



Development of *In vitro* protocols for mass propagation of *Pueraria phaseoloides* (Roxb.) Benth. from the Chittagong Hill Tracts of Bangladesh

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Abstract

Pueraria phaseoloides (Roxb.) Benth. Commonly known as tropical kudzu, is an ethno-medicinally significant climbing perennial legume of the Fabaceae family. Endemic to regions including the Chittagong Hill Tracts (CHT) of Bangladesh, it is heavily exploited for its bioactive iso-flavonoids, notably puerarin. The species currently faces localized threats due to overharvesting and low natural regeneration rates. To mitigate these conservation challenges and establish a sustainable supply for the pharmaceutical industry, an efficient *in vitro* seed germination and direct micropropagation protocol was established. Seed germination was optimized on Murashige and Skoog (MS) medium, achieving a maximum germination rate of 100% within 30-33 days when supplemented with 1.0 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l α -naphthaleneacetic acid (NAA). For multiple shoot buds (MSBs) induction *via* direct organogenesis, nodal segments exhibited the highest morphogenic response (80%) on MS medium fortified with 2.0 mg/l BAP and 2.0 mg/l indole-3-acetic acid (IAA), producing a maximum of 2.29 ± 0.41 MSBs per explant. Optimal shoot elongation (an increase of 3.07 ± 0.18 cm after 30d of culture) was recorded on MS medium containing 2.0 mg/l BAP and 1.0 mg/l NAA. For rhizogenesis, half strength MS medium supplemented with 2.0 mg/l indole-3-butyric acid (IBA) proved superior, inducing a 90% rooting frequency with an average of 6.17 ± 0.19 number of roots per shoot and a maximum root length of 3.26 ± 0.22 cm. Fully regenerated seedlings were successfully acclimatized *ex vitro* with a 70% survival rate. This highly reproducible propagation protocol provides a robust platform for the rapid multiplication, conservation and commercial cultivation of *P. phaseoloides*.

Keywords: Acclimatization, direct organogenesis, micropropagation, *Pueraria phaseoloides*, rhizogenesis.

Introduction

Medicinal plants have historically served as the cornerstone of traditional and complementary healthcare systems globally, providing an expansive reservoir of therapeutically active secondary metabolites [1-2]. Among these, the Fabaceae family contains numerous species of immense pharmacological value. *Pueraria phaseoloides* (Roxb.) Benth. Commonly recognized as tropical kudzu, is a climbing perennial legume widely distributed across South and Southeast Asia, including the Chittagong Hill Tracts (CHT) of Bangladesh. Morphologically, the plant is distinguished by alternate, trifoliolate leaves with dark green, hairy surfaces and axillary racemes bearing mauve to deep purple flowers that develop into linear, cylindrical pods containing small black seeds.

Phytochemical profiling of *P. phaseoloides* reveals a dense concentration of alkaloids, flavonoids, phlobatannins, saponins, steroids, tannins, terpenoids and critical phenolic compounds [3]. The roots, in particular, are highly valued in traditional Chinese and regional folk medicine due to their rich accumulation of bioactive isoflavonoids, such as puerarin [4]. Pharmacological studies validate the ethno-medicinal use of *Pueraria* species, demonstrating their profound efficacy in exhibiting hypothermic, spasmolytic, antihypertensive and antiarrhythmic properties [5]. Furthermore, organic extracts of its leaves have demonstrated notable antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, alongside estrogenic and anxiolytic effects that

highlight its potential in managing menopausal and neurological disorders [6-7].

Despite its robust pharmacological profile, the natural populations of *P. phaseoloides* in the wild, particularly in the subtropical environments of Bangladesh, are facing rapid depletion [8]. In countries like Bangladesh, rural and tribal communities, particularly in the Chittagong Hill Tracts, heavily rely on these native plant resources for primary healthcare, placing immense pressure on wild populations [9-10]. Over exploitation by local communities and modern pharmaceutical entities, coupled with severe habitat fragmentation and deforestation, has placed immense pressure on its natural reserves. The global and domestic market for herbal medicines is expanding rapidly, necessitating sustainable raw material sources to prevent localized extinction [11-12]. Conventional propagation *via* seeds is severely restricted by inconsistent seed viability, prolonged dormancy and variable germination rates in natural ecosystems [13].

In vitro tissue culture, particularly direct micropropagation, offers a highly viable and necessary biotechnological intervention to overcome these conventional constraints [14-15]. Micropropagation facilitates the rapid, large scale and season independent generation of disease free, genetically uniform plantlets. Micropropagation has been successfully applied to other medicinal legumes, such as *Mucuna pruriens* [16], validating its utility for the Fabaceae family. While previous studies have documented the micropropagation of various medicinal legumes, there

remains a critical gap in standardized, highly efficient direct organogenesis protocols specifically tailored for *P. phaseoloides* sourced from the CHT region.

Therefore, the primary objective of this research was to develop and standardize a highly reproducible *in vitro* protocol for the mass propagation of *P. phaseoloides*. This study focused strictly on optimizing *in vitro* seed germination kinetics, identifying the precise plant growth regulators (PGRs) combinations for maximizing direct multiple shoot buds (MSBs) induction from nodal segments, standardizing elongation metrics and achieving successful rhizogenesis and subsequent *ex vitro* acclimatization.

Materials and Methods

1. Source of plant material and explant preparation

Mature seeds and nodal segments of healthy, field grown *Pueraria phaseoloides* (Roxb.) Benth. were collected from the Chittagong Hill Tracts (CHT), Bangladesh. The collected explants were washed thoroughly under continuous running tap water to remove superficial dust and debris. The materials were treated with 1% Savlon (ACI Pharma, Bangladesh) combined with a few drops of Tween-20 for 5-10 minutes under constant agitation, followed by 3-4 successive rinses with sterile distilled water. Within a sterilized laminar airflow cabinet, the explants were immersed in 70% ethanol for 60 seconds. Final surface sterilization was executed using a 0.1% (w/v) mercuric chloride (HgCl₂) solution. Following sterilization, the explants were rinsed 4-5 times with autoclaved distilled water to eliminate any residual sterilant before inoculation.

2. Basal Media Preparation

Murashige and Skoog (MS) basal medium^[17] was employed across all morphogenic phases. To prepare the medium, analytical grade macro and micro nutrients, vitamins and 3.0% (w/v) sucrose were dissolved in glass distilled water. The pH of the medium was digitally adjusted to 5.8 using 1N NaOH or 1N HCl prior to the addition of 0.8% (w/v) agar (Himedia, India) as the gelling agent. The medium was dispensed into culture vessels (test tubes or conical flasks), tightly plugged with non-absorbent cotton, sealed with aluminum foil and sterilized *via* autoclaving at 121°C under 1.9 kg/cm² pressure for 20 minutes.

3. Preparation of plant growth regulators (PGRs)

Stock solutions of auxins including α -Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), and Indole-3-butyric acid (IBA) and cytokinins including 6-Benzyl aminopurine (BAP) and Kinetin (Kn) were prepared by dissolving the concentrated powders in appropriate solvents (1N NaOH for auxins; dilute HCl for cytokinins) before bringing them to final volume with distilled water. These PGRs were incorporated into the autoclaved MS media at various concentrations (0.5 to 2.0 mg/l) depending on the experimental phase.

4. Culture conditions

All inoculated culture vessels were maintained in a highly controlled environmental growth room. Cultures were incubated under a 14-hour continuous light and 10-hour dark photoperiod cycle. Illumination was provided by cool

white fluorescent tubes maintaining a light intensity of 2000-3000 lux, with the ambient temperature strictly regulated at $25 \pm 2^\circ\text{C}$.

5. Micropropagation and rhizogenesis procedures

For *in vitro* seed germination, sterilized seeds were inoculated onto MS medium supplemented with singular or combined concentrations of BAP, Kn and NAA. For the direct induction of multiple shoot buds (MSBs), nodal segments excised from *in vitro* raised seedlings were cultured on MS media fortified with varying matrices of BAP or Kn combined with NAA or IAA. Rescued shoot buds were subsequently transferred to specific elongation media to monitor longitudinal growth. For rooting (rhizogenesis), elongated mini plantlets measuring 2-3 cm in height were excised and transferred to half strength MS basal media containing various concentrations of IBA, IAA and NAA.

6. Acclimatization

Well rooted *in vitro* seedlings were subjected to a gradual *ex vitro* acclimatization phase. Culture vessels were uncapped inside the culture room for one day, followed by exposure to standard room temperature conditions for 6 to 12 hours over successive days. The seedlings were carefully extracted and the roots were gently washed under running tap water to remove adhering agar. They were transplanted into plastic pots containing a sterilized mixture of garden soil and compost (2:1 ratio), pre-treated with 0.1% Agrosan fungicide. High humidity was maintained through daily misting. Following the emergence of new leaves (approximately 10 days), the hardened seedlings were transferred to open environmental soil.

7. Experimental design and statistical analysis

The experiments were arranged in a completely randomized design. Morphogenic data, including germination percentages, shoot counts, elongation lengths and root metrics, were recorded systematically after required culture days. Data represent the mean of replicates \pm Standard Error (SE), calculated as $SE = SD / \sqrt{N}$, where SD is the standard deviation and N is the number of observations (5 to 10 replicates per treatment).

Results

1. *In vitro* seed germination

The initial phase of the study assessed the efficacy of cytokinins (BAP, Kn) individually and in combination with an auxin (NAA) on the *in vitro* germination of *P. phaseoloides* seeds. The germination responses varied significantly based on the PGRs composition of the medium (Table 1).

PGR free half strength and full strength MS media yielded limited responses, with only 30% and 50% germination occurring after prolonged periods of 40-43 and 39-42 days, respectively. The introduction of cytokinins alone improved the rates; 1.0 mg/l and 2.0 mg/l BAP both achieved 70% and 80% seed germination accordingly. However, the synergistic application of BAP and NAA proved overwhelmingly superior. The highest germination rate of 100% was recorded on MS medium supplemented with 1.0

mg/l BAP and 0.5 mg/l NAA (Fig 1), which also drastically reduced the required germination time to 30-33 days. Kinetin (Kn) combinations were comparatively less effective, with 1.0 mg/l Kn + 0.5 mg/l NAA achieving only 60% germination. The data clearly demonstrates that the BAP and NAA synergy is optimal for triggering rapid and uniform green seedling development.

2. Direct induction and proliferation of multiple shoot buds (MSBs)

Nodal segments collected from the naturally grown in the field were utilized to induce direct MSBs. Various matrices of BAP and Kn, either singularly or paired with NAA or IAA, were evaluated (Table 2).

BAP combinations heavily outperformed than Kn combinations in inducing direct organogenesis. The maximum morphogenic response where 80% of the nodal explants underwent proliferation was observed on MS medium fortified with 2.0 mg/l BAP and 2.0 mg/l IAA (Fig. 2). This specific formulation yielded the highest average of 2.29 ± 0.41 MSBs per nodal segment within the shortest induction time (22-24 days) followed by the second optimal response was noted on MS medium with 2.0 mg/l BAP + 2.0 mg/l NAA, yielding a 75% response rate and 2.19 ± 0.44 MSBs after 23-25d of culture (Fig. 3). Conversely, single treatment of Kinetin exhibited the lowest (40%) organogenic efficiency. MS medium with 1.0 mg/L Kn induced proliferation in only 40% of explants, generating a minimal 1.08 ± 0.30 MSBs per explant over an extended period of 30-32 days.

3. *In vitro* callus induction from *in vitro* raised leaf segment

When *in vitro* derived leaf segment were cultured on MS medium fortified with 2, 4-D individually and in combination with BAP, Kn, NAA and IAA (Table 3). In the tested media, *in vitro* raised leaf segment produced greenish compact callus (GCC) and whitish compact callus (WCC). The percentage of explant producing callus were recorded after 18-29d of culture.

The maximum number of greenish compact callus (GCC) from leaf segment (85%) was produced when cultured in MS medium containing 1.0 mg/l 2, 4-D + 1.0 mg/l BAP after 18-20d of inoculation (Fig. 4) followed by MS + 1.0 mg/l 2,4-D + 1.0 mg/l IAA (80%; 19-21d, WCC) and MS + 0.5 mg/l 2,4-D + 1.0 mg/l BAP (80%; 20-22d; GCC).

On the other hand, the lowest number of leaf segment responses in proliferation (45%) and produced whitish compact callus (WCC) when grown on the MS medium supplemented with 1.0 mg/l 2, 4-D within 27-29 days of culture.

4. *In vitro* shoot elongation

To facilitate robust longitudinal growth, rescued mini plantlets were transferred to specific elongation media. The elongation dynamics were recorded over a 30d of culture (Graph 1). The combination of BAP and NAA proved exceptionally effective for shoot elongation. The peak longitudinal increment was observed on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA, which facilitated a remarkable average length increase of 3.07 ± 0.18 cm, bringing the final mean shoot length to 4.66 ± 0.19 cm (Fig 5). This was closely followed by the 1.0 mg/l BAP + 0.5 mg/l NAA treatment, generating a 3.03 ± 0.15 cm increase. Kinetin based formulations consistently suppressed elongation kinetics; MS medium containing 1.0 mg/l Kn resulted in a meager 0.82 ± 0.15 cm length increase over the 30 days monitoring phase.

5. Rhizogenesis and *ex vitro* acclimatization

Elongated plantlets (2-3 cm) were subcultured onto half strength MS medium augmented with various auxins to induce rooting. The application of IBA profoundly influenced rhizogenic success compared to IAA or NAA. The highest root induction frequency (90%) occurred on ½ MS medium containing 2.0 mg/l IBA (Graph 2, Fig. 6), which simultaneously produced the maximum root count (6.17 ± 0.19 roots per shoot) and the greatest (3.26 ± 0.22 cm) root elongation (Graph 3). A combined treatment of 0.5 mg/l IAA + 0.5 mg/l IBA was also highly effective, securing an 85% rooting response with 5.98 ± 0.14 roots per shoot. In stark contrast, PGR free ½ MS media exhibited severely constrained rhizogenesis, yielding only a 45% induction rate with an average increase of 3.52 ± 0.14 number roots and 1.24 ± 0.23 cm length.

Following successful root formation, the healthy seedlings were advanced to the acclimatization phase. Utilizing the 2:1 garden soil to compost matrix and systematic atmospheric humidity step-down, 70% of the *in vitro* generated *P. phaseoloides* seedlings successfully established themselves in the *ex vitro* environment, displaying normal phenotypic morphology and robust growth.

Table 1: Effect of BAP and Kn individually and in combination with NAA on *in vitro* seeds germination of *P. phaseoloides*.

Media	PGRs Conc. (mg/l)	Total no. of seeds	No. of germinated seeds	% of germinated seeds	Time required for germination	Remarks
½ MS without PGRs	-	10	3	30	40-43	GS
Full MS without PGRs	-	10	5	50	39-42	GS
BAP	1.0	10	7	70	34-37	GS
	2.0	10	8	80	35-38	GS
Kn	1.0	10	6	60	38-41	GS
	2.0	10	7	70	36-39	GS
BAP + NAA	1.0 + 0.5	10	10	100	30-33	GS
	2.0 + 0.5	10	9	90	32-35	GS
Kn + NAA	1.0 + 0.5	10	6	60	37-40	GS
	2.0 + 0.5	10	7	70	35-38	GS

*d = days; GS = Green Seedlings; **values are the means \pm SE of each experiment consist of five replicates.

Table 2: Effect of different concentrations and combinations of BAP and Kn individually and in combination with NAA and IAA on induction of MSBs from natural grown nodal segment of *P. phaseoloides*.

PGRs supplement in the media (mg/l)		% of explants showing proliferation	time(d) required for induction of shoot buds	No. of MSBs Produced (mean \pm SE)
BAP	1.0	50	28-30	1.25 \pm 0.38
BAP	2.0	55	27-29	1.42 \pm 0.41
Kn	1.0	40	30-32	1.08 \pm 0.30
Kn	2.0	45	29-31	1.13 \pm 0.33
BAP+ NAA	1.0 + 1.0	70	24-26	2.06 \pm 0.43
	2.0 + 2.0	75	23-25	2.19 \pm 0.44
BAP + IAA	1.0 + 1.0	70	24-26	2.01 \pm 0.37
	2.0 + 2.0	80	22-24	2.29 \pm 0.41
Kn + NAA	1.0 + 1.0	60	26-28	1.72 \pm 0.39
	2.0 + 2.0	65	25-27	1.87 \pm 0.54
Kn + IAA	1.0 + 1.0	55	27-29	1.43 \pm 0.43
	2.0 + 2.0	50	28-30	1.25 \pm 0.34

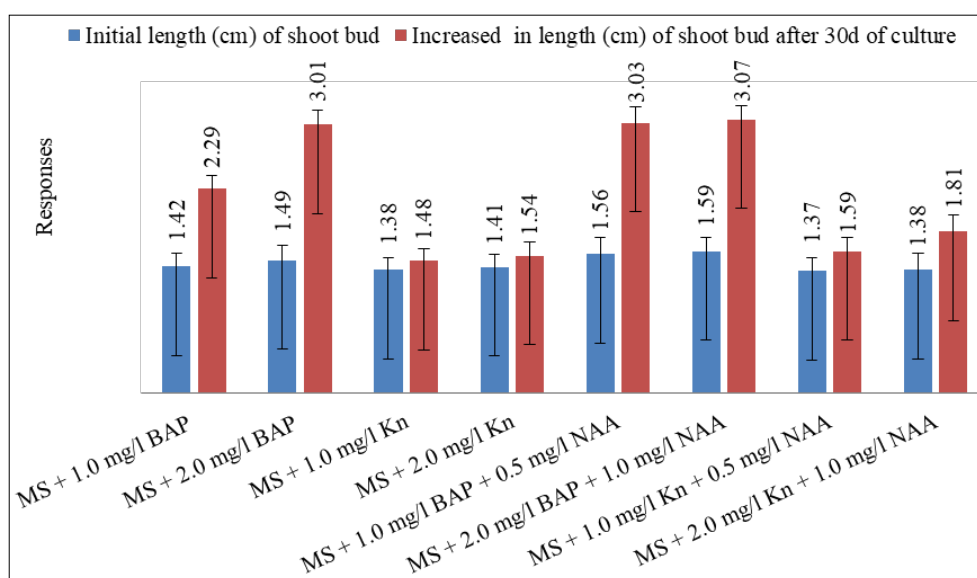
*d = days; MSBs = Multiple Shoot Buds; **values are the means \pm SE of each experiment consist of ten replicates.

Table 3: Effect of 2, 4-D individually and in combination with BAP, Kn, NAA and IAA on induction of callus tissue from the *in vitro* derived leaf segments of *P. phaseoloides* on 0.8% (w/v) agar solidified MS medium.

Medium	PGRs conc. (mg/l)	% of leaf producing callus	Time (d) required for callus induction	Color and texture of induced callus
MS + 2,4-D	1.0	45	27-29	WCC
	2.0	50	26-28	WCC
MS + 2,4-D + BAP	0.5 + 1.0	80	20-22	GCC
	1.0 + 1.0	85	18-20	GCC
MS + 2,4-D + Kn	0.5 + 1.0	55	25-27	GCC
	1.0 + 1.0	60	24-26	GCC
MS + 2,4-D + NAA	0.5 + 1.0	65	23-25	GCC
	1.0 + 1.0	75	21-23	GCC
MS + 2,4-D + IAA	0.5 + 1.0	70	22-24	WCC
	1.0 + 1.0	80	19-21	WCC

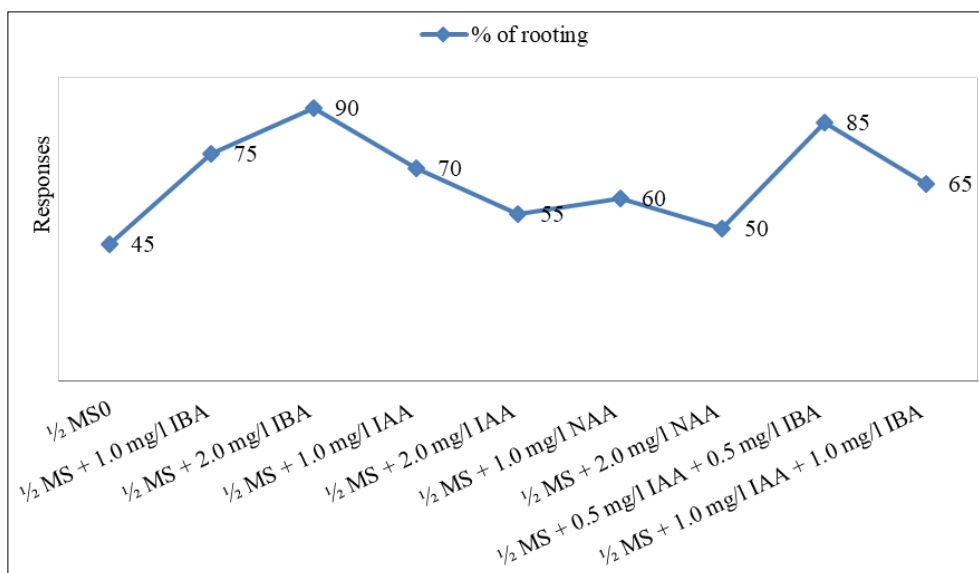
*d = days; GCC = green compact callus; WCC = whitish compact callus;

**values are the means \pm SE of each experiment consist of five replicates.



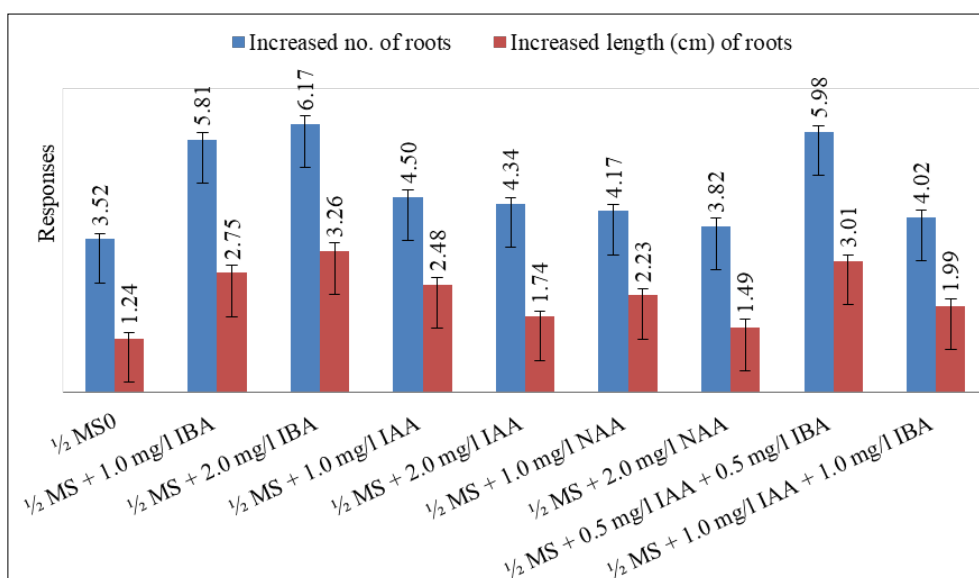
*d = days; values are the means \pm SE of each experiment consist of five replicates.

Graph 1: Elongation of directly produced germinated seedlings of *P. phaseoloides* on MS medium fortified with different concentrations and combinations of PGRs.



*values are the means \pm SE of each experiment consist of ten replicates.

Graph 2: Percentage of rooting in *in vitro* germinated plantlet of *P. phaseoloides* on PGR free half MS and PGRs supplemented half strength MS medium.



*values are the means \pm SE of each experiment consist of ten replicates.

Graph 3: Increased number and length of roots in *in vitro* elongated plantlet of *P. phaseoloides* on PGR free half MS and PGRs supplemented half strength MS medium.



Fig. 1: Green seedlings were produced from seed germination in *P. phaseoloides* on MS + 1.0 mg/l BAP + 0.5 mg/l NAA.



Fig. 2: Induction of MSBs of *P. phaseoloides* from natural grown nodal segment on MS + 2.0 mg/l BAP + 2.0 mg/l IAA.



Fig. 3: Induction of MSBs of *P. phaseoloides* from natural grown nodal segment on MS + 2.0 mg/l BAP + 2.0 mg/l NAA.



Fig. 4: Induction of callus from *in vitro* developed leaf segments of *P. phaseoloides* on MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP.



Fig. 5: Elongation of germinated mini plantlet *P. phaseoloides* on MS + 2.0 mg/l BAP + 1.0 mg/l NAA.



Fig. 6: Induction of roots in elongated plantlet of *P. phaseoloides* on 1/2 MS + 2.0 mg/l IBA.

Discussion

The establishment of a highly efficient direct propagation protocol for *Pueraria phaseoloides* is a critical advancement for both its conservation and pharmaceutical exploitation. This study systematically mapped the morphogenic thresholds of the species across varying concentrations of plant growth regulators.

The *in vitro* seed germination kinetics revealed a profound dependency on the synergistic interaction between a cytokinin (BAP) and an auxin (NAA). The peak 100% germination rate achieved with 1.0 mg/l BAP and 0.5 mg/l NAA demonstrates that balanced exogenous PGRs supplementation can forcefully break innate seed dormancy mechanisms while accelerating embryonic tissue expansion. This aligns with fundamental botanical principles, where BAP catalyzes the disruption of apical dominance and invigorates early cellular division [18], while low concentrations of NAA structurally guide nascent cellular differentiation [19].

During the induction of direct multiple shoot buds (MSBs) from nodal segments, the cytokinin-auxin synergy was once again paramount. The superior efficacy of BAP combined with IAA (achieving 80% response and 2.29 MSBs/ nodal segment) highlights BAP's dominant role in promoting axillary bud proliferation. Kinetin (Kn) exhibited an observably weaker regulatory influence on *P. phaseoloides* tissues, generating the lowest shoot counts. The pronounced morphogenic superiority of BAP over other cytokinins, due to its slower metabolic degradation rate within plant tissues, ensures a sustained supply of active cytokinin to the explant. The specific success of BAP with IAA or NAA for MSBs induction has been well corroborated in literature concerning medicinally potent families; analogous findings have been reported for *Plumbago indica* and *Boerhaavia diffusa*, where equivalent hormonal matrices induced prolific shoot multiplication [20-22]. Furthermore, similar morphogenic efficacy with BAP combinations has been reported in other important Fabaceae and Asteraceae members, such as *Mucuna pruriens* [16] and *Wedelia sinensis* [23], underscoring the universality of this hormonal synergy in triggering high frequency organogenesis.

Shoot elongation patterns mirrored the MSBs induction responses, with the BAP and NAA matrix dictating the

highest longitudinal growth (3.07 cm increase after 30d of culture). The data unequivocally confirms that an elevated cytokinin to lower auxin ratio maintains active intercalary meristem function while preventing premature tissue hardening in *P. phaseoloides* micro shoots.

Rhizogenesis represents the most critical hurdle in *in vitro* protocols prior to *ex vitro* integration. In this study, IBA (2.0 mg/l) drastically outperformed securing a 90% rooting frequency on half strength MS medium. The physiological mechanism driving IBA's superiority relates to its structural stability; IBA functions as an inherent slow releasing auxin. It undergoes delayed enzymatic degradation in the culture medium, thereby exerting a continuous and sustained morphogenic stimulus that strongly favors adventitious root primordia initiation [24]. This slow release dynamic prevents the immediate toxicity often seen with high dose IAA while securing prolonged cellular division at the root apex. Similar efficacies of IBA utilizing half strength basal matrices have been extensively documented across various threatened medicinal herbs, such as *Heliotropium indicum* [25], *Mentha viridis* [26] and *Catharanthus roseus* [27] confirming its universal applicability in stimulating robust root architecture required for field survival [28].

Finally, the 70% *ex vitro* acclimatization survival rate underscores the structural competence of the *in vitro* generated roots and stomatal apparatus. The gradual humidity transition phase successfully mitigated transplantation shock, proving that this protocol yields physiologically resilient seedlings ready for commercial propagation and ecosystem reintroduction.

Conclusion

This study successfully establishes a highly reproducible, robust *in vitro* micropropagation and seed germination protocol for *Pueraria phaseoloides* (Roxb.) Benth., a heavily exploited medicinal legume native to Bangladesh. The optimized parameters specifically the utilization of 1.0 mg/l BAP + 0.5 mg/l NAA for 100% seed germination, 2.0 mg/l BAP + 2.0 mg/l IAA for maximal direct shoot proliferation and half strength MS with 2.0 mg/l IBA for robust rhizogenesis provide a scalable blueprint for mass production. By bypassing natural reproductive limitations and eliminating wild harvesting pressures, this

biotechnological framework ensures sustainable commercial cultivation to support the global pharmaceutical demand for puerarin and iso-flavonoids, while simultaneously securing the ecological preservation of this valuable species in the Chittagong Hill Tracts.

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