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Development of efficient *in vitro* protocol for mass scale propagation of *Achyranthes aspera* L. - A medicinal plant species of Bangladesh

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Abstract
Achyranthes aspera L. is a highly valuable medicinal plant in Bangladesh, traditionally exploited for its bioactive secondary metabolites. Over harvesting from natural habitats necessitates the development of a reliable, large scale propagation protocol. This study establishes an efficient, highly reproducible *in vitro* seed germination and direct micropropagation pathway for *A. aspera* utilizing nodal segments and shoot apices. The maximum *in vitro* seed germination rate (100%) was achieved on Murashige and Skoog (MS) medium supplemented with 2.0 mg/l 6-benzyl amino purine (BAP) within 6-9 days. For multiple shoot buds (MSBs) induction, shoot apices exhibited superior organogenic potential compared to nodal segments. The highest frequency of MSBs induction (94%) and maximum number of shoots per explant (5.10 ± 0.37) were recorded on MS medium fortified with 2.0 mg/l BAP and 1.0 mg/l Indole-3-acetic acid (IAA) within 11-14 days. Nodal segments also performed optimally under BAP influence, yielding 4.33 ± 0.32 shoots per explant (92% induction) on MS medium with 2.0 mg/l BAP and 1.0 mg/l α-Naphthalene acetic acid (NAA). For shoot elongation, MS medium containing 1.0 mg/l BAP and 0.5 mg/l NAA facilitated the maximum longitudinal growth (4.68 ± 0.32 cm). Vigorous *in vitro* rhizogenesis (94% rooting frequency, 5.46 ± 0.20 roots per shoot, 3.98 ± 0.17 cm increased length) was achieved on half-strength MS medium supplemented with 1.0 mg/l Indole-3-butyric acid (IBA) after 30d of culture. Successfully rooted seedlings were acclimatized *ex vitro* with an 80% survival rate, displaying normal morphological development without somaclonal variations. This optimized protocol provides a robust foundation for the commercial cultivation, pharmaceutical exploitation and germplasm conservation of *A. aspera*.

Keywords: *Achyranthes aspera*, direct organogenesis, *in vitro* seed germination, micropropagation, plant growth regulators

Introduction
The utilization of medicinal plants forms the cornerstone of traditional and modern pharmacognosy, contributing significantly to global healthcare systems. *Achyranthes aspera* L., commonly known as "Apang" or "Upothlengra" in Bangladesh, is an annual, erect, stiff herbaceous weed belonging to the Amaranthaceae family. Geographically distributed across the tropical and subtropical regions, it holds immense therapeutic significance in indigenous ethno-medicine [1-2]. Pharmacological profiling of *A. aspera* reveals a rich matrix of secondary metabolites, predominantly triterpenoid saponins (e.g. achyranthine), ecdysterone, alkaloids, flavonoids and essential phenolic compounds [3]. These bioactive compounds confer potent antimicrobial, anti-inflammatory, antioxidant, diuretic and wound healing properties, driving its high demand in the rapidly expanding pharmaceutical industry [4-5].
Despite its tremendous medicinal value, the commercial supply of *A. aspera* relies entirely on indiscriminate wild harvesting. This unregulated exploitation, compounded by habitat fragmentation, urbanization and poor natural seed viability under fluctuating climatic constraints, poses a severe threat to its localized populations in Bangladesh. Consequently, natural reserves are rapidly depleting, positioning the species under ecological stress [6-7]. Conventional propagation *via* seeds is frequently hampered by low germination percentages, high susceptibility to soil borne pathogens and genetic heterogeneity [8]. Thus, there is an urgent requisite for an alternative, efficient and rapid multiplication strategy.
In vitro micropropagation serves as a powerful biotechnological intervention for the mass scale clonal multiplication and *ex situ* conservation of elite medicinal germplasm, independent of seasonal constraints [9-10]. Direct organogenesis, in particular, ensures the genetic fidelity of the regenerants by bypassing the intermediate callus phase, making it the preferred pathway for pharmaceutical cultivation [11]. While micropropagation protocols have been documented for various medicinal taxa within the Amaranthaceae family and allied groups [12-13], comprehensive, data driven frameworks optimizing direct shoot bud's proliferation and seed germination kinetics specifically for the Bangladeshi ecotype of *A. aspera* remain profoundly lacking.
The current investigation was designed to address this critical research gap. The primary objective was to establish a highly reproducible, robust and economically viable protocol for the rapid *in vitro* seed germination and direct mass scale micropropagation of *Achyranthes aspera* L. By systematically evaluating the synergistic effects of various exogenous cytokinins and auxins on nodal segments and shoot apices, this study aims to facilitate sustainable biomass production, supporting both pharmaceutical industries and ecological restoration initiatives.

Materials and Methods
Source of plant material and explant preparation
Healthy, disease free seeds, mature shoot apices and nodal segments of *Achyranthes aspera* L. (Fig. 1) were collected from naturally growing in the experimental field. The collected materials were thoroughly washed under

continuous running tap water for 30 minutes to remove surface debris and microbial loads. The explants were subsequently treated with a 1% Savlon solution (ACI Pharma, Bangladesh) containing a few drops of Tween-20 for 5-10 minutes under constant agitation. To ensure complete removal of the surfactant, the explants were rinsed 3-4 times with sterile distilled water.

Aseptic manipulations were performed inside a laminar airflow cabinet. The explants were subjected to a brief immersion (60 seconds) in 70% (v/v) ethanol, followed by surface sterilization using a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution. The duration of the $HgCl_2$ treatment was optimized for each explant type to maximize decontamination while preserving tissue viability. Finally, the sterilant was completely eliminated by 4-5 consecutive washes in sterile double distilled water. The sterilized shoot apices and nodal segments were excised into uniform pieces (0.5-1.0 cm) using a sterile surgical scalpel for immediate inoculation.

Basal media preparation and culture conditions

The standard Murashige and Skoog (MS) basal medium [14] was utilized for all morphogenic phases, including seed germination, shoot proliferation and elongation. For rhizogenesis, half-strength (1/2 MS) medium was employed. The media were enriched with 3% (ω/υ) analytical-grade sucrose and solidified with 0.8% (ω/υ) agar (Himedia, India). The pH of the culture media was digitally adjusted to 5.80 ± 0.02 using 1N NaOH or 1N HCl prior to the addition of agar.

The media were dispensed into appropriate culture vessels (test tubes or conical flasks) and autoclaved at 121 ºC under 1.5 kg/cm^2 pressure for 30 minutes. All cultures were incubated in a controlled growth chamber maintained at a temperature of 25 ± 2 ºC. The photoperiod was regulated at a 14-hour continuous light / 10-hour dark cycle, illuminated by cool white fluorescent tubes emitting a light intensity of 2000-3000 lux.

Plant Growth Regulators (PGRs)

To induce varied morphogenic responses, the basal MS media were fortified with highly precise configurations of exogenous cytokinins and auxins. Stock solutions of 6-benzyl amino purine (BAP) and Kinetin (Kn) were dissolved in dilute HCl, whereas Indole-3-acetic acid (IAA), α-Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) were dissolved in 1 N NaOH.

- ***In vitro* seed germination:** MS media were tested without PGRs (MS0) and with varying concentrations of BAP, Kn, IAA and NAA (1.0-2.0 mg/l), individually and in combination.

- **Direct MSBs proliferation:** MS media were supplemented with BAP or Kn (1.0-2.0 mg/l) individually, or synergistically combined with auxins (NAA or IAA at 0.5-1.0 mg/l) exclusively for direct organogenesis from shoot apices and nodal segments.

- **Shoot elongation:** Elongation matrices consisted of MS medium supplemented with BAP or Kn (1.0-2.0 mg/l) combined with NAA or IAA (1.0 mg/l).

- **Rhizogenesis:** Elongated micro shoots (2.0-3.0 cm) were transferred to half strength MS media fortified with IBA, IAA, or NAA (0.5-2.0 mg/l) to induce root architecture.

Acclimatization and ex *vitro* transfer

Seedlings developing a profound and robust root system *in vitro* were selected for hardening. The culture vessels were initially kept open within the culture room for 24 hours, then transferred outside the culture room for progressively increasing durations (6 to 12 hours) over 5 days to adapt to ambient humidity and temperature. The agar matrix was gently washed from the root system using running tap water. The seedlings were transplanted into small plastic pots containing a sterile combination of garden soil and compost (1:1 ratio), pre-treated with a 0.1% Agrosan fungicidal solution. High humidity was maintained by spraying water every 24 hours. After successful establishment approximately 10 days, the hardened plants were transferred to open field conditions.

Experimental design and statistical analysis

All experiments were conducted utilizing a Completely Randomized Design (CRD). Each treatment condition comprised exactly 5-10 replicates. Quantitative data pertaining to germination frequency, shoot multiplication rate, shoot length, root count and root length were recorded chronologically. The statistical variance is expressed as the Mean ± Standard Error (SE), calculated using standard deviation formulas for empirical accuracy.

Results

***In vitro* seed germination**

The morphological response of *A. aspera* seeds cultured on varying MS formulations is detailed in Table 1. Initial radicle protrusion and subsequent green seedling emergence demonstrated high sensitivity to the PGRs matrix. Half strength MS0 medium showed 50% seed germination after 12-15 days while PGR free MS basal medium yielded a moderate germination rate of 70% requiring 7-9 days, cytokinin supplementation markedly accelerated the kinetic timeline and success frequency.

The absolute maximum seed germination rate (100%) was achieved on MS medium fortified exclusively with 2.0 mg/l BAP, effectively breaking dormancy and generating robust green seedlings within a narrow 6-9-day timeline (Fig. 1). Kinetin formulations also proved highly effective, with 2.0 mg/l Kn facilitating a 90% germination rate within 7-9 days (Fig. 2). Conversely, isolated auxin treatments (IAA and NAA) suppressed the germination velocity, extending the induction period up to 15 days and restricting the germination percentage to 60-80%. Combined synergistic treatments of BAP with IAA or NAA (1.0 mg/l each) yielded moderate success (80%).

Direct multiple shoot buds (MSBs) proliferation

Direct organogenesis was strictly observed, devoid of any intermediary callus phase, validating the genetic stability of the morphogenic sequence. The differential response of natural shoot apices and nodal segments to cytokinin-auxin matrices over a 45 days of culture span is quantifying in Table 2.

Shoot apices culture

Natural shoot apices exhibited exceptional morphogenic plasticity. The maximum proliferation frequency (94%) was

recorded on MS medium supplemented with a specific synergy of 2.0 mg/l BAP + 1.0 mg/l IAA (Fig. 3). This optimal formulation induced MSBs rapidly within 11-14 days, culminating in the highest shoot density of 5.10 ± 0.37 buds per explant. Substituting BAP with Kn (2.0 mg/l Kn + 1.0 mg/l IAA) slightly reduced the morphogenic output to 87% with 4.90 ± 0.37 buds per explant. The minimum response (75%) for shoot apices was observed on singly 1.0 mg/l Kn, yielding only 2.22 ± 0.20 shoots per explant over a delayed 18-23 days induction period.

Nodal segment culture

Natural nodal explants demonstrated highly efficient, albeit slightly lower, multiplication rates compared to shoot apices. The highest morphogenic frequency (92%) and maximum MSBs count (4.33 ± 0.32 per explant) occurred on MS medium integrated with 2.0 mg/l BAP + 1.0 mg/l NAA within a rapid 12-15 days window (Fig. 4). Kinetin based formulations (e.g. 2.0 mg/l Kn + 1.0 mg/l IAA) resulted in 3.82 ± 0.40 shoots per explant. The lowest proliferation parameters for nodal segments (65% frequency, 2.33 ± 0.24 shoots) were recorded on singly 1.0 mg/l BAP treatments. Overall, BAP demonstrated decisive superiority over Kinetin in overcoming apical dominance and initiating lateral bud break.

Shoot bud elongation

To secure anatomically viable plantlets, the densely clustered MSBs were excised and sub-cultured onto elongation media for 30 days. The longitudinal developmental metrics are presented in Graph 1.

The most profound longitudinal extension was observed when BAP was paired with NAA. The maximum net increase in shoot length (4.68 ± 0.32 cm) was achieved on MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l NAA, generating robust elongation (Fig. 5) followed by MS + 2.0 mg/l BAP + 1.0 mg/l NAA medium (4.26 ± 0.21 cm, Fig. 6). Kinetin also demonstrated strong elongation capabilities; 1.0 mg/l Kn paired with 0.5 mg/l NAA yielded a net length increase of 4.31 ± 0.27 cm (Fig. 7) followed by MS + 2.0 mg/l Kn + 1.0 mg/l NAA medium (3.91 ± 0.24 cm, Fig. 8). Conversely, the basal substitution of singly BAP (1.0 mg/l) suppressed elongation, resulting in the minimal recorded growth increment (1.92 ± 0.24 cm).

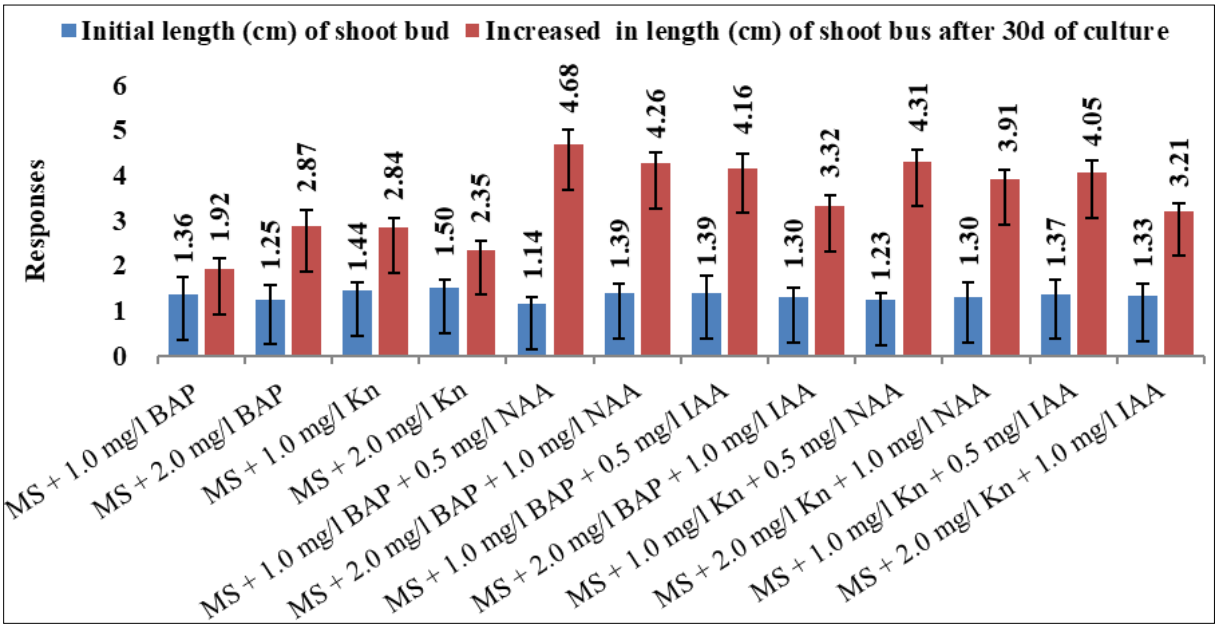
Rhizogenesis

The induction of a vigorous adventitious root system is the most critical determinant for successful ex *vitro* survival. Elongated micro shoots (2.0-3.0 cm) were cultured on half strength MS media fortified with varying auxins (Graph 2 and Graph 3).

IBA decisively outperformed IAA and NAA in orchestrating rhizogenesis. The highest root induction frequency (94%) was achieved on half strength MS medium supplemented with 1.0 mg/l IBA. This formulation generated the most robust root architecture, recording a maximum of 5.56 ± 0.20 roots per shoot with an average longitudinal extension of 3.98 ± 0.32 cm within 30 days of cvulture (Fig. 9). IAA treatments (1.0 mg/l) yielded the second-best response (87% rooting, 4.83 ± 0.24 roots per shoot, 3.03± 0.24 cm increased in root length). NAA proved to be the least effective rhizogenic agent; elevated concentrations (1.0 mg/l NAA) severely depressed root induction to 73%, producing a minimum of 3.47 ± 0.20 roots with a restricted length of 2.17 ± 0.23 cm.

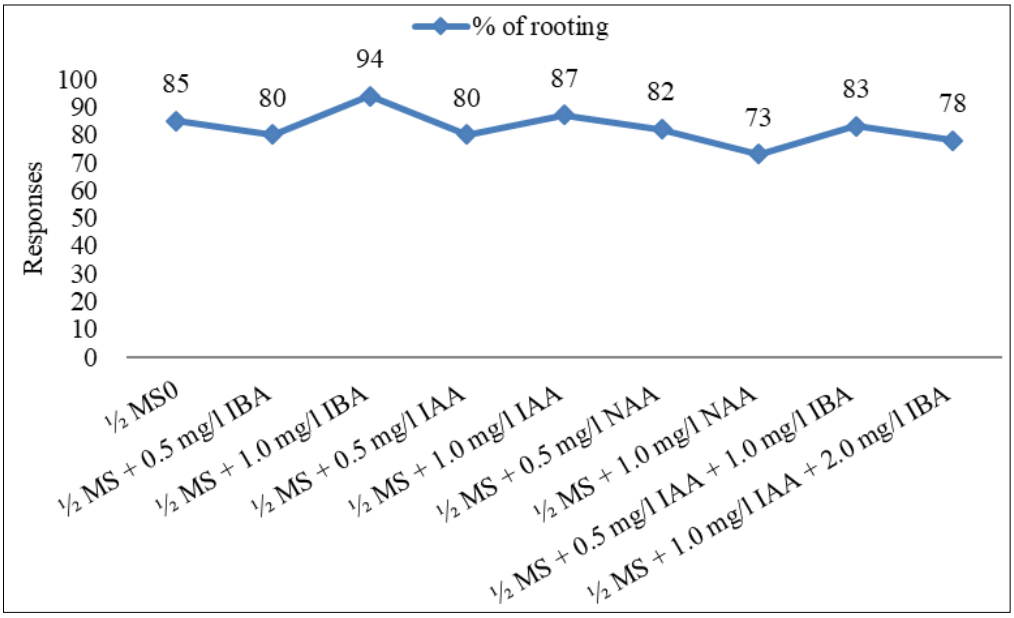
Acclimatization and ex *vitro* survival

Chronological hardening processes were highly successful for the *in vitro* generated *A. aspera* seedlings. Seedlings featuring profound root architectures derived from the optimal IBA media were transferred to the soil compost (1:1) matrix. The gradual exposure to ambient ex *vitro* humidity and photoperiods mitigated transplant shock. Ultimately, a commendable 80% survival rate was recorded. The acclimatized seedlings exhibited normal phenotypic morphology, vibrant photosynthetic tissue and standard growth vigor, devoid of any discernible somaclonal or anatomical aberrations.



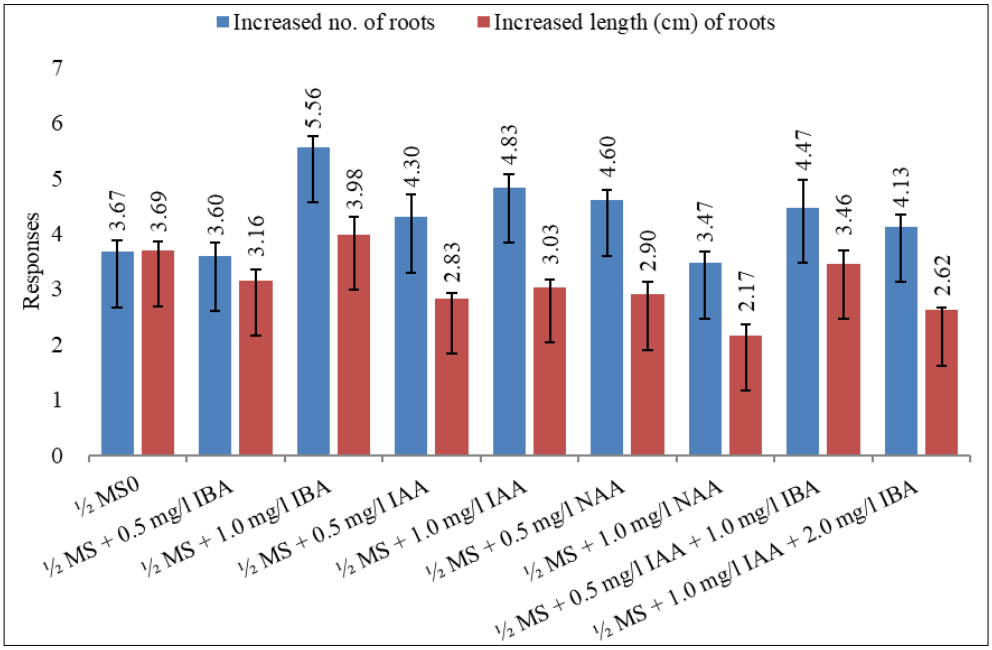
*d = days; MSBs = Multiple shoot buds; **values are the means ± SE of each experiment consist of five replicates.

Graph 1: Elongation of directly produced MSBs of *A. aspera* on agar solidified MS medium supplemented with different PGRs



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

Graph 2: Percentage of rooting in elongated shoot buds of *A. aspera* 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 seedlings of *A. aspera* on PGR free half MS and PGRs supplemented half strength MS medium

Fig 1: *In vitro* seed germination of *A. aspera* on MS medium with 2.0 mg/l BAP

Fig 2: *In vitro* seed germination of *A. aspera* on MS medium with 2.0 mg/l Kn

Fig 3: Development of MSBs from shoot apices of *A. aspera* on MS + 2.0 mg/l BAP + 1.0 mg/l IAA

Fig 4: Induction of MSBs from nodal segment of *A. aspera* on MS + 2.0 mg/l BAP + 1.0 mg/l NAA

Fig 5: Elongation of MSBs derived from shoot apices of *A. aspera* on MS + 1.0 mg/l BAP + 0.5 mg/l NAA

Fig 6: Elongation of shoot bud raised from shoot apices of *A. aspera* on MS + 2.0 mg/l BAP + 1.0 mg/l NAA

Fig 7: Elongation of MSBs derived from shoot apices of *A. aspera* on MS + 1.0 mg/l Kn + 0.5 mg/l NAA

Fig 8: Elongation of shoot bud raised from shoot apices of *A. aspera* on MS + 2.0 mg/l Kn + 1.0 mg/l NAA

Fig 9: Induction of roots in elongated plantlet of *A. aspera* on ½ MS + 1.0 mg/l IBA

Discussion

The development of an efficient, direct organogenesis protocol requires the precise manipulation of the *in vitro* microenvironment, predominantly *via* the delicate balance of exogenous plant growth regulators. This study successfully delineates a high frequency, mass scale multiplication pathway for *Achyranthes aspera* L., prioritizing genetic stability by completely bypassing the dedifferentiated callus stage.

The germination of seeds *in vitro* is regulated by the complex interplay of moisture, osmotic potential and hormonal signaling. In our investigation, MS medium fortified with 2.0 mg/l BAP achieved a superlative 100% germination rate within a tightly compressed timeline. Cytokinins like BAP are scientifically documented to antagonize abscisic acid (ABA) pathways within the seed endosperm, effectively breaking embryo dormancy and mobilizing nutritional reserves for rapid radicle protrusion [15-16]. The accelerated kinetics observed here align perfectly with physiological models where exogenous BAP supersedes the inherent PGRs limitations of wildly harvested Amaranthaceae seeds.

In the context of direct multiple shoot buds (MSBs) proliferation, natural shoot apices exhibited higher organogenic plasticity compared to natural nodal segments. This physiological variance can be attributed to the dense concentration of totipotent meristematic cells naturally localized in the apical dome [17]. BAP consistently demonstrated profound superiority over kinetin in initiating morphogenic responses across both explants. This superiority of BAP is widely acknowledged in global tissue culture literature due to its rapid cellular uptake, resistance to enzymatic degradation by cytokinin oxidases and its potent ability to dismantle apical dominance, thereby forcing lateral axillary meristems into active division [18-19]. Crucially, the highest MSBs yields necessitated synergistic auxin integration (2.0 mg/l BAP + 1.0 mg/l IAA for shoot apices; 2.0 mg/l BAP + 1.0 mg/l NAA for nodal segments). The inclusion of a low concentration auxin alongside a high concentration cytokinin creates an optimal intracellular PGRs gradient that regulates the cell cycle and directs the morphogenic destiny towards shoot pole formation [20-21]. Similar synergistic requirements for direct shoot proliferation have been reported in highly valuable medicinal taxa such as *Centella asiatica* [22], *Catharanthus roseus* [12] and *Boerhaavia diffusa* [23].

Longitudinal elongation of the clustered shoots was highly responsive to a balanced equi-molar ratio of cytokinin and auxin (1.0 mg/l BAP + 0.5 mg/l NAA). While high cytokinins are required for bud multiplication, their prolonged presence severely stunts internodal elongation. The reduction of BAP concentration and the introduction of NAA orchestrates cell wall relaxation and osmotic water uptake, driving cell elongation along the longitudinal axis [24].

The architecture of rhizogenesis was unequivocally dominated by IBA in half strength MS medium. The use of half strength MS reduces total nitrogen, imposing a mild osmotic and nutrient stress that physiological studies confirm promotes resource allocation towards root foraging behavior [25]. The superlative efficacy of IBA (1.0 mg/l yielding 5.56 ± 0.20 roots) over IAA and NAA is deeply rooted in its molecular biochemistry. IBA functions as a slow releasing auxin precursor; it is protected from rapid enzymatic oxidation *via* IAA oxidases and undergoes steady β-oxidation to convert into active IAA precisely at the site

of adventitious root initiation [26-27]. This slow, sustained PGRs release generates a continuous morphogenic signal, preventing the disorganized callus formation commonly induced by harsh synthetic auxins like NAA and instead promoting highly organized root meristems. This phenomenon is highly consistent with rooting architectures observed in *Bacopa monnieri* [28] and *Boerhaavia diffusa* [29]. The transition from the heterotrophic, highly humid *in vitro* environment to the autotrophic, desiccating ex *vitro* environment is a severe physiological bottleneck. The impressive 80% acclimatization success observed in this study underscores the anatomical competence (e.g. functional stomatal regulation and well developed epicuticular waxes) of the generated plantlets.

Conclusion

This investigation establishes a highly efficient, rigorously optimized protocol for the rapid *in vitro* seed germination and direct micropropagation of the vital medicinal plant, *Achyranthes aspera* L. By strictly utilizing direct organogenesis from seeds, shoot apices and nodal segments, the genetic integrity of the clones is ensured. The protocol leverages the exceptional organogenic potency of BAP paired with IAA/NAA for shoot multiplication and the sustained rhizogenic action of IBA for root development. Yielding a robust 80% ex *vitro* survival rate, this propagation framework provides an immediate, highly reproducible solution to mitigate the destructive wild harvesting of *A. aspera* in Bangladesh. It facilitates the continuous, season independent supply of elite, homogenous biomass necessary to support the commercial pharmaceutical industry while successfully advancing the *ex situ* conservation of this ecologically threatened botanical asset.

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