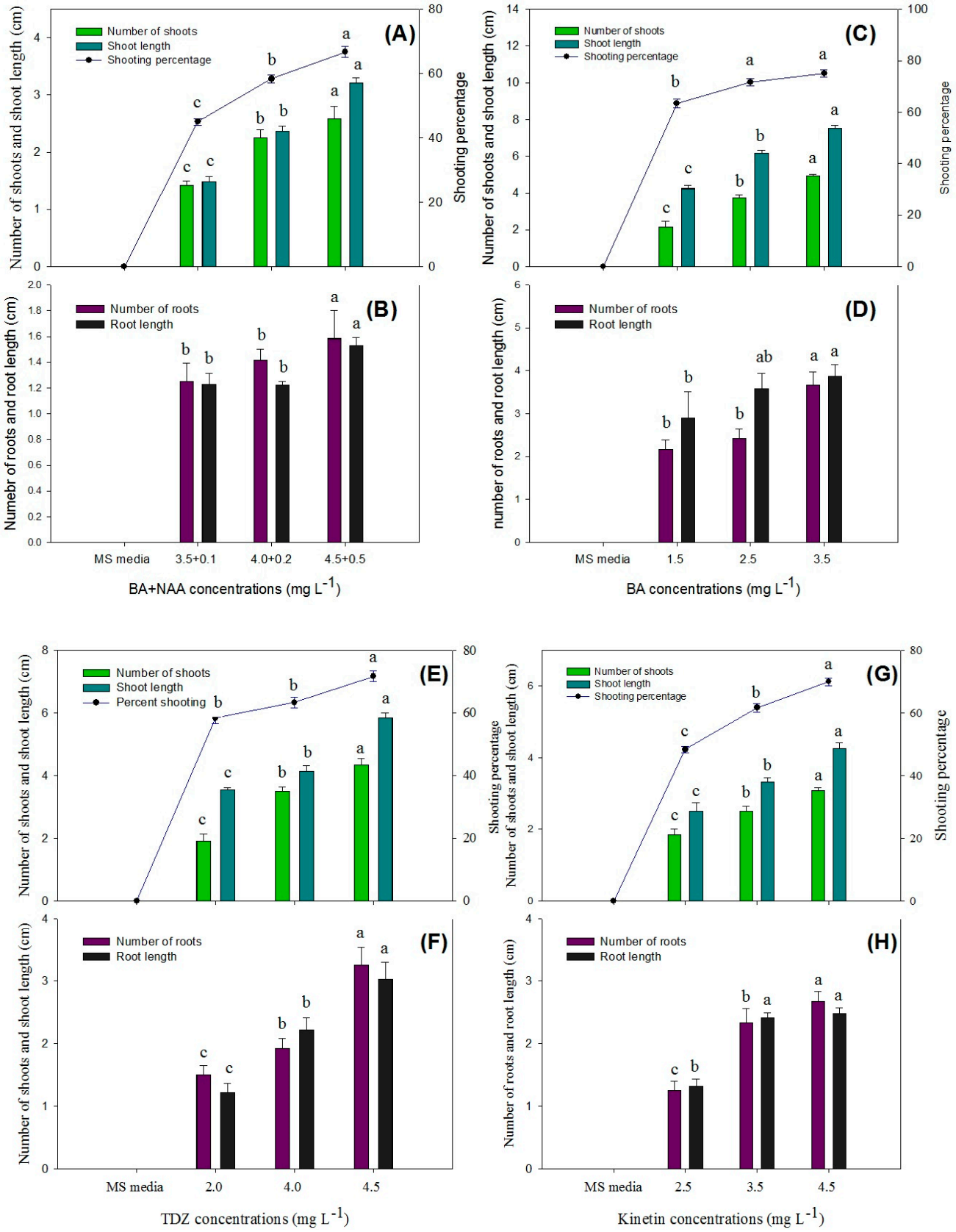

Figure 7. *Cont.*

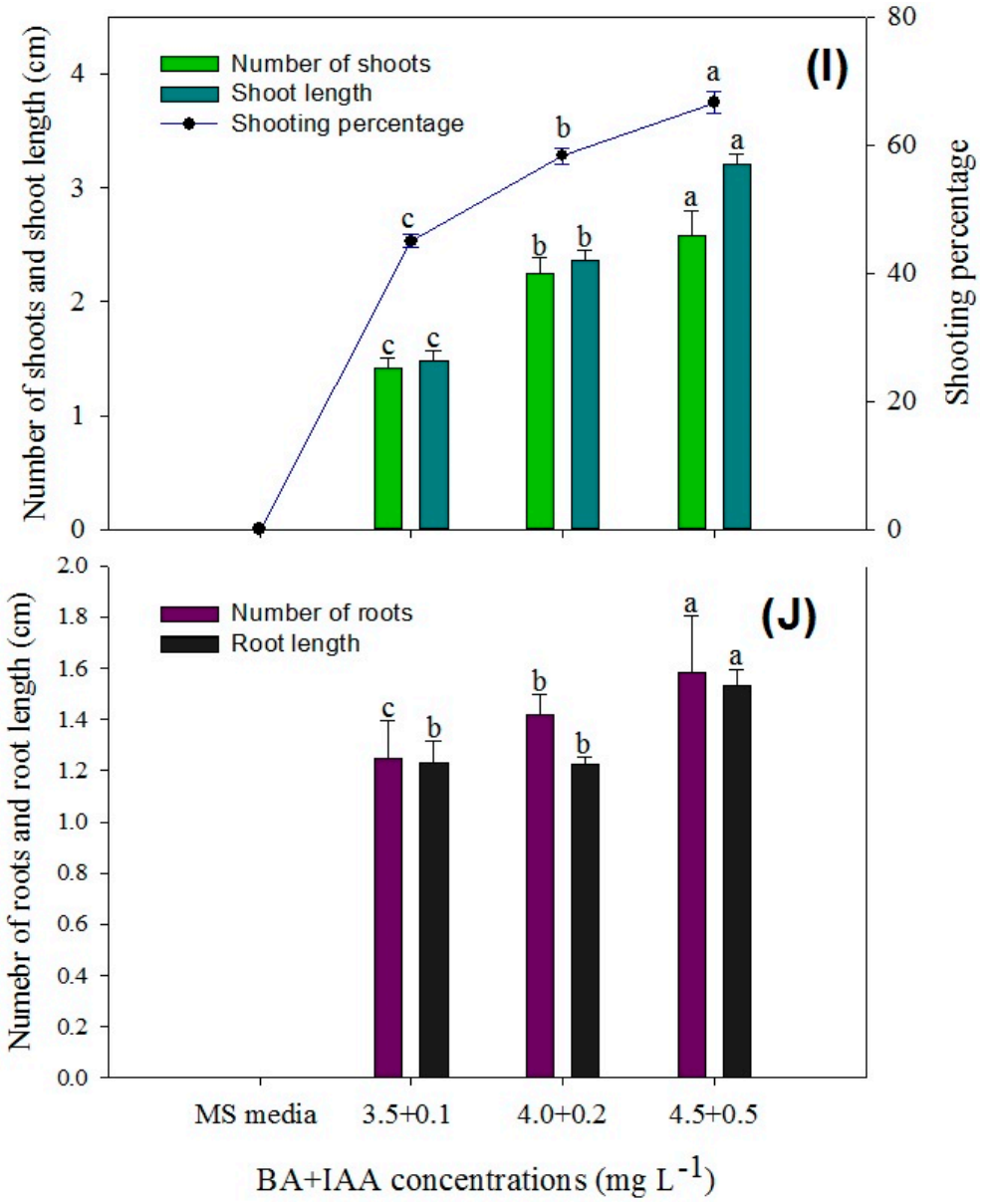

Figure 7. Comparison of the number of shoots, shoot length, shoot induction percentage, number of roots, and root length in various explants of *P. hexandrum* during indirect organogenesis under different concentrations of BA + NAA (**A**,**B**), BA (**C**,**D**), TDZ (**E**,**F**), KIN (**G**,**H**), and BA + IAA (**I**,**J**). Small letters represent data mean comparisons at $p \leq 0.05\%$. Error bars represent the standard error.



Figure 8. Direct organogenesis from rhizomes having active buds on MS medium. (**A**,**F**) Supplemented with 4 mg L^{-1} BA + 0.5 mg L^{-1} NAA. (**B**,**G**) Shoot propagation from rhizomes on MS medium supplemented with 3.5 mg L^{-1} BA. (**C**,**H**) Shoot propagation from rhizomes on MS medium supplemented with 4.5 mg L^{-1} TDZ. (**D**,**I**) Shoot propagation from rhizomes on MS medium supplemented with 4.5 mg L^{-1} KIN. (**E**,**I**,**J**) Shoot propagation from rhizomes on MS medium supplemented with 4 mg L^{-1} BA + 0.5 mg L^{-1} IAA. (**K**) Shoot propagation from rhizomes on MS medium supplemented with BA + NAA, BA, TDZ, KIN, and BA + NAA.

3.6. Hardening off and Acclimatization

In this study, after the completion of the four-week culture period on the rooting medium, plantlets were carefully selected that exhibited not only robust and well-established root systems but also optimal physiological parameters indicative of their readiness for acclimatization. These carefully chosen plantlets underwent a gradual acclimatization process, which aimed to facilitate their transition from the synthetic media to soil and from the controlled environment of the growth chamber to the natural environment of the greenhouse. This acclimatization phase commenced with the methodical

removal of the polyethylene bags, thereby exposing the plantlets to the fluctuating light and temperature patterns characteristic of outdoor settings.

Furthermore, as part of the acclimatization regimen, ambient humidity levels were gradually introduced to the plantlets, thereby simulating the moisture conditions they would encounter in their natural habitat. Subsequently, the plantlets were transplanted into larger pots filled with a suitable substrate, ensuring adequate space for root expansion and nutrient uptake. These pots were then placed within a greenhouse facility where environmental parameters such as temperature, humidity, and light intensity could be carefully regulated to facilitate the plantlets' adjustment to their new surroundings.

Over time, under the nurturing care provided within the greenhouse, the plantlets continued to develop and thrive, exhibiting signs of healthy growth and adaptation to ex vivo conditions. Notably, the culmination of this acclimatization process was reflected in the remarkable achievement of an impressive survival rate of 87% among the transplanted plantlets, underscoring their successful transition and establishment in the natural environment (Figure 9).



Figure 9. *P. hexandrum* successfully acclimatized to outdoor conditions after a hardening off period of 4 to 6 weeks under ex vivo conditions.

4. Discussion

4.1. Seed Sterilization and Growth Response

Seed sterilization is crucial to prevent microbial damage and enhance germination [23]. Aseptic conditions are essential in tissue culture to prevent contamination. Various disinfectants, including $HgCl_2$, can improve germination rates, but microbial infestation can hinder growth. Surface sterilization with mercuric chloride is important but may affect biological characteristics [24]. $HgCl_2$ is an effective disinfectant for reducing bacterial and fungal contamination in tissue culture [25].

In this study, treating *P. hexandrum* seeds with 0.1% $HgCl_2$ for 5 min resulted in an 85.43% germination rate, suggesting its efficacy in seed sterilization and the enhancement of germination [26–28]. However, concentrations beyond 0.1% cased browning and the eventual death of seeds and explants. Sterilization outcomes varied significantly based on $HgCl_2$ concentration and exposure duration, with higher concentrations and longer exposures reducing bacterial contamination but leading to rapid explant necrosis [29,30]. Previous research supports $HgCl_2$ and Bavistin's effectiveness as a disinfectant for various plant species. Its efficacy depends on factors such as concentration, contact time, explant type, and microbial species [31,32]. Optimal concentrations and exposure times range from 0.1% for 3–15 min for some species. However, $HgCl_2$ treatment can induce issues like browning and toxicity, which can be mitigated with certain agents [33]. Comparatively, $HgCl_2$ is more effective than NaOCl in combating microbial contamination. Therefore, optimizing $HgCl_2$ concentration and duration is crucial for effective decontamination without compromising seed viability [22,34].

4.2. Callus Induction

Callus induction varies with explant type and forms undifferentiated callus tissue due to the hormone's effects [35,36]. The balance of auxin and cytokinin influences de-differentiation and re-differentiation, inducing callus formation in specific medium ratios. This study found that leaf explants were the most efficient in callus formation, while root explants were the least responsive, reflecting the role of auxins and cytokinins in cell division and differentiation [37].

Previous studies have reported *P. hexandrum* callus induction and proliferation using different explants and PGRs [15,17,38]. Optimal PGR combinations (BA + 2,4-D, BA + NAA) resulted in varied callus textures at different concentrations, aligning with findings on callus formation and micropropagation in *P. hexandrum* [39]. Similar callogenesis results were observed by Sharma et al [6] and Chawla et al [36,40–42] in *P. hexandrum* and Roylea elegans using the same PGRs. However, studies on hypocotyl and root explants showed different outcomes in callus induction and podophyllotoxin production [38], possibly due to variations in sources, genotypes, explant treatments, and extraction methods [16,33,34].

In this study, shoot and leaf explants of *P. hexandrum* produced yellow, white, and greenish compact calluses in all treatments. However, leaf and stem explants displayed different colors on similar media with 2,4-D and BA; BA and NAA; and TDZ and NAA. Leaf explants produced brown-white, greenish, and friable calluses, while stem explants produced yellow-white, greenish, and friable calluses. Different colors in calluses result from variations in hormone types, concentrations, and conditions for different explants [43]. Callus morphology and color also depend on explant type, plant growth regulators, and pigment accumulation influenced by light, temperature, pH, and stress [34,44]. The induced calluses are capable of conservation, subculture, and further development into embryos or organs with the use of different PGRs [45].

4.3. Shoot Induction of P. hexandrum from Calluses via Indirect Organogenesis

In vitro plant regeneration, involving callus and direct explant methods, is influenced by various factors [13,20,34]. The agar-based culture medium initially caused shoot browning, impeding growth and proliferation. This issue was addressed by augmenting the MS medium with activated charcoal supplementation. This study successfully established a protocol for *P. hexandrum* shoot multiplication from calluses, incorporating different PGRs and activated charcoal. Initially facing shoot browning on the agar-based culture medium, the addition of 3 mg L^{-1} activated charcoal to the MS medium effectively controlled necrosis, resulting in robust and healthy shoot formations. The most effective shoot induction in *P. hexandrum* was achieved with the MS medium containing BA (4 mg L^{-1}) (Figure 4D) + NAA (0.5 mg L^{-1}) and 3 mg L^{-1} activated charcoal, resulting in the highest shoot formation and length. BA in the shooting medium favored in vitro shoot multiplication, consistent with previous studies [6,41,46].

The study investigated the effects of BA and TDZ, both individually and in combination with auxins, on indirect organogenesis and shoot regeneration. Notably, BA at a concentration of 2.0 mg L^{-1} demonstrated high efficacy in promoting shoot formation. This finding contrasts with the work of Chen et al. [35], who reported that IAA exhibited optimal efficiency compared to other plant growth regulators (PGRs). However, our study revealed that IAA combinations had limitations in terms of shoot multiplication. Specifically, lower concentrations of BA resulted in fewer shoots, leading us to expect that higher BA concentrations would increase shoot numbers. Interestingly, in *Vitex negundo* L., higher BA concentrations actually reduced shoot production, emphasizing the need for species-specific optimization in tissue culture protocols [39,40]. In contrast, our study on *P. hexandrum* showed a significant increase in shoot multiplication with BA concentrations exceeding 0.5 mg L^{-1}. BA's impact on natural hormone metabolism, tissue penetration, and cell uptake enhanced shoot formation and quality in *P. hexandrum* in vitro. The type and concentration of PGR also influenced shoot quality and quantity in the explants, with a significant amount of readily accessible BA contributing to the observed effects [39,40]. Geng et al. [45] found BA to be more effective than thidiazuron (TDZ) for increasing shoot

number and elongation at concentrations of 0 to 2.0 mg L^{-1} [47–49]. In summary, our study highlights BA as the most effective cytokinin for indirect organogenesis among those tested.

4.4. In Vitro Root Induction in Calluses of P. hexandrum

Root induction is vital for in vitro propagation of hard-to-root plants. Auxins are key hormones for root initiation and development, but their optimal type and concentration depend on the plant and the culture conditions [50]. NAA's effectiveness is attributed to its influence on cell division, enlargement, meristematic activity, and nucleic acid and protein synthesis [51].

The results align with previous studies emphasizing NAA's role in successful root induction in *P. hexandrum* and other plant species [52]. NAA's stability and mobility further facilitate its uptake and translocation within plant tissues. The study aligns with previous research highlighting the importance of NAA supplementation for successful root induction in the in vitro culture of different plant species [53]. The findings contribute to the optimization of in vitro propagation techniques for *P. hexandrum* and underline the importance of selecting the right type and concentration of plant growth regulators for enhancing roots. IAA resulted in the lowest rooting percentage due to its rapid degradation and potential inhibition of root elongation. The findings contributed to the optimization of in vitro propagation techniques for *P. hexandrum* and demonstrate the importance of selecting the right PGR types and concentrations for enhancing shoot and root organogenesis and propagation.

4.5. In Vitro Shoot Proliferation from Explants via Direct Organogenesis

Direct organogenesis is crucial for genetic manipulations and the micropropagation of plants [15]. However, some plant species, including *P. hexandrum*, face challenges in shoot organogenesis due to the slow growth rate and overexploitation, as the plant contains secondary metabolites. This study successfully established the in vitro regeneration and proliferation of *P. hexandrum* from rhizome explants through direct organogenesis. These findings are in line with prior research that has achieved regeneration from various explants, including seeds, cotyledons, hypocotyls, and leaves, using different PGRs such as BA, TDZ, NAA, and IAA [6,23].

In overcoming challenges in shoot organogenesis, antioxidants and charcoal were employed, aligning with prior research achieving regeneration from different explants using various PGRs [45]. Different cytokinins (BA with NAA, TDZ, and KIN) in the media induced distinct responses, enhancing in vitro regeneration of *P. hexandrum* [15,16]. The results contrast with studies reporting low or no shoot regeneration from rhizome explants of *P. hexandrum*, potentially influenced by varied plant growth regulators (IBA, IAA, 2iP, BA, and NAA) and the absence of activated charcoal [6,21,27]. This study underlines the need for suitable PGR combinations; BA and NAA with activated charcoal proves most promising for efficient shoot multiplication and longer shoot lengths in *P. hexandrum*.

In conclusion, the BA + NAA treatment demonstrated the most favorable outcomes in terms of shoot induction in this experiment, leading to high shoot multiplication and longer shoot lengths. The study emphasized *P. hexandrum*'s responsiveness to organogenesis, highlighting the role of plant growth regulators (PGRs) in regulating shoot induction through cell division and elongation.

5. Conclusions

P. hexandrum is an endangered but valuable medicinal plant native to the temperate and cold climates of the Himalayan region. It provides socioeconomic, medicinal, ecological, and traditional benefits, but faces threats from overexploitation, habitat loss, and environmental adversities. This study developed an efficient in vitro propagation and conservation protocol using plant growth regulators (PGRs) and various explant sources. We optimized conditions for seed germination, sterilization, callus formation, direct and indirect organogenesis, and acclimatization of *P. hexandrum*. Among the PGR combinations tested, 1 mg L^{-1} BA combined with 2 mg L^{-1} (2,4-D) yielded the highest callus induction and growth. For direct organogenesis, BA was the most effective in promoting shoot

growth and regeneration, while for indirect organogenesis, BA and NAA produced the maximum number of shoots from rhizomes. The plantlets were successfully acclimatized and transferred to ex vitro conditions with a high survival rate. This protocol can facilitate the mass propagation and preservation of *P. hexandrum*, providing large numbers of uniform and disease-free plants in a short time. Future research should focus on field trials, quality assessment, genetic diversity analysis, and biotechnological production of podophyllotoxin. This study contributes to plant biotechnology, sustainable utilization, and conservation biology for endangered or economically important plant species.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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