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In Vitro Micropropagation of *Acorus Calamus* L. Using Rhizome Nodal Segments: Optimization of Surface Sterilization, Hormonal Regimes, And Acclimatization

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Abstract- ***Acorus calamus L. (family Acoraceae), commonly known as Sweet Flag or 'Vacha', is a pharmaceutically important semi-aquatic perennial herb whose bioactive rhizomes are extensively exploited in Ayurveda, Unani, and Traditional Chinese Medicine. Conventional propagation through rhizome segments is severely limited by low multiplication rates and susceptibility to soilborne pathogens, necessitating the development of reliable micropropagation protocols. The present study, conducted at the State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, established a reproducible and efficient in vitro propagation protocol for A. calamus using rhizome nodal segments as explants. An optimized two-step surface sterilization regimen 1.0% Bavistin (carbendazim) for 10 minutes followed by 0.1% $HgCl_2$ for 3 minutes effectively eliminated microbial contamination while preserving explant viability. Shoot induction was most effectively achieved on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 6-benzylaminopurine (BAP), yielding a maximum of 4.8 ± 0.6 shoots per explant within approximately 9 days. Shoot multiplication was optimized on MS medium containing 3.0 mg/L BAP combined with 3.0 mg/L naphthaleneacetic acid (NAA), producing 9.4 ± 1.2 shoots per explant with a mean shoot length of 5.1 ± 0.9 cm. In vitro rooting was best accomplished on half-strength MS medium supplemented with 1.0 mg/L NAA, generating fibrous, well-differentiated roots within 8 days. Ex vitro acclimatization of in vitro-raised plantlets in a soil : sand : organic manure (1:1:1) substrate under stepwise humidity reduction achieved an 85% survival rate after four weeks under greenhouse conditions. The protocol developed provides a scalable tool for the clonal propagation of A. calamus, with applications in conservation of threatened natural populations, commercial cultivation for the essential oil and pharmaceutical industries, and production of certified disease-free planting material.***

Keywords: Acorus calamus, micropropagation, rhizome nodal explant, BAP, NAA, shoot multiplication, acclimatization, Ayurvedic medicinal plant

I. INTRODUCTION

Acorus calamus L. (Family: Acoraceae), commonly known as Sweet Flag or 'Vacha' in Sanskrit, is a perennial semi-aquatic herb distributed widely across Asia, Europe, and North America, with its center of origin in India (Verma & Singh, 2012). The plant thrives in wetlands, marshy habitats, and riverbanks, ascending up to 2200 m altitude, and is cultivated across diverse Indian states including Jammu & Kashmir, Himachal Pradesh, Manipur, Uttarakhand, Tamil Nadu, and Maharashtra. Its sword-shaped leaves and horizontally creeping rhizome are its most distinctive morphological features, with the rhizome representing the primary site of pharmaceutical significance (Balakumbahan et al., 2010).

The rhizomes of A. calamus are rich in bioactive phytoconstituents including essential oils, α-asarone, β-asarone, flavonoids, tannins, and saponins (Muthusamy et al., 2022). These compounds confer a diverse array of biological activities such as antimicrobial, anti-inflammatory, analgesic, neuroprotective, antioxidant, and insecticidal properties (Sharma et al., 2014; Quraishi et al., 2017; Sharma et al., 2020). The plant has been extensively employed in traditional systems of medicine including Ayurveda, Unani, and Traditional Chinese Medicine for the treatment of cognitive impairment, epilepsy, asthma, bronchitis, digestive disorders, and

rheumatism (Khwairakpam et al., 2018). Its classification as a Medhya Rasayana in Ayurvedaa group of plants enhancing memory and intellect reflects its long-standing recognition as a neurologically active herb (Sharma et al., 2014). Additionally, its aromatic volatile constituents, particularly asarone and arylaldehydes, have established its commercial value in the perfume and cosmetic industries (Babar et al., 2020; Ahmed et al., 2010).

Despite its considerable medicinal and economic importance, large-scale cultivation of A. calamus is severely constrained by its predominantly asexual mode of propagation through rhizome segments. This conventional method results in inherently low multiplication rates and significantly increases the risk of pathogen transmission, particularly bacterial diseases such as leaf blight and rhizome rot (Sabitha et al., 2000). Furthermore, indiscriminate overharvesting from natural populations combined with progressive habitat destruction has led to a marked decline in wild populations of this valuable species (Wulansari et al., 2023). These challenges collectively underscore the urgent need for alternative, scalable propagation strategies that ensure both genetic fidelity and phytochemical consistency of the planting material.

Plant tissue culture, specifically micropropagation, offers a scientifically validated and practically viable solution to address these constraints. Micropropagation enables the rapid clonal multiplication of genetically uniform, pathogen-free plantlets under aseptic in vitro conditions, irrespective of season or geographic limitations (Rajasekharan et al., 2010; Tikendra et al., 2022). The success of any in vitro propagation protocol is fundamentally dependent on the establishment of effective surface sterilization procedures that minimize microbial contamination while preserving explant viability and regenerative potential (Devi et al., 2012). This is particularly critical for rhizomatous medicinal plants like A. calamus, which harbor endogenous microflora within their tissues and are prone to fungal and bacterial contamination during culture initiation (Khan et al., 2016). Optimization of sterilization protocols using agents such as sodium hypochlorite (NaOCl), mercuric chloride ($HgCl_2$), and ethanol, at appropriate concentrations and exposure durations, forms the foundational step toward successful culture establishment (Meetei et al., 2024).

Beyond sterilization, the development of a reproducible in vitro propagation protocol requires systematic optimization of basal culture media and plant growth regulator (PGR) combinations. Cytokinins such as 6-benzylaminopurine (BAP) and kinetin, alone or in synergy with auxins like naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA), play a pivotal role in governing shoot proliferation and root induction in medicinal plant tissue culture (Tikendra et al., 2022; Devi et al., 2012). For A. calamus specifically, shoot tip and rhizome explants have been identified as suitable explant sources owing to their high meristematic activity and regeneration competence (Rajasekharan et al., 2010). However, a comprehensive, reproducible protocol integrating all stages from explant sterilization and shoot multiplication to rooting and ex vitro hardening is yet to be fully standardized for this species.

The final and equally critical stage of micropropagation is the successful transfer of in vitro-raised plantlets to ex vitro conditions. Plantlets regenerated in tissue culture are physiologically adapted to high humidity, low light, and heterotrophic nutrition; their direct transplantation to natural environments typically results in high mortality due to their inability to withstand water stress and autotrophic conditions (Meetei et al., 2024). A systematic hardening and acclimatization procedure, employing suitable rooting substrates and a gradual reduction in humidity, is therefore essential for ensuring high survival rates and successful field establishment of micropropagated plantlets.

In this context, the present investigation was undertaken with the following specific objectives: (i) to standardize effective sterilization techniques for the successful initiation of aseptic cultures of A. calamus, minimizing contamination during culture establishment; (ii) to develop a reliable and reproducible protocol for in vitro propagation of A. calamus under controlled laboratory conditions (iii) to perform hardening and acclimatization of in vitro

raised plantlets under ex vitro conditions. The findings of this study are expected to contribute toward large-scale commercial production, conservation of natural populations, and a consistent supply of high-quality planting material of this pharmaceutically important medicinal herb.

II. MATERIALS AND METHODS

2.1 Plant Material and Explants Selection

Healthy, disease-free plants of Acorus calamus L. were collected from the Medicinal Garden of the State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. Rhizome segments bearing nodal regions (1.0–1.5 cm) were selected as explants for in vitro culture initiation, owing to their high meristematic activity and regenerative competence.

2.2 Surface Sterilization of Explants

Surface sterilization was carried out in a sequential multi-step procedure. Explants were first washed under running tap water for 30 minutes with a few drops of Tween 20 (surfactant) to remove dust and surface debris. They were then rinsed 3–4 times with sterile distilled water, followed by treatment with 1% (w/v) Bavistin (carbendazim) solution for 5 minutes to eliminate fungal contaminants, with subsequent rinsing 3–5 times with sterile distilled water.

Further sterilization was performed inside a Laminar Air Flow (LAF) cabinet. Explants were treated with freshly prepared 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10–12 minutes, followed by thorough rinsing 3–4 times with sterile double-distilled water to completely remove all traces of the sterilant. Explant survival rate and contamination frequency were recorded to assess the efficacy of the sterilization protocol.

2.3 Preparation of Culture Media

Murashige and Skoog (1962) basal medium (MS medium) was used throughout the study. Stock solutions of macronutrients (20×), micronutrients (200×), iron source (20×), vitamins (200×), and potassium iodide were prepared separately in distilled water and stored in clean glass bottles at 4°C. Inositol was added freshly at the time of media preparation.

For preparation of 500 mL MS medium, respective volumes of stock solutions were combined, sucrose was added at 3% (w/v) as carbon source, and the pH was adjusted to 5.7–5.8 using 0.1 N NaOH or HCl. Agar (0.8%, w/v) was added as solidifying agent and dissolved completely by heating in a microwave oven. Plant growth regulators (PGRs) BAP (6-benzylaminopurine) and NAA (naphthaleneacetic acid) were added at required concentrations from stock solutions (100 mg/L) prepared in ethanol/KOH/NaOH as appropriate. The medium was dispensed into sterile glass culture bottles and test tubes, sealed, and autoclaved at 121°C and 15 psi for 20 minutes. Sterilized media were allowed to cool and solidify overnight in the LAF chamber before use.

2.4 Inoculation and Culture Conditions

Inoculation was performed inside a LAF cabinet pre-sterilized under UV light for 30 minutes and wiped with 70% ethanol. Sterilized explants were aseptically transferred onto the prepared MS medium using sterile forceps near a flame. Cultures were maintained in a culture room at 25 ± 2°C under a 16-hour light/8-hour dark photoperiod provided by cool white fluorescent tubes at approximately 3000 lux. Subcultures were performed at regular intervals to promote proliferation and shoot multiplication.

2.5 Hardening and Acclimatization

In vitro-raised rooted plantlets were carefully removed from culture vessels and the adhering agar was gently washed off with distilled water to prevent fungal growth. Plantlets were transferred to plastic pots containing a sterilized rooting substrate and maintained under high humidity conditions (covered with polythene bags) to facilitate gradual acclimatization. Humidity was progressively reduced over a period of 2–3 weeks to allow adaptation to ex vitro conditions. Survival rate and morphological performance of acclimatized plantlets were recorded to evaluate hardening success.

2.6 Statistical Analysis

All experiments were arranged in a Completely Randomized Design (CRD) and each treatment was replicated a minimum of three times (n = 10 explants per replication). Quantitative data were expressed as mean ± standard error (SE). Analysis of variance (ANOVA) was performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA) or

GraphPad Prism (version 9.0). When ANOVA indicated significant treatment effects, means were compared using Duncan's Multiple Range Test (DMRT) at a significance level of $p \leq 0.05$ [16]. Percentage data (contamination, survival, rooting percentage) were subjected to arcsine square root transformation prior to statistical analysis to normalize the distribution [17]. Pearson's correlation analysis was performed to determine relationships between growth regulator concentrations and morphological response parameters.

III. RESULTS AND DISCUSSION

3.1 Response of Explants under In Vitro Culture Conditions

The present investigation was aimed at developing a reliable protocol for in vitro micropropagation of Acorus calamus L. using rhizome segments bearing nodal regions as explants. Healthy rhizome segments (1.0–1.5 cm in length) collected from young donor plants responded positively under in vitro conditions, confirming their suitability for culture establishment. The explants were successfully stabilized on Murashige and Skoog (MS, 1962) basal medium under aseptic conditions following an optimized two-step surface sterilization procedure.

Visible swelling of explants commenced within 5–7 days of inoculation, followed by shoot initiation in 10–15 days on hormone-supplemented media. The majority of healthy, properly sterilized explants showed greening and active growth, whereas those subjected to mechanical injury during sterilization or excessive chemical exposure exhibited necrosis or failed to respond. Similar early growth responses from nodal explants have been reported in other monocotyledonous marsh plants (Vyas et al., 2003; Gantait et al., 2011).

3.2 Effect of Bavistin Pre-treatment on Decontamination

Fungal contamination is a major constraint in establishing aseptic cultures from field-collected explants of semi-aquatic and aromatic plants such as A. calamus (Kapoor et al., 2011). To address this, Bavistin (carbendazim), a systemic broad-spectrum fungicide, was evaluated at 1.0% (w/v) for varying exposure durations prior to $HgCl_2$ surface sterilization. The results are presented in Table 1.

Table 1. Effect of Bavistin (In 1%) treatment for decontamination of Acorus calamus L. rhizome nodal explants

Treatment	Duration (min)	Observation
T1	0	Heavy fungal contamination; no culture establishment
T2	5	Moderate contamination; partial culture establishment
T3	10	Best response: clean cultures, healthy growth, no contamination
T4	15	No contamination but slight tissue damage and browning observed

Values represent pooled observations from three independent experiments (n = 20 explants per treatment).

The untreated control (T1) exhibited heavy fungal contamination with almost complete loss of explants, confirming the high endogenous microbial load characteristic of field-sourced rhizomes. Exposure to 1.0% Bavistin for 5 minutes (T2) reduced but did not eliminate contamination. The treatment involving 1.0% Bavistin for 10 minutes (T3) yielded the best outcome: clean, uncontaminated cultures with vigorous explant growth, indicating an optimal balance between fungicidal efficacy and tissue compatibility. Prolonged treatment (T4, 15 min) eliminated contamination but induced visible phytotoxic effects including browning and reduced regeneration capacity, consistent with reports that excessive fungicide exposure compromises cell viability in sensitive tissues (Bhatt and Dhar, 2000). Rhizome nodal segments demonstrated comparatively greater tolerance to Bavistin than

softer tissues, likely attributable to the lignified epidermal layers of the rhizome.

3.3 Effect of $HgCl_2$ Concentration and Duration on Surface Sterilization

Following Bavistin pre-treatment, mercuric chloride ($HgCl_2$) at 0.1% (w/v) was employed as a terminal chemical sterilant to eliminate residual surface microorganisms. Four exposure durations were evaluated (Table 2). Although $HgCl_2$ is a powerful surface sterilant, it is also strongly phytotoxic; therefore, optimization of exposure time is critical (Chaturvedi and Razdan, 1997).

Table 2. Effect of $HgCl_2$ (0.1 %) treatment with different duration on surface sterilization efficiency of Acorus calamus L. rhizome nodal explants

Treatment	Duration (min)	Observation
T1	1	Inadequate sterilization; contamination evident in majority of cultures
T2	3	Optimal result: clean explants, healthy growth, no browning observed
T3	5	Good sterilization; slight browning noted in some explants
T4	7	Severe tissue damage and browning; reduced explant viability

Values represent pooled observations from three independent experiments (n = 20 explants per treatment).

An exposure of 1 minute (T1) was insufficient for complete surface decontamination, with contamination persisting in a majority of cultures. An exposure of 3 minutes (T2) produced optimal results, yielding clean, undamaged explants with high culture establishment efficiency. At 5 minutes (T3), sterilization was effective but mild browning was observed in a proportion of explants. At 7 minutes (T4), severe tissue damage and generalized browning resulted in marked reduction of explant viability. These findings corroborate the results of Baskaran and Jayabalan (2005) and Singh et al. (2016) who reported similar thresholds for $HgCl_2$ toxicity in rhizomatous monocots. The combined sequential sterilization protocol (1.0% Bavistin, 10 min → 0.1% $HgCl_2$, 3 min) is therefore recommended for routine culture initiation of A. calamus.

3.4 Effect of BAP on In Vitro Shoot Induction

The role of cytokinins, particularly 6-benzylaminopurine (BAP), in promoting axillary bud break and shoot proliferation from nodal explants is well established (Murashige, 1974; Skoog and Miller, 1957). MS medium supplemented with varying concentrations of BAP (0.5–3.0 mg/L) was evaluated for its effect on shoot induction from A. calamus rhizome nodal explants. Data are presented in Table 3.

Table 3. Effect of BAP concentration on in vitro shoot induction from Acorus calamus L. rhizome nodal explants on MS medium

Treatment (MS Medium)	Days to Shoot Initiation	No. of Shoots/Explant	Observation
MS + 0.5 mg/L BAP	14 ± 1.2	1.2 ± 0.3	Minimal response; few shoots initiated
MS + 1.0 mg/L BAP	12 ± 0.9	2.1 ± 0.4	Moderate shoot induction; shoots slender
MS + 1.5 mg/L BAP	10 ± 0.8	3.4 ± 0.5	Good shoot induction; healthy shoots
MS + 2.0 mg/L BAP	9 ± 0.7	4.8 ± 0.6	Best result: vigorous shoots within 15 days

Treatment (MS Medium)	Days to Shoot Initiation	No. of Shoots/Explant	Observation
MS + 2.5 mg/L BAP	10 ± 1.0	4.2 ± 0.7	Good induction; slight callus at base
MS + 3.0 mg/L BAP	11 ± 1.1	3.5 ± 0.5	Moderate; abnormal growth in some explants

Mean ± Standard Error; n = 15 explants per treatment; data recorded at 30 days of culture. Values with different superscripts differ significantly at $p \leq 0.05$ (ANOVA followed by Duncan's Multiple Range Test).

Shoot induction was observed across all BAP concentrations tested, but the response was concentration-dependent. The lowest BAP concentration (0.5 mg/L) produced minimal response with only 1.2 ± 0.3 shoots per explant. Shoot number progressively increased with BAP concentration, reaching a peak of 4.8 ± 0.6 shoots/explant at 2.0 mg/L BAP, with shoots initiation occurring in 9 ± 0.7 days. At 2.0–3.0 mg/L BAP, shoots were healthy, elongated, and exhibited normal morphology; however, concentrations above 2.0 mg/L showed a declining trend in shoot number with signs of abnormal morphology and basal callusing at 3.0 mg/L. The stimulatory effect of BAP on shoot proliferation at optimum concentrations and its inhibitory effect at higher concentrations have been documented for several aromatic medicinal plants, including Valeriana jatamansi (Wawrosch et al., 1999), Acorus gramineus (Huang et al., 2011), and Curcuma longa (Salvi et al., 2002). The optimal concentration of 2.0 mg/L BAP observed in the present study is consistent with published protocols for monocotyledonous rhizomatous species.

3.5 Effect of BAP and NAA Combinations on Shoot Multiplication

Initiated shoots were sub-cultured onto MS medium containing a fixed concentration of BAP (3.0 mg/L) combined with increasing concentrations of NAA (0.5–3.0 mg/L) to evaluate the effect on shoot multiplication rate and shoot length. The interaction of cytokinin and auxin is known to modulate organogenesis in tissue culture systems (George et al., 2008). The results are summarized in Table 4.

Table 4. Effect of BAP and NAA combinations on shoot multiplication of Acorus calamus L. on MS medium

Treatment (BAP + NAA)	Shoots/Explant (Mean ± SE)	Shoot Length (cm)	Observation
3.0 mg/L BAP + 0.5 mg/L NAA	4.1 ± 0.6	2.8 ± 0.4	Moderate multiplication; shoots healthy
3.0 mg/L BAP + 1.0 mg/L NAA	5.6 ± 0.7	3.2 ± 0.5	Good multiplication; compact shoots
3.0 mg/L BAP + 1.5 mg/L NAA	6.3 ± 0.8	3.7 ± 0.6	High multiplication; some callus
3.0 mg/L BAP + 2.0 mg/L NAA	7.2 ± 0.9	4.1 ± 0.7	Very good; vigorous shoots
3.0 mg/L BAP + 2.5	8.1 ± 1.0	4.5 ± 0.8	Excellent multiplication; healthy plantlets

Treatment (BAP + NAA)	Shoots/Explant (Mean ± SE)	Shoot Length (cm)	Observation
mg/L NAA			
3.0 mg/L BAP + 3.0 mg/L NAA	9.4 ± 1.2	5.1 ± 0.9	Best: highest multiplication rate, vigorous growth

Mean ± Standard Error; n = 15 explants per treatment; data recorded at 30 days of subculture.

The number of shoots per explant increased progressively with increasing NAA concentration in the presence of 3.0 mg/L BAP. The treatment combination of 3.0 mg/L BAP + 3.0 mg/L NAA yielded the highest multiplication rate (9.4 ± 1.2 shoots/explant) with an average shoot length of 5.1 ± 0.9 cm. At lower NAA concentrations (0.5–1.0 mg/L), multiplication rates were comparatively modest. No callus induction was observed at the optimal combination, and shoots displayed normal leaf morphology. The synergistic interaction between BAP and NAA in promoting shoot proliferation has been previously documented in Costus speciosus (Vinitha and Rajasekaran, 2009) and Zingiberaceae members (Renuka and Devi, 2010). The results suggest that NAA at higher concentrations complements BAP in sustaining active cell division and axillary bud activation without compromising phenotypic fidelity.

3.6 In Vitro Root Induction and Plantlet Development

Well-developed shoots (3–4 cm) were excised and transferred to half-strength MS (½ MS) medium supplemented with NAA at concentrations ranging from 0.5 to 2.5 mg/L for root induction. Auxins, particularly NAA and IBA, are widely used for in vitro rhizogenesis (Zimmerman and Stoutemeyer, 1935; Lloyd and McCown, 1980). The results are presented in Table 5.

Table 5. Effect of NAA concentration on in vitro root induction from Acorus calamus L. microshoots on ½ MS medium

Treatment (½ MS + NAA)	Days to Root Initiation	No. of Roots/Plantlet	Observation
½ MS + 0.5 mg/L NAA	10 ± 0.8	1.8 ± 0.3	Poor rooting; few thin roots
½ MS + 1.0 mg/L NAA	8 ± 0.7	3.2 ± 0.4	Good rooting; healthy fibrous roots
½ MS + 1.5 mg/L NAA	9 ± 0.8	2.9 ± 0.4	Moderate; slight root thickening
½ MS + 2.0 mg/L NAA	10 ± 0.9	2.4 ± 0.5	Reduced root quality; thick callused roots
½ MS + 2.5 mg/L NAA	12 ± 1.0	1.9 ± 0.4	Callus formation; poor rooting efficiency

Mean ± Standard Error; n = 15 shoots per treatment; data recorded at 21 days of culture.

Root induction was achieved across all NAA concentrations tested. The treatment ½ MS + 1.0 mg/L NAA yielded the best rooting response, with 3.2 ± 0.4 roots per plantlet initiated within 8 ± 0.7 days. Roots were fibrous, white, and well-differentiated, indicating efficient auxin-mediated rhizogenesis. At sub-optimal concentrations (0.5 mg/L NAA), rooting was sparse and delayed. Conversely, concentrations of 2.0 mg/L and above resulted in callus formation at the root base and the development of thick, shortened roots with reduced functionality — a well-documented auxin toxicity effect (Bhatt and Dhar, 2000; Nower, 2014). The

superior performance of 1.0 mg/L NAA on ½ MS in the present study is in agreement with reports for Acorus gramineus (Huang et al., 2011) and other semi-aquatic monocots.

3.7 Hardening and Ex Vitro Acclimatization

In vitro-raised plantlets with well-developed shoots and roots were subjected to a stepwise acclimatization procedure to facilitate successful transition to ex vitro conditions. The abrupt shift from the high-humidity, low-light, and sugar-supplemented in vitro environment to the ex-vitro environment frequently results in desiccation and transplant shock (Pospíšilová et al., 1999). To mitigate this, plantlets were first washed to remove agar, treated with 1.0% Bavistin for 10–15 minutes as a prophylactic antifungal measure, and transplanted into portray trays containing a sterilized substrate of soil: sand: organic manure (1:1:1). Acclimatization data are summarized in Table 6.

Table 6. Acclimatization and survival of in vitro-raised Acorus calamus L. plantlets under greenhouse conditions

Stage	Conditions	Observation	Survival Rate
Week 1	High humidity (>90%), shade	Acclimatization begins; no wilting	92%
Week 2	Gradual humidity reduction (~75%)	New leaf emergence; root growth	89%
Week 3–4	Normal greenhouse conditions	Vigorous growth; well-established	85%

Observations recorded under controlled greenhouse conditions at SFRI, Jabalpur; n = 50 plantlets.

Plantlets maintained under high-humidity (>90%) shaded conditions during the first week showed a survival rate of 92%. Gradual exposure to ambient humidity and light over weeks 2–4 was accompanied by progressive root ramification and new leaf emergence. After four weeks, an overall survival rate of 85% was recorded, which is considered satisfactory for in vitro-derived monocotyledonous plantlets (Pinto et al., 2010). The use of an organic manure-enriched substrate likely facilitated nutrient availability and root growth, contributing to successful establishment. These results are comparable to acclimatization success rates reported for Elettaria cardamomum (80–88%) and Hedychium coronarium (82–90%) (Kumar et al., 2008).

IV. CONCLUSION

The present study, conducted at the State Forest Research Institute (SFRI), Jabalpur, successfully standardize a reproducible and efficient protocol for the in vitro micropropagation of Acorus calamus L. (family Acoraceae), an economically and medicinally important aromatic perennial herb commonly known as Bach or Sweet Flag. Conventional vegetative propagation of this species through rhizomes is constrained by slow multiplication rates and vulnerability to soil borne fungal pathogens, including Colletotrichum falcatum and Fusarium spp., which cause leaf spot and leaf blight diseases. The present micropropagation protocol offers a viable alternative for the rapid, large-scale production of disease-free planting material.

Rhizome segments bearing nodal regions served as highly responsive and reproducible explants. An optimized two-step sterilization regime comprising 1.0% Bavistin (carbendazim) for 10 minutes followed by 0.1% $HgCl_2$ for 3 minutes effectively minimized contamination without compromising explant viability. Shoot induction was most efficiently achieved on MS medium supplemented with 2.0 mg/L BAP, producing 4.8 ± 0.6 healthy shoots per explant within approximately 9 days. Shoot multiplication was maximized on MS medium containing 3.0 mg/L BAP + 3.0 mg/L NAA, yielding 9.4 ± 1.2 shoots per explant with a mean shoot length of 5.1 ± 0.9 cm. In vitro rooting was best accomplished on ½ MS medium supplemented with 1.0 mg/L NAA, producing fibrous, well-differentiated roots suitable for transplantation. Ex vitro acclimatization under stepwise humidity reduction achieved a survival rate of 85% after four weeks.

The optimized concentrations of BAP and NAA played critical and complementary roles in enhancing shoot induction, multiplication, and rooting efficiency. The results confirm that proper sterilization, cytokinin-auxin balance, and a gradual hardening protocol are collectively essential for successful in vitro culture of A. calamus. This protocol provides a promising and scalable tool for the clonal propagation of A. calamus, with significant applications in conservation of natural populations, commercial cultivation for the essential oil and pharmaceutical industries, and the production of certified disease-free planting stock for restoration programs. Future investigations should evaluate somaclonal variation in micropropagated plants, long-term ex vitro performance, and the feasibility of cryopreservation for germplasm conservation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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