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Standardization of *in vitro* techniques for mass scale propagation of *Asclepias curassavica* L. - A medicinally important plant species of Bangladesh

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Abstract

Asclepias curassavica L., commonly known as tropical milkweed is a highly valuable medicinal plant belonging to the Apocynaceae family. Despite its rich repository of therapeutic secondary metabolites including cardenolides and polyphenols; wild populations in Bangladesh face severe depletion due to over exploitation, habitat fragmentation and poor natural seed viability. This study aimed to establish a highly efficient, reproducible and standardized *in vitro* direct propagation protocol for the mass scale conservation and commercial production of *A. curassavica*. Utilizing nodal segments and shoot apices, direct multiple shoot buds (MSBs) proliferation, shoot elongation and rhizogenesis were systematically evaluated across varying concentrations of Plant Growth Regulators (PGRs). The highest frequency of MSBs induction from nodal explants (85%) and the maximum number of shoots per explant (2.40 ± 0.85) were achieved within 21-23 days on MS medium supplemented with 2.0 mg/l BAP and 2.0 mg/l NAA. For combined evaluation of explant types, nodal segments significantly outperformed shoot apices. In the callogenesis assays, the highest frequency of greenish compact callus (90%) was recorded from internode explants cultured on MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l BAP within 20-22 days. Shoot elongation was optimized on MS medium fortified with 2.0 mg/l BAP and 1.0 mg/l NAA, yielding a maximum length increase of 3.18 ± 0.32 cm after 30d of culture. Efficient *in vitro* rhizogenesis was obtained on half strength MS medium containing 2.0 mg/l IBA, which induced the highest rooting percentage (95%), root count (6.35 ± 0.31/shoot) and root length (3.67 ± 0.21 cm). Successfully rooted seedlings were systematically acclimatized and transferred to *ex vitro* field conditions, demonstrating a high survival rate of 80%. This optimized protocol ensures the sustainable, year round production of genetically uniform and disease free *A. curassavica* seedlings, mitigating the pressure on wild habitats and fulfilling the demands of the global pharmaceutical industry.

Keywords: *Asclepias curassavica*, medicinal plant, *In vitro* propagation, callogenesis, rhizogenesis

Introduction

Medicinal plants have historically formed the foundational infrastructure of traditional healthcare systems globally, providing an indispensable source of therapeutic agents and precursor molecules for modern drug synthesis [1]. The World Health Organization (WHO) estimates that approximately 80% of the population in developing countries relies on traditional plant-based medicine for their primary healthcare needs [2]. In Bangladesh, the integration of traditional modalities such as Ayurveda, Unani and ethno-medicine is deeply embedded in rural and tribal healthcare strategies [3]. This escalating reliance on herbal formulations has propelled the global botanical market, prompting pharmaceutical industries to commercialize plant based therapeutics heavily [4].

Asclepias curassavica L. under the family Apocynaceae, locally recognized as wild ipecacuanha or tropical milkweed, is an erect perennial undershrub of immense pharmacological significance. Distributed sporadically across the Chittagong, Noakhali and Barisal regions of Bangladesh, this species is traditionally utilized for its potent emetic, purgative, antipyretic, anti-inflammatory, and styptic properties [5-6]. Phytochemical analyses have revealed that the aerial and subterranean parts of the plant are rich in bioactive cardenolides (such as asclepin, calotropin and uzarigenin), triterpenoids and crucial polyphenols including quercetin, kaempferol and rutin [7]. These secondary metabolites render the plant highly effective in treating diverse ailments ranging from bronchitis and pneumonia to dermatological infections and intestinal parasites.

Despite its high therapeutic quotient, the long term sustainability of *A. curassavica* resources is acutely threatened. Heavy demand for its pharmaceutical resources, compounded by population pressure, unregulated wild harvesting, and the degradation of natural ecosystems in Bangladesh, has driven this species toward alarming vulnerability [8]. Furthermore, natural propagation of *A. curassavica via* seeds is often hindered by low seed viability, strict germination dormancy constraints and high seedling mortality under fluctuating environmental conditions [9]. Consequently, there is an urgent and critical need to develop alternative, biotechnology-driven conservation strategies.

In vitro tissue culture offers a robust and reliable pathway to overcome these ecological and physiological bottlenecks. Micropropagation through direct organogenesis ensures the rapid, mass scale and year round generation of disease free, genetically uniform clones, independent of seasonal and environmental limitations [10]. In parallel, the establishment of controlled callogenic systems facilitates key cellular and tissue-level investigations, serving as an essential starting material for somatic embryogenesis, cell suspension cultures and the standardized bioreactor scale bio-manufacturing of highly valuable cardenolides and cardiotonic glycosides [11].

Therefore, the present investigation was specifically designed to establish a standardized and highly efficient *in vitro* propagation and callus induction protocol for *Asclepias curassavica* L. The study strictly evaluated the morphogenic responses of nodal segments, shoot apices, internodes and leaf explants to various concentrations and combinations of specific auxins and cytokinins to optimize multiple shoot buds (MSBs) induction, callogenesis, shoot elongation and rhizogenesis, culminating in successful *ex vitro* acclimatization.

Materials and Methods

1. Source of explants and surface sterilization

Fresh, healthy nodal segments, leaf parts and shoot apices of *Asclepias curassavica* L. were excised from field grown mature plants sourced from the Chittagong region of Bangladesh. The excised plant materials were subjected to a rigorous, multi-step surface sterilization protocol under aseptic laboratory conditions to eliminate microbial contaminants. Initially, the explants were thoroughly washed under continuous running tap water to remove superficial dust and debris. The materials were then treated with a 1% Savlon (ACI Pharma, Bangladesh) solution mixed with a few drops of Tween-20 for 5-10 minutes with constant agitation. Following this, the explants were rinsed 3-4 times with sterile distilled water to ensure complete removal of the detergent. The materials were subsequently transferred to a sterile laminar airflow cabinet, briefly rinsed in 70% ethanol for less than 60 seconds and immersed in a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for a specific duration tailored to the explant type (typically 5-8 minutes). Finally, to eliminate all traces of the toxic sterilant, the explants were washed 4-5 times with sterile double distilled water.

2. Preparation of basal media and plant growth regulators

Murashige and Skoog (MS) basal medium [12] (Murashige and Skoog 1962) was utilized for all *in vitro* culture phases, including initial shoot induction, cell callogenesis, shoot elongation and proliferation. For rhizogenesis, half strength MS medium (½MS) was prepared to induce physiological stress conducive to rooting. The media were fortified with 3% (w/v) analytical grade sucrose as a carbon source.

Plant growth regulators (PGRs) encompassing cytokinins: 6-benzylaminopurine (BAP), Kinetin (Kn) and auxins: Indole-3-acetic acid (IAA), α-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) were prepared as precise stock solutions and supplemented into the media either individually or in synergistic combinations prior to sterilization. For the callogenesis experiments, 2,4-D was used at concentrations of 1.0-2.0 mg/l, both alone and in structural combinations with cytokinins (BAP, Kn) and auxins (NAA, IAA). The pH of all culture media was digitally adjusted to 5.8 using 1N NaOH or 1N HCl. The media were subsequently solidified using 0.8% (w/v) agar (Himedia, India). Aliquots of 30 ml and 40 ml of the media were dispensed into 2 cm × 15 cm test tubes and 100 ml conical flasks, respectively. The culture vessels were tightly sealed with cotton plugs and aluminum foil and autoclaved at 121°C at a pressure of 1.9 kg/cm² for 20 minutes.

3. Culture incubation conditions

Inoculations were strictly performed within the sterilized environment of the laminar airflow cabinet using flame sterilized surgical scalpels and forceps. The cultured explants were transferred to a controlled culture room maintained at a temperature of 25 ± 2°C. The photoperiod was regulated at a 14-hour continuous light and 10-hour continuous dark phase, powered by cool white fluorescent tubes emitting a light intensity of 2000-3000 lux.

4. Subculture, elongation and rooting protocols

Proliferating primary multiple shoot buds (MSBs) were aseptically rescued and subcultured at 15-20 day intervals onto fresh media containing optimal PGR combinations to promote further multiplication and elongation. Callus tissue obtained from the inoculation of leaf and internode segments was systematically subcultured at 20-day intervals on identical or PGRs adjusted media to monitor texture and proliferation growth patterns. Elongated micro shoots attaining a length of 2.5-4.0 cm were individually excised and transferred to freshly prepared half strength MS rooting media containing various concentrations of IBA, IAA or NAA to initiate rhizogenesis.

5. Acclimatization and Transplantation

In vitro generated plantlets possessing a well-developed root system after 30 days of rooting culture were subjected to a gradual *ex vitro* acclimatization process. Initially, the lids of the culture vessels were loosened and maintained in the culture room for 24 hours. The vessels were subsequently transferred outside the culture room for 6 hours, followed by 12 hours the next day. The seedlings were then carefully extracted and the roots were gently washed under running tap water to remove residual agar. The seedlings were transplanted into small plastic pots containing a sterilized substrate mixture of garden soil and compost in a 2:1 ratio. The soil was pre-treated with 0.1% Agrosan (fungicide) to prevent pathogenic fungal infections. To maintain a high micro humidity environment, the pots were misted with water every 24 hours. Following the emergence of new foliage 10 days approximately, the fully hardened seedlings were successfully transferred to the open field.

6. Experimental design and statistical analysis

All experiments were structured using a Completely Randomized Design (CRD). For shoot induction and callogenesis parameters, each experiment consisted of five to twelve replicates. Data on morphogenic responses, including the percentage of explant response, days required for shoot or callus induction, average number of MSBs per explant, callus texture/color, shoot elongation (cm), rooting percentage and root lengths (cm), were recorded systematically. The collected data were statistically analyzed and the results were expressed as the Mean ± Standard Error (SE) using standard deviation calculations.

Results

1. Direct Multiple Shoot Buds (MSBs) induction from nodal segments

The morphogenic efficacy of full strength MS medium supplemented with various concentrations of BAP and Kn, both individually and in combination with NAA, was evaluated for direct MSBs induction from nodal explants of *A. curassavica*. PGR free full strength and half strength MS media were also utilized.

The data, summarized in Table 1, indicate that PGR free half strength MS medium completely failed to elicit any morphogenic response. Full strength MS medium without PGRs yielded a negligible response, producing only 3.17 ± 0.29 shoots per explant in 50% of the cultures after a prolonged 25-28 days. In stark contrast, the inclusion of cytokinins significantly accelerated and multiplied shoot bud differentiation. The highest frequency of MSBs induction (92%) and the maximum number of shoots per nodal segment (5.50 ± 0.22) were recorded on MS medium supplemented with 2.0 mg/l BAP combined with 0.5 mg/l NAA (Fig. 1). Under this optimal combination, shoot induction was initiated rapidly within 16-19 days of culture. This was closely followed by the combination of 1.0 mg/l BAP + 0.5 mg/l NAA, which yielded an 83% response with an average of 5.17 ± 0.17 MSBs per explant. Comparatively, combinations utilizing Kn and NAA were noticeably less effective, with 2.0 mg/l Kn + 0.5 mg/l NAA producing a maximum of 4.83 ± 0.31 shoots in 75% of the cultured explants.

2. Comparative MSBs proliferation between nodal segments and shoot apices

To determine explant superiority and evaluate altered auxin-cytokinin matrices, nodal segments and shoot apices were cultured on MS media supplemented with equal ratio high concentrations of BAP or Kn paired with NAA or IAA (Table 2).

In vitro developed Nodal segments consistently demonstrated superior morphogenic competence over *in vitro* raised shoot apices across all treatments. For nodal segments, the maximum induction rate (85%) and the highest average MSBs count (2.40 ± 0.35) were observed on MS medium containing 2.0 mg/l BAP + 2.0 mg/l NAA within 21-23 days (Fig. 2). When IAA was substituted for NAA the response marginally declined to 80%, yielding 2.28 ± 0.31 shoots per explant. Shoot apices exhibited a parallel but slightly subdued trend; the highest proliferation from shoot apices (75%) was recorded on 2.0 mg/l BAP + 2.0 mg/l NAA, yielding 2.33 ± 0.35 shoots within 23-25 days (Fig. 3). The lowest response for both explant types was consistently observed in media supplemented exclusively with Kn (1.0 mg/l), validating the absolute superiority of BAP combined with strong auxins like NAA for direct organogenesis in *A. curassavica*.

3. Effect of 2,4-D individually and in combination with BAP and Kn on callus induction from *in vitro* developed leaf and internode explants

To evaluate somatic cell plasticity and organize dedifferentiation tissues, *in vitro* derived leaf and internode segments were cultured on 0.8% (w/v) agar solidified MS medium fortified with 2,4-D individually and in structural combinations with cytokinins (BAP, Kn) and auxiliary auxins (NAA, IAA). Within 20-30 days of inoculation, cell division at the wound edges gave rise to either Greenish Compact Callus (GCC) or Whitish Compact Callus (WCC). The quantitative results, compiled after 20 days of cultivation, are presented in Table 3.

For leaf segment explants, the maximum callogenic response (75%) was achieved on MS medium containing 1.0 mg/l 2,4-D + 1.0 mg/l BAP, yielding a healthy greenish compact callus within 22-24 days (Fig. 4). This was followed by the combination of 1.0 mg/l 2,4-D + 1.0 mg/l NAA, which recorded a 70% callus formation frequency with GCC characteristics in 23-25 days. The lowest percentage of leaf explants initiating callus (50%) occurred on MS medium fortified exclusively with 1.0 mg/l 2,4-D after a prolonged delay of 28-30 days.

For internode explants, callogenesis was significantly more pronounced and accelerated. The highest percentage of greenish compact callus development (90%) was successfully generated on MS medium supplemented with 1.0 mg/l 2,4-D + 1.0 mg/l BAP, initiating callus cell division within 20-22 days of culture (Fig. 5). This optimized response was followed closely by MS medium supplemented with 1.0 mg/l 2,4-D + 1.0 mg/l NAA, showing an 85% callus response with a GCC phenotype within 21-23 days. The lowest callogenic frequency from internode tissues (65%) was found on MS medium containing 1.0 mg/l 2,4-D alone after 26-29 days of culture.

4. Longitudinal shoot elongation dynamics

Following primary proliferation, the *in vitro* regenerated shoot buds required secondary cultivation to achieve functional anatomical length prior to rooting. The micro shoots were transferred to MS elongation matrices fortified with distinct cytokinins (BAP, Kn) and NAA (Table 4).

Over a 30days culture period, the combination of BAP and NAA demonstrated dramatic longitudinal elongation superiority. The absolute highest incremental increase in shoot length (3.18 ± 0.32 cm) was attained on MS medium containing 2.0 mg/l BAP + 1.0 mg/l NAA, finalizing at a total average length of 4.88 ± 0.32 cm (Fig. 6). Conversely, media formulations relying solely on Kn proved highly inefficient for extension growth, with 1.0 mg/l Kn yielding the absolute lowest net length increase of just 0.87 ± 0.18 cm over the same 30-day temporal window (Fig. 7).

5. Rhizogenesis architecture

Elongated shoots (2-3 cm) were individually excised and inoculated into half strength MS basal media fortified with IBA, IAA and NAA to stimulate adventitious root formation. Data captured 30 days post inoculation (Graph 1-3) unequivocally established the superiority of IBA for rhizogenesis in *A. curassavica*.

The peak rooting percentage (95%) was recorded on half strength MS medium supplemented with 2.0 mg/l IBA (Graph 1). This medium architecture also induced the maximum density of root primordia, achieving 6.35 ± 0.31 roots per shoot and facilitated the maximum (3.67 ± 0.21 cm) longitudinal root extension (Graph 2-3, Fig. 8). A synergistic combination of 0.5 mg/l IAA + 0.5 mg/l IBA also performed robustly, yielding a 90% rooting frequency with 6.17 ± 0.17 roots per shoot. In sharp contrast, auxin free half strength MS medium resulted in poor rhizogenic competence, logging only a 50% rooting rate with sparse and stunted root architecture (3.83 ± 0.17 roots; 1.33 ± 0.21 cm length).

6. *Ex vitro* acclimatization

Following successful *in vitro* rhizogenesis, the seedlings equipped with dense, functional root networks underwent a highly controlled, phased hardening process. Following transfer to the semi controlled nursery environment in plastic pots containing the 2:1 garden soil and compost mixture, the seedlings demonstrated vigorous vegetative growth. New leaf emergence was validated within 10 days, ultimately resulting in an excellent final *ex vitro* survival rate of 80% upon field transfer (Fig. 9).

Table 1: Development of MSBs from nodal explant of *A. curassavica* on half MS, full MS and MS medium supplemented with different PGRs.

Media	PGRs Conc. (mg/l)	% of explant response	Time (d) required for induction of MSBs	Average no. of MSBs/ explants (mean ± SE)
½ MS0	-	-	-	-
MS0	-	50	25-28	3.17 ± 0.29
BAP	1.0	58	23-26	3.83 ± 0.21
	2.0	75	19-22	4.66 ± 0.34
Kn	1.0	58	24-27	3.33 ± 0.31
	2.0	67	21-24	4.00 ± 0.21
BAP + NAA	1.0 + 0.5	83	17-20	5.17 ± 0.17
	2.0 + 0.5	92	16-19	5.50 ± 0.22
Kn + NAA	1.0 + 0.5	67	20-23	4.33 ± 0.22
	2.0 + 0.5	75	18-21	4.83 ± 0.31

*d = days; **MSBs = Multiple Shoot Buds; ***values are the mean ± SE of each experiment consist of 12 replicates.

Table 2: Effect of different concentrations and combinations of BAP and Kn individually and in combination with NAA and IAA on MS medium fortified for induction of MSBs from *in vitro* developed nodal segment and shoot apices of *A. curassavica*.

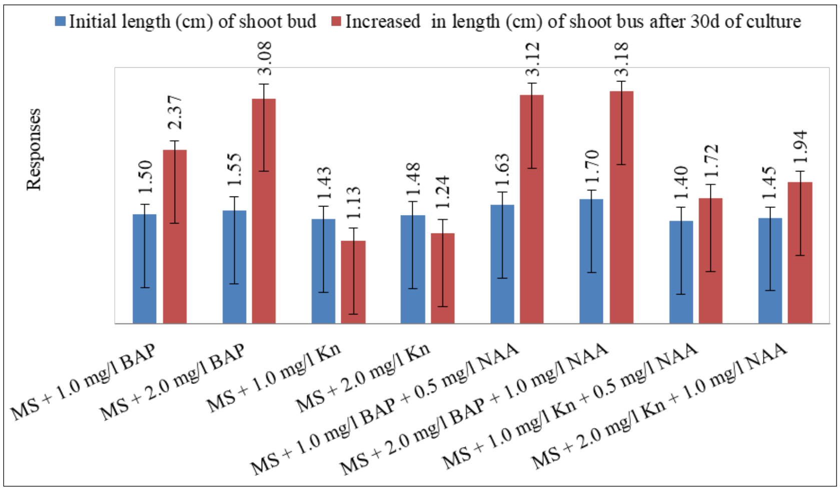
Explant	PGRs supplement in the media (mg/l)		% of explants showing proliferation	time(d) required for induction of shoot buds	No. of MSBs Produced (mean ± SE)
Nodal segment	BAP	1.0	55	27-29	1.33 ± 0.34
	BAP	2.0	60	26-28	1.51 ± 0.39
	Kn	1.0	45	29-31	1.16 ± 0.33
	Kn	2.0	50	28-30	1.22 ± 0.39
	BAP+ NAA	1.0 + 1.0	75	23-25	2.14 ± 0.32
		2.0 + 2.0	85	21-23	2.40 ± 0.35
	BAP + IAA	1.0 + 1.0	75	23-25	2.08 ± 0.36
		2.0 + 2.0	80	22-24	2.28 ± 0.31
	Kn + NAA	1.0 + 1.0	65	25-27	1.83 ± 0.36
		2.0 + 2.0	70	24-26	1.97 ± 0.35
	Kn + IAA	1.0 + 1.0	60	26-28	1.54 ± 0.24
		2.0 + 2.0	55	27-29	1.36 ± 0.27
Shoot apices	BAP	1.0	45	29-31	1.22 ± 0.38
	BAP	2.0	50	28-30	1.46 ± 0.32
	Kn	1.0	35	31-33	1.05 ± 0.24
	Kn	2.0	40	30-32	1.17 ± 0.35
	BAP+ NAA	1.0 + 1.0	65	25-27	2.07 ± 0.31
		2.0 + 2.0	75	23-25	2.33 ± 0.35
	BAP + IAA	1.0 + 1.0	65	25-27	1.93 ± 0.35
		2.0 + 2.0	70	24-26	2.21 ± 0.32
	Kn + NAA	1.0 + 1.0	55	27-29	1.72 ± 0.24
		2.0 + 2.0	60	26-28	1.89 ± 0.23
	Kn + IAA	1.0 + 1.0	50	28-30	1.46 ± 0.28
		2.0 + 2.0	45	29-31	1.27 ± 0.31

*d = days; **MSBs = Multiple Shoot Buds;
***values are the mean ± SE of each experiment consist of 10 replicates.

Table 3: Effect of 2, 4-D individually and in combination with BAP and Kn on induction of callus tissue from *in vitro* raised leaf and internode of *A. curassavica* on 0.8% (w/v) agar solidified MS medium.

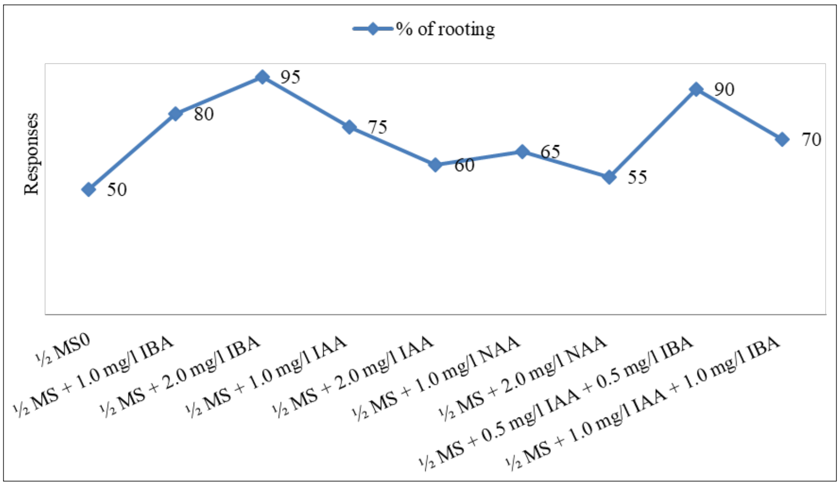
Explant	PGRs concentrations (mg/l)		% of leaf/ internode producing callus	Time (d) required for callus induction	Color and texture of induced callus
Leaf segment	2,4-D	1.0	50	28-30	GCC
		2.0	55	26-28	GCC
	2,4-D + BAP	0.5 + 1.0	65	24-26	GCC
		1.0 + 1.0	75	22-24	GCC
	2,4-D + Kn	0.5 + 1.0	60	25-27	WCC
		1.0 + 1.0	65	24-26	WCC
	2,4-D + NAA	0.5 + 1.0	65	24-26	GCC
		1.0 + 1.0	70	23-25	GCC
	2,4-D + IAA	0.5 + 1.0	60	25-27	WCC
		1.0 + 1.0	65	24-26	WCC
Internode	2,4-D	1.0	65	26-29	GCC
		2.0	70	25-28	GCC
	2,4-D + BAP	0.5 + 1.0	80	22-25	GCC
		1.0 + 1.0	90	20-22	GCC
	2,4-D + Kn	0.5 + 1.0	75	23-26	WCC
		1.0 + 1.0	80	22-25	WCC
	2,4-D + NAA	0.5 + 1.0	75	23-26	GCC
		1.0 + 1.0	85	21-23	GCC
	2,4-D + IAA	0.5 + 1.0	70	25-28	WCC
		1.0 + 1.0	75	23-26	WCC

*d = days; GC = Greenish Compact Callus; WC = Whitish Compact Callus;
**values are the mean ± SE of each experiment consist of five replicates.



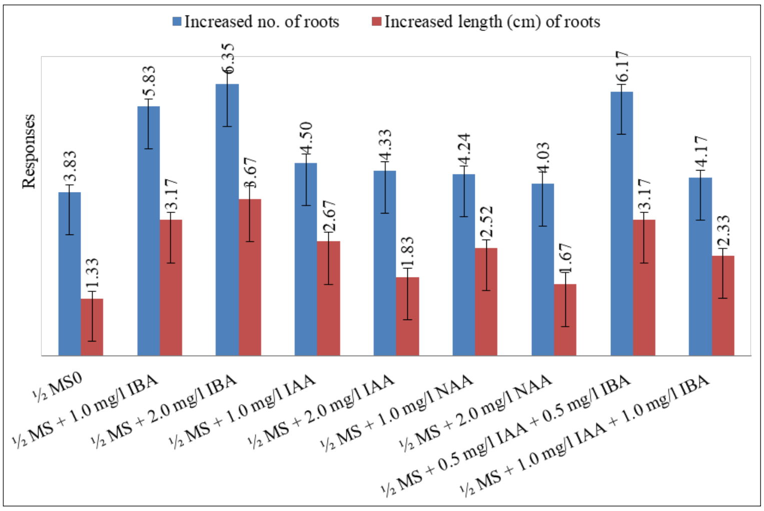
*d = days; values are the means ± SE of each experiment consist of five replicates.

Graph 1: Elongation of directly *in vitro* developed shoot buds of *A. curassavica* on MS medium fortified with different concentrations and combinations of PGRs.



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

Graph 2: Percentage of rooting in *in vitro* elongated shoot bud of *A. currasavica* on PGR free half MS and PGRs supplemented half strength MS medium.



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

Graph 3: Increased number and length of roots in *in vitro* elongated shoot bud of *A. currasavica* on PGR free half MS and PGRs supplemented half strength MS medium.

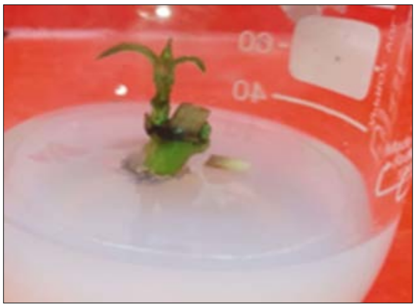


Fig. 1: Initiation of MSBs of *A. curassavica* from nodal segment on MS + 2.0 mg/l BAP + 0.5 mg/l NAA

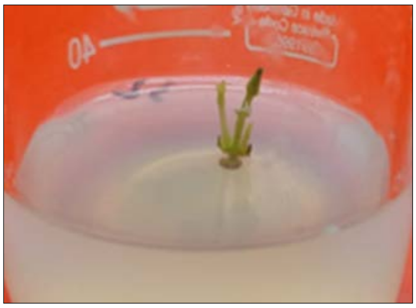


Fig. 2: Development of MSBs of *A. curassavica* from *in vitro* derived nodal segment on MS + 2.0 mg/l BAP + 2.0 mg/l NAA

Fig. 3: Maximum proliferation of MSBs of *A. curassavica* from *in vitro* raised shoot apices on MS + 2.0 mg/l BAP + 2.0 mg/l NAA

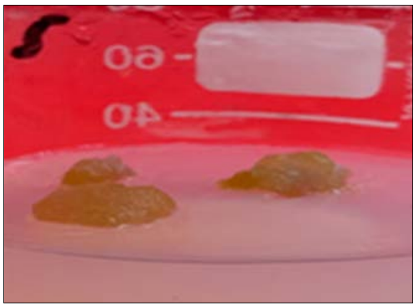


Fig. 4: Initiation of greenish compact callus in *A. curassavica* from *in vitro* derived leaf segment on MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP

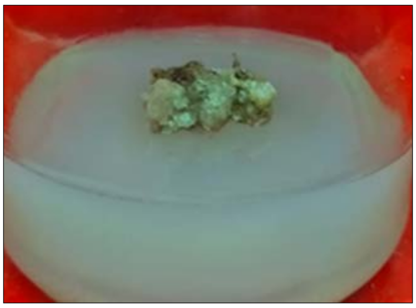


Fig. 5: Development of greenish compact callus in *A. curassavica* from *in vitro* raised internode on MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP

Fig. 6: Elongation of *A. curassavica* on MS + 2.0 mg/l BAP + 1.0 mg/l NAA

Fig. 7: Elongation of *A. curassavica* on MS + 1.0 mg/l Kn

Fig. 8: Induction of roots in elongated shoot buds of *A. currasavica* with ½ MS + 2.0 mg/l IBA

Fig. 9: Acclimatization of *A. currasavica* in outside the environment

Discussion

The development of a robust, standardized micropropagation pathway is an absolute prerequisite for the commercial exploitation and ecological conservation of highly targeted medicinal species like *Asclepias curassavica*. Due to the persistent accumulation of valuable cardenolides and phenolics, wild populations are frequently decimated. This study successfully mapped a complete *in vitro* direct organogenesis and callus induction protocol, circumventing the physiological constraints of natural seed germination and establishing a secondary pathway for biosynthetic evaluation of dedifferentiated cells.

The results clearly establish that direct multiple shoot buds (MSBs) induction is strongly governed by the exogenous application of cytokinins, specifically BAP. The pronounced superiority of BAP over Kinetin observed in *A. curassavica* nodal cultures aligns precisely with the physiological role of BAP. Exogenous BAP rapidly disrupts apical dominance, aggressively promoting the activation and subsequent proliferation of dormant lateral meristems [13]. Furthermore, BAP exhibits higher *in vitro* molecular stability and resists enzymatic degradation by plant tissues much more efficiently than other purine derivative cytokinins.

However, maximum morphogenic expression was not achieved with BAP alone, but rather through a carefully calibrated synergistic interaction between BAP and a moderate concentration of the synthetic auxin, NAA (2.0 mg/l BAP + 0.5 mg/l NAA). This specific PGR matrix triggered the highest proliferation rate (92%) and the highest shoot count (5.50 ± 0.22). This observation implies that while cytokinins initiate cell division and meristem organization, a baseline threshold of auxin is required to modulate vascular tissue differentiation and stabilize the growing primordial [14]. This potent BAP-NAA synergy is well documented in the direct organogenesis of other critical medicinal plants within the Apocynaceae and affiliated families, confirming the foundational validity of this response mechanism [15-17].

Between the two explants typologies evaluated for direct shoot production, nodal segments demonstrated a distinct organogenic superiority over shoot apices. Nodal tissues naturally harbor axillary meristems which are pre-programmed for shoot generation. When stimulated by optimal *in vitro* conditions, these existing meristematic centers rapidly transition into active shoot buds without requiring extensive cellular reprogramming [18]. Shoot apices, conversely, tend to expend energy maintaining primary apical extension rather than maximizing lateral axillary multiplication.

In addition to direct multiplication, somatic cell dedifferentiation was evaluated through callogenesis experiments utilizing 2,4-D. Callus proliferation was highly sensitive to the combination of 2,4-D (auxin) and BAP (cytokinin), where internode explants exhibited superior callus induction rates (90%) and accelerated timelines compared to leaf explants. The synergism of auxins (such as 2,4-D) with cytokinins (BAP) in inducing cell dedifferentiation is a classical phenomenon in somatic tissue plasticity [19]. 2,4-D acts as a strong synthetic auxin that triggers chromatin decondensation, causing mature, differentiated somatic cells to lose their specialized functions and return to a highly proliferative state. When balanced by BAP, cellular replication is significantly accelerated, forming compact, chlorophyll rich greenish parenchymatous masses (GCC) or whitish compact masses (WCC) where Kn or other PGRs were substituted. This callogenic efficiency is consistent with patterns observed in other medicinal plants, including *Aristolochia indica* [20] and *Stephania pierrei* [21]. The marked superiority of internode tissues over leaves for callogenesis is likely due to the proximity of actively dividing vascular cambial layers in stem structures, which undergo cellular dedifferentiation more readily than highly specialized, mature leaf mesophyll tissues.

For shoot elongation, achieving sufficient internodal extension is a mandatory step before root induction to ensure proper *ex vitro* physiological function and photosynthetic capacity. The combination of 2.0 mg/l BAP and 1.0 mg/l NAA facilitated maximum longitudinal growth. While elevated BAP initiates shoot multiplication, maintaining a sustained but balanced BAP:NAA ratio allows the micro shoots to absorb nutrients actively and expand their cell walls longitudinally, a trend parallel to the elongation dynamics reported for *Rauvolfia serpentina* [22] and *Aloe vera* [23].

Successful rhizogenesis is the ultimate determinant of a micropropagation protocol's viability. In this study, ½ MS media fortified with IBA (2.0 mg/l) significantly outperformed IAA, NAA and hormone free controls. The specific structural chemistry of Indole-3-butyric acid (IBA) makes it less sensitive to rapid photo oxidation and endogenous enzymatic degradation compared to IAA [24]. Operating as a "slow release" auxin repository, IBA maintains continuous, steady-state molecular signaling at the basal cut ends of the micro-shoots, orchestrating massive adventitious root primordia initiation and subsequent rapid cellular elongation. Similar superior rhizogenic behaviors of IBA have been extensively reported in diverse botanical tissues, including *Bacopa monnieri* [25] and *Solanum nigrum* [26], strongly validating the results obtained for *A. curassavica*. The highly successful acclimatization rate (80%) of these well rooted *A. curassavica* seedlings guarantees that this protocol can be seamlessly scaled to an industrial level.

Conclusion

This study successfully engineered a highly efficient, reproducible and standardized direct propagation and callus induction protocol for *Asclepias curassavica* L., by passing the ecological and physiological constraints of wild seed germination. The precise calibration of BAP and NAA on nodal explants stimulated massive multiple shoot buds (MSBs) proliferation, while the application of 1.0 mg/l 2,4-D and 1.0 mg/l BAP on leaf and internode explants generated a highly robust callogenic response. Furthermore, the application of 2.0 mg/l IBA on half strength MS medium triggered a highly robust adventitious rooting response. The 80% survival rate during *ex vitro* acclimatization highlights the industrial scalability of this pathway. By ensuring a continuous, year round supply of uniform, disease free clones and standardized callus tissues, this protocol fundamentally safeguards the natural biodiversity of Bangladesh while providing a strong biotechnological foundation for secondary metabolite analysis, cell suspension studies, and commercial cultivation for the pharmaceutical and herbal industries.

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