

***In vitro* Micropropagation of *Asparagus racemosus* Willd. Using Nodal Segment Explants Optimization of Surface Sterilization, Shoot Root Induction and Acclimatization Protocol**

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ABSTRACT:

Asparagus racemosus Willd. (family Asparagaceae), popularly known as Shatavari and revered as the 'Queen of Herbs' in Ayurveda, is a high-value medicinal plant of critical pharmacological importance, now listed as an endangered species due to over-exploitation and habitat loss. The present study was undertaken to develop an optimized in vitro micropropagation protocol using nodal segment explants (5th–6th node from shoot apex) on Murashige and Skoog (MS) basal medium. Two critical variables were systematically evaluated: (i) surface sterilization efficacy, and (ii) plant growth regulator (PGR)-mediated shoot organogenesis. For surface sterilization, Bavistin (Carbendazim, 1%) applied for 8 minutes (B5) yielded the maximum contamination-free rate of 78% without phytotoxicity; subsequent treatment with 0.01% HgCl₂ for 0.1 minutes (H7) achieved 90% contamination-free explants with fully preserved tissue viability. Among eight NAA × BAP combinations evaluated over three consecutive weeks, Treatment T4 (NAA 3.0 mg/L + BAP 4.0 mg/L) produced the maximum shoot height (6–8 cm) and shoot number (6 shoots/explant) by Week 3, while Treatment T6 (NAA 3.0 mg/L + BAP 2.0 mg/L) offered the most stable and contamination-free multiplication (4 shoots/explant, 5–7 cm). Both treatments confirmed that elevated cytokinin-to-auxin ratios drive vigorous axillary bud activation in this species. Two successive hardening trials, however, failed entirely due to the complete absence of in vitro root formation in the transferred plantlets, identifying root induction as the single most critical unresolved step in the protocol. Future work should prioritize a dedicated in vitro rooting phase using 0.5–1.0 mg/L IBA on half-strength MS medium, followed by evaluation of acclimatization substrates, to complete a commercially scalable and reproducible micropropagation protocol for this medicinally essential species.

KEY WORDS: *Asparagus Racemosus*, Shatavari, Micropropagation, Nodal Explant, BAP, NAA, Surface Sterilization, Acclimatization, Ayurveda.

INTRODUCTION

Asparagus racemosus Willd. (family Asparagaceae), commonly known as Shatavari, holds a paramount position in the traditional Indian system of medicine, Ayurveda, where it is

revered as the 'Queen of Herbs' [1]. The name Shatavari, derived from Sanskrit, translates to 'she who possesses a hundred husbands,' reflecting the plant's profound association with fertility, vitality, and reproductive health [2]. Widely distributed across tropical and subtropical regions of Asia, Africa, and Australia, this perennial climbing shrub is recognized not

only for its ethnomedicinal importance but also for its rich phytochemical composition, particularly its steroidal saponins (Shatavarins I–IV), which are responsible for a diverse array of pharmacological activities including immunomodulatory, antioxidant, antidiabetic, anti-inflammatory, galactagogue, and adaptogenic effects [3,4,5].

Classically documented in foundational Ayurvedic texts such as the Charak Samhita and Ashtang Hridayam, *A. racemosus* has been prescribed for centuries to address disorders of the female reproductive system, gastrointestinal complaints, nervous conditions, and respiratory ailments [6]. Modern pharmacological research has corroborated many of these traditional claims, with studies demonstrating its significant antiulcer activity, galactagogue effects through elevation of prolactin levels, immunostimulatory potential, antidiabetic effects through stimulation of insulinotropic pathways, antitussive properties comparable to codeine phosphate, and anti-inflammatory effects comparable to dexamethasone in experimental models [7,8,9,10,11,12]. These findings cement the plant's relevance in contemporary herbal medicine and nutraceutical industries worldwide.

However, the escalating commercial demand for its tuberous roots, compounded by destructive harvesting practices and progressive habitat loss due to deforestation, has rendered the species endangered in several of its natural habitats, raising urgent concerns about its long-term conservation and sustainable availability [13,14,2]. The plant is now listed among threatened medicinal species in India, and conventional propagation methods have proven inadequate to meet the rapidly growing demand from pharmaceutical and Ayurvedic industries [15].

In response to this growing threat, micropropagation through *in vitro* plant tissue culture has emerged as a reliable and efficient strategy for the large-scale, clonal multiplication of medicinally important plants [16,17]. Tissue culture techniques not only facilitate the rapid propagation of elite, disease-free planting material but also offer a controlled platform for studying plant developmental physiology and the role of plant growth regulators in organogenesis [18]. The judicious application of cytokinins such as 6-Benzylaminopurine (BAP) and auxins such as Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA), typically on Murashige and Skoog (1962) basal medium, has been shown to critically influence shoot induction, multiple shoot proliferation, and root regeneration in a variety of medicinal plant species [17,19].

Despite the recognized medicinal significance of *A. racemosus*, systematic micropropagation protocols for this species remain limited and require further optimization [20,21]. In particular, the effects of different plant growth regulators on explant response, shoot multiplication efficiency, rooting capacity, and successful acclimatization under *ex vitro* conditions warrant detailed investigation [22,23]. Surface sterilization of explants is also a critical determinant of tissue culture success, as

inadequate sterilization leads to microbial contamination that compromises culture establishment [16]. The establishment of an efficient and reproducible tissue culture protocol for *A. racemosus* would represent a significant step toward both the conservation of this endangered species and the reliable supply of high-quality planting material for medicinal and commercial purposes [20].

With this background, the present study was undertaken with the following objectives: (i) to evaluate the effect of different plant growth regulators (BAP, NAA, and IBA) on shoot induction from selected explants; (ii) to achieve multiple shoot formation under controlled *in vitro* conditions; (iii) to induce root formation in regenerated shoots; (iv) to assess the effectiveness of the surface sterilization process; and (v) to perform hardening and acclimatization of *in vitro* raised plantlets under *ex vitro* conditions.

MATERIAL AND METHODS

2.1 Plant Material: Healthy donor plants of *Asparagus racemosus* Willd. were sourced from the Medicinal Plant Nursery, State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. Nodal segments from the 5th–6th node from the shoot apex were selected as explants, as these regions contain active axillary meristems with high organogenic potential [2,24]. Only phenotypically vigorous, disease-free plants were used as explant donors.

2.2 Explant Surface Sterilization: Explants were subjected to a sequential sterilization protocol to eliminate surface microbial flora while preserving tissue viability. All steps from chemical treatment onward were performed inside a laminar air flow (LAF) cabinet maintained under UV irradiation for 30 minutes prior to use [25]. The complete sterilization procedure is summarized in Table 1.

Glassware was pre-sterilized by autoclaving at 121°C and 15 psi for 20 minutes, followed by hot-air oven treatment at 160°C for 2 hours. All media, sterile water, and reusable utensils were autoclaved at 121°C and 15 psi for 20 minutes before use [26].

2.3 Culture Medium Composition: Murashige and Skoog (1962) basal medium (MS medium) was used throughout the study as the standard nutrient formulation, selected for its broad applicability and proven efficacy across numerous plant species and culture systems [19]. The complete composition of the MS medium employed is presented in Table 2.

2.4 Preparation of Stock Solutions: Concentrated stock solutions were prepared for convenience and stored at 4°C in amber glass bottles to prevent photodegradation. Macronutrient stocks (Stock A) were prepared at 20× concentration; micronutrient, iron, and vitamin stocks (B, C, D) at 200×. All chemicals were weighed individually on a calibrated digital analytical balance and dissolved sequentially in double-distilled water (DDW) to a final volume of 500 mL per stock.

The iron stock ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ + Na_2EDTA) required gentle warming to ensure complete dissolution. Myo-inositol was

weighed fresh and added directly during medium preparation [17,27]. Stock compositions are given in Table 2.

Table 1: Sequential Surface Sterilization Protocol for *Asparagus racemosus* Nodal Explants

Agent / Method	Concentration / Condition	Duration	Application
Running tap water + Tween-20	Few drops per 100 mL	30 min	Initial surface wash to remove debris
Bavistin (Carbendazim)	1% (w/v)	5 min	Broad-spectrum antifungal pre-treatment
Sterile distilled water rinse	—	4–5×	Removal of chemical residues
HgCl_2 (Mercuric chloride)	0.1% (w/v)	5–8 min	Surface antimicrobial sterilization (LAF)
Sterile distilled water rinse	—	3–4×	Complete removal of HgCl_2 residues
70% Ethanol	70% (v/v)	Brief wipe	LAF bench and instrument disinfection
Autoclave (Moist heat)	121°C, 15 psi	20 min	Media, glassware, sterile water
Hot air oven (Dry heat)	160°C	2 h	Empty glassware, metal instruments
UV radiation	—	30 min pre-use	LAF cabinet internal surfaces

Table 2: Concentrated Stock Solution Compositions for MS Medium Preparation

Stock	Multiplier	Compound	Concentration (mg/L)
Stock A – Macronutrients	20×	NH_4NO_3 / KNO_3 / $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ / $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ / KH_2PO_4	33000 / 38000 / 8800 / 7400 / 3400
Stock B – Micronutrients	200×	KI / H_3BO_3 / $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ / $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ / Na_2MoO_4 / CuSO_4 / CoCl_2	166 / 1240 / 4460 / 1720 / 50 / 5 / 5
Stock C – Iron	200×	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ / $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	5560 / 7460
Stock D – Vitamins	200×	Myo-Inositol / Nicotinic acid / Pyridoxine-HCl / Glycine / Thiamine-HCl	20000 / 100 / 100 / 400 / 20

Table 3: Plant Growth Regulators (PGRs) Used in Plant Tissue Culture: Types, Concentrations, and Primary Functions

PGR Class	Compound	Conc. Range (mg/L)	Primary Function in Culture
Auxin	IAA	0.1–2.0	Natural auxin; mild rooting, cell elongation
Auxin	IBA	0.1–2.0	Adventitious root induction (preferred for rooting)
Auxin	NAA	0.1–2.0	Callus induction, root initiation; combined with cytokinins
Auxin	2,4-D	0.01–5.0	Potent callus induction; somatic embryogenesis
Cytokinin	BAP	0.1–5.0	Primary shoot multiplication, axillary bud proliferation
Cytokinin	Kinetin	0.1–3.0	Shoot induction; less potent than BAP
Cytokinin	TDZ	0.001–1.0	High-potency shoot organogenesis, woody/medicinal plants
Cytokinin	Zeatin	0.1–2.0	Natural cytokinin; meristem and sensitive species
Gibberellin	GA_3	0.1–1.0	Shoot elongation, internode extension, dormancy breaking

2.5 MS Medium Preparation: For 500 mL of MS medium, 25 mL of Stock A (20×), 2.5 mL each of Stocks B, C, and D (200×) were combined in a 500 mL conical flask and made up to approximately 450 mL with DDW. Sucrose (15 g, 3% w/v) was dissolved completely under continuous stirring. The pH was adjusted to 5.6–5.8 using 1N HCl or 1N NaOH, as verified with a

calibrated digital pH meter. Plant growth regulators (PGRs) were added at the required concentrations from their stock solutions. Agar (4 g, 0.8% w/v) was added and the volume made up to 500 mL, then heated in a microwave oven until complete dissolution. The hot medium was dispensed into sterile glass bottles and test tubes, sealed, and autoclaved at 121°C and 15 psi for 20 minutes.

Sterilized vessels were placed overnight in the LAF cabinet to cool and solidify before inoculation.

2.6 Plant Growth Regulators (PGRs): Stock solutions of all PGRs were prepared at 100 mg/L by dissolving 10 mg of each compound in a minimal volume of the appropriate solvent (IAA and IBA in 70% ethanol; BAP and kinetin in 1N KOH; NAA and 2,4-D in 1N NaOH; GA₃ in ethanol) and making up to 100 mL with DDW. Working concentrations were calculated using the dilution formula $S_1V_1 = S_2V_2$ and added to media prior to autoclaving [28]. In the present study, BAP and NAA were used as the primary cytokinin and auxin, respectively. PGR types, concentrations, and their primary functions in tissue culture are summarized in Table 3.

The auxin-to-cytokinin ratio governs morphogenic outcome: a high ratio promotes root induction and callus formation, while a low ratio (high cytokinin) drives shoot proliferation and axillary bud development [17]. The specific concentrations of BAP and NAA applied in individual treatments are detailed in the Results section.

2.7 Inoculation: Sterilized explants (0.5–2.0 cm) were trimmed at both ends using a sterile scalpel and inoculated vertically onto the solidified MS medium in culture vessels, inside the LAF cabinet. All transfers were performed adjacent to a burner flame using sterile forceps. Culture vessels were sealed with polypropylene

caps and labelled with explant identity, medium treatment, and inoculation date.

2.8 Culture Conditions: Inoculated cultures were incubated in a controlled-environment culture room maintained at $25 \pm 2^\circ\text{C}$ under a 16-hour photoperiod (cool-white fluorescent lamps, 3000 lux) and 8-hour dark period. Cultures were monitored regularly for contamination, bud emergence, shoot elongation, and root development. Sub-culturing was performed every 3–4 weeks by transferring regenerated shoots onto fresh medium of identical composition.

RESULTS AND DISCUSSION

3.1 Culture Establishment: Nodal segment explants of *Asparagus racemosus* Willd. (approximately 0.5–2.0 cm, harvested from the 5th–6th node) were successfully established on MS basal medium under aseptic conditions following a standardized sequential sterilization protocol. Explants responded positively to *in vitro* conditions, showing no signs of necrosis or senescence in optimally sterilized treatments, confirming the suitability of nodal segments as explant source material for this species. The strong inherent regenerative capacity of *A. racemosus* nodal tissue, attributable to preformed axillary meristems, was evident from shoot emergence in the control (T1) without exogenous PGR supplementation, consistent with earlier reports [29].

Table 4. Effect of Bavistin (Carbendazim, 1%) Treatment Duration on Fungal Contamination and Explant Viability in *Asparagus racemosus* Nodal Segment Cultures

Treatment	Conc. (%)	Duration (min)	Contaminated (%)	Contamination-free (%)	Observation
B1 (Control)	0.00	0	100	0	Heavy fungal contamination; no sterilization
B2	1.00	2	80	20	Insufficient; contamination present
B3	1.00	4	60	40	Partial reduction; contamination persists
B4	1.00	5	45	55	Low contamination; explants healthy
B5 □	1.00	8	22	78	OPTIMAL: minimal contamination, healthy tissue
B6	1.00	10	25	75	Low contamination; incipient tissue damage
B7	1.00	15	85	15	Severe phytotoxicity; tissue damage observed
B8	1.00	20	90	10	Complete tissue necrosis; not viable

□ B5 (1% Bavistin, 8 min): optimal treatment — 78% contamination-free explants with fully viable tissue.

Note: All treatments followed by 3× rinse with sterile distilled water. Values represent means from replicated trials.

3.2 Effect of Bavistin Treatment on Explant Sterilization: Fungal contamination represents one of the primary causes of culture failure in plant tissue culture protocols. In the present study, nodal explants of *A. racemosus* were treated with 1% Bavistin (active ingredient: Carbendazim, a broad-spectrum benzimidazole fungicide) at six different exposure durations to determine the optimal treatment that minimized contamination without inducing phytotoxicity. Results are presented in Table 4.

The untreated control (B1) exhibited 100% contamination, confirming the mandatory requirement for antifungal pre-treatment. A progressive reduction in contamination was observed from B2 through B5 as exposure duration increased from 2 to 8 minutes. Treatment B5 (1% Bavistin, 8 min) was identified as the optimal treatment, yielding 78% contamination-free explants with healthy, viable tissue. Beyond this threshold, treatments B6, B7, and B8 showed increasing phytotoxic effects, with B8 (20 min) resulting in severe tissue necrosis and complete loss of

regenerative capacity. These results indicate that Carbendazim at 1% is effective within a narrow therapeutic window (5–8 minutes) for nodal explants of *A. racemosus*. The tissue-type dependency of sterilization tolerance is a well-documented phenomenon in tissue culture. These findings align with those of [30], who reported highest survival rates (85%) in *A. racemosus* using a combined sterilization regime of Bavistin, Streptomycin, Kanamycin, HgCl₂, and ethanol.

3.3 Effect of HgCl₂ on Surface Sterilization: Following Bavistin pre-treatment, nodal explants were subjected to surface sterilization with Mercuric Chloride (HgCl₂) inside the Laminar Air Flow cabinet. Two concentrations (0.10% and 0.01%) across a range of exposure durations were evaluated. Results are presented in Table 5.

Table 5: Effect of HgCl₂ Concentration and Exposure Duration on Surface Sterilization Efficacy and Explant Viability in *Asparagus racemosus*

Treatment	HgCl ₂ Conc. (%)	Duration (min)	Contamination (%)	Contamination-free (%)	Efficacy free (%)	Observation
H1 (Control)	0.00	0	95	5	Nil	Heavy contamination
H2	0.10	1	15	85	Good	Low contamination; no damage
H3	0.10	3	3	97	Good	Near-complete; tissue damage evident
H4	0.10	4	2	98	Good	Near-complete; tissue damage
H5	0.10	6	1	99	Excess	Phytotoxic; explants died post-transfer
H6	0.10	5	0	100	Excess	Severe phytotoxicity; death
H7 □	0.01	0.1	10	90	Optimal	Best balance: no tissue damage
H8	0.01	0.5	12	88	Good	Low contamination; healthy survival

□ H7 (0.01% HgCl₂, 0.1 min): optimal treatment — 90% contamination-free explants with no tissue damage.

Note: All sterilizations performed inside the LAF cabinet. Tissue damage observed at ≥3 min exposure at 0.10% concentration.

Table 6: Effect of NAA and BAP Concentrations on Shoot Height and Multiplication in *Asparagus racemosus* Nodal Segment Cultures over Three Weeks on MS Basal Medium

Trt	NAA (mg/L)	BAP (mg/L)	Wk1 Ht (cm)	Wk1 Shoots	Wk2 Ht (cm)	Wk2 Shoots	Wk3 Ht (cm)	Wk3 Observation
T1 (Ctrl)	0	0	3–4	3	3–4	3	4–5	Healthy; callus at base from Wk 2
T2	1.00	1.00	2–3	2	2–3	2	3–4	Moderate growth; well-developed shoots
T3	2.00	2.00	3	2	4	2	4	Healthy shoot growth; no callus
T4 □	3.00	4.00	5–7	3	6–8	5	6–8	OPTIMAL: max height + shoots; no callus
T5	2.00	3.00	1.5–3	4	1.5–3	5	3–4	High count; callus + contamination
T6 □	3.00	2.00	5–6	4	5–7	4	5–7	Consistent elongation; no callus
T7	0.30	0.30	4–5	2	4–5	2	4–5	Healthy but limited multiplication
T8	0.10	0.20	2–3	1	2–3	1	2–3	Minimal response; single shoot; callus at tip

□ T4 (NAA 3.0 + BAP 4.0 mg/L) and T6 (NAA 3.0 + BAP 2.0 mg/L): identified as optimal treatments for shoot multiplication.

Note: NAA = Naphthalene Acetic Acid; BAP = 6-Benzylaminopurine. All cultures on MS basal medium, pH 5.8, 25±2°C, 16/8 h photoperiod, 3000 lux.

The untreated control (H1) showed 95% contamination, confirming the inadequacy of upstream Bavistin treatment alone for complete surface sterilization. At 0.10% HgCl₂, increasing exposure caused progressive tissue damage culminating in explant death at exposures ≥5 minutes. In contrast, 0.01% HgCl₂ produced superior outcomes: Treatment H7 (0.01%, 0.1 min) achieved 90% contamination-free explants with fully intact, healthy tissue, and

was identified as the optimal treatment. The markedly different outcomes between 0.10% and 0.01% HgCl₂ illustrate that for delicate monocot nodal tissue such as that of *A. racemosus*, lower HgCl₂ concentration with brief contact time is more appropriate than higher concentrations typically recommended for woody or herbaceous dicot species [25,17].

3.4 Effect of NAA and BAP on Shoot Induction and Multiplication Nodal explants were cultured on MS medium supplemented with eight NAA × BAP combinations (T1–T8) and monitored over three consecutive weeks. Shoot height and number were recorded weekly; results are consolidated in Table 6.

3.4.1 Week 1 Response: By the end of Week 1, shoot emergence was observed across all treatments. The control (T1, no PGRs) produced 3 shoots of 3–4 cm, confirming the strong endogenous regenerative capacity of *A. racemosus* nodal meristems. T4 (NAA 3.0 + BAP 4.0 mg/L) and T6 (NAA 3.0 + BAP 2.0 mg/L) were the strongest performers in Week 1, achieving shoot heights of 5–7 cm and 5–6 cm respectively. T5 (NAA 2.0 + BAP 3.0 mg/L) produced the highest shoot number (4 shoots/explant) at this stage, though with the onset of visible basal callus, indicating the more balanced auxin-to-cytokinin ratio in this treatment was initiating dedifferentiation concurrently with organogenesis.

3.4.2 Week 2 Response: Growth differences between treatments intensified in Week 2. T4 progressed to 6–8 cm height with 5 shoots/explant, while T6 maintained 5–7 cm with 4 shoots/explant both showing consistent elongation and proliferation. T5 reached 5 shoots/explant but exhibited early signs of contamination alongside continued callus development. The control (T1) produced incipient basal callus from Week 2 onward, attributable to accumulation of endogenous auxin at the wound site of the cut nodal segment [31].

3.4.3 Week 3 Response and Treatment Ranking: By Week 3, T4 produced the maximum shoot height (6–8 cm) and shoot number (6 shoots/explant) with vigorous proliferation and no callus, establishing it as the most productive treatment. T6 maintained consistent 5–7 cm shoots (4/explant) with clean, callus-free cultures, ranking it as the most stable and reliable treatment. The overall pattern across all treatments strongly corroborates the foundational principle established by Skoog and Miller (1957) that a high cytokinin-to-auxin ratio promotes shoot organogenesis [31]. These findings are consistent with [29], who reported effective shoot development in *A. racemosus* on NAA+BAP supplemented MS medium, and with [6], who documented maximum shoot proliferation efficiency of 12.16 shoots/explant at 1.5 mg/L BAP + 2.0 mg/L Kinetin in the same species.

3.5 Callus Formation: Callus formation was observed in two treatments: T5 (NAA 2.0 mg/L + BAP 3.0 mg/L) from Week 1, and T1 (control) from Week 2. In T5, the callus manifested as a soft, whitish, friable swelling at the cut base of the explant, proliferating gradually alongside the induced shoots. This response is mechanistically consistent with the relatively balanced auxin-to-cytokinin ratio in T5, which simultaneously promotes both shoot organogenesis and undirected cell division [31,17]. Although callus induction was not a primary objective of the present study, T5-derived callus represents potentially valuable material for future investigations into indirect organogenesis, somatic embryogenesis, and *in vitro* production of steroidal saponins the primary bioactive constituents of *A. racemosus* [32].

3.6 Hardening and Acclimatization: Successful transfer of *in vitro*-raised plantlets to ex vitro conditions requires a functional root system capable of water and nutrient uptake from the substrate. *In vitro*-grown shoots typically develop poorly functional cuticular wax, non-operational stomata, and limited photosynthetic competence due to the high humidity, low light, and constant nutrient availability in the culture environment [33]. Acclimatization therefore necessitates not only a gradual reduction in ambient humidity but, critically, prior establishment of an *in vitro* root system.

3.6.1 First Hardening Attempt: Four explants from T5 (NAA 2.0 + BAP 3.0 mg/L) were transferred to a sand rooting substrate after 28 days of *in vitro* culture. None of the four explants survived. Post-transfer examination confirmed the complete absence of root formation in all T5 plantlets. The high cytokinin-to-auxin ratio maintained throughout the T5 culture period consistently favoured shoot organogenesis at the expense of root induction. In the absence of a functional root system, the transferred shoots were entirely unable to absorb water from the substrate, rendering them acutely vulnerable to desiccation stress [33,34].

3.6.2 Second Hardening Attempt: A second hardening trial was conducted three weeks later using explants from T1 (control) and T6 (NAA 3.0 + BAP 2.0 mg/L). Both T1 and T6 shoots appeared visually healthy and green at the time of transfer. However, root formation was again entirely absent in all transferred plantlets, and all transfers failed due to progressive water stress and desiccation. The collective failure of both hardening attempts unambiguously identifies the absence of an *in vitro* rooting phase as the critical limiting factor in the present micropropagation protocol. This finding contrasts directly with successful acclimatization reports in the literature: [30] achieved an 85% survival rate in *A. racemosus* using sphagnum peat moss and perlite (1:1) under greenhouse conditions, following prior *in vitro* rooting; [35] achieved approximately 70% survival by first establishing *in vitro* roots on half-strength MS medium, then potting in vermiculite:peat moss (1:1). Both studies confirm that prior root establishment is the single most decisive determinant of acclimatization success. Future optimization efforts must therefore incorporate a dedicated *in vitro* rooting phase using auxins such as IBA on half-strength MS medium prior to hardening [17,36].

CONCLUSION

The present study established a reproducible sequential protocol for the *in vitro* culture of *Asparagus racemosus* Willd. using nodal segment explants, and systematically evaluated the critical variables of surface sterilization and PGR-mediated shoot organogenesis. Optimal surface sterilization was achieved through a two-stage protocol: 1% Bavistin (Carbendazim) for 8 minutes (B5), yielding 78% contamination-free explants with fully viable tissue, followed by 0.01% HgCl₂ for 0.1 minutes (H7), achieving 90% contamination-free explants without phytotoxicity.

Among eight NAA × BAP treatment combinations evaluated over three weeks, T4 (NAA 3.0 mg/L + BAP 4.0 mg/L) produced the

maximum shoot height (6–8 cm) and shoot number (6 shoots/explant) by Week 3, establishing it as the most productive treatment for shoot multiplication. T6 (NAA 3.0 mg/L + BAP 2.0 mg/L) was the most stable and reliable treatment, producing consistent 5–7 cm shoots (4/explant) across all three weeks without callus or contamination. Both treatments confirm that a high cytokinin-to-auxin ratio drives vigorous axillary bud activation in *A. racemosus*, consistent with established tissue culture theory [31].

The two successive hardening failures, attributable exclusively to the absence of *in vitro* root formation, constitute the principal limitation of the present study. They simultaneously provide its most important actionable finding: the protocol requires a dedicated *in vitro* rooting stage — specifically, transfer of regenerated shoots to half-strength MS medium supplemented with 0.5–1.0 mg/L IBA prior to *ex vitro* hardening before a complete and functional micropropagation protocol for *A. racemosus* can be realized. This recommendation is strongly supported by successful rooting and acclimatization data reported for this species in the literature [30,35,36].

In summary, the present study has established optimized sterilization parameters and identified superior PGR combinations for shoot induction in *A. racemosus*, providing a solid and scientifically validated foundation upon which a complete, commercially scalable micropropagation protocol can be constructed. Future work should focus on: (i) *in vitro* rooting protocol optimization using IBA on ½ MS medium; (ii) acclimatization substrate evaluation (peat moss:perlite vs. vermiculite:soil); and (iii) assessment of genetic fidelity of regenerated plantlets using molecular markers to confirm clonal uniformity for pharmaceutical-grade production of this high-value medicinal species.

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this manuscript. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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