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Optimization of vegetative propagation and micropropagation protocols for the conservation and large-scale cultivation of *Aquilegia fragrans* Benth

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Abstract

The present study aimed to establish a robust propagation protocol for the endangered medicinal species *Aquilegia fragrans* through rhizome cuttings and *in vitro* micropropagation techniques. The effects of various plant growth regulators (PGRs) such as GA₃, BAP, and IBA on vegetative propagation, including sprouting, root and shoot development, were investigated. Rhizome cuttings treated with 150 ppm GA₃ exhibited the highest sprouting percentage (83.34%) and significant enhancement in growth parameters, including shoot and root length, leaf number, and rhizome development. Additionally, the study assessed the influence of different soil compositions on sprouting success, with a mixture of soil, sand, and vermicompost (1:1:1) yielding the highest sprouting rate and optimal morphological traits. The micropropagation protocol was further optimized by evaluating surface sterilization techniques and the effect of cytokinins, particularly Meta-topolin and TDZ, in combination with auxins (IAA, IBA, and NAA). The combination of TDZ (3.5 mg L⁻¹) and IBA (1.25 mg L⁻¹) was found to induce the highest regeneration frequency (82.00%), with a maximum of 6 shoots per explant and a reduced induction period. Acclimatization of *in vitro*-derived plantlets under greenhouse conditions resulted in a 75% survival rate, confirming the feasibility of this propagation method for large-scale cultivation and conservation.

Keywords *A. fragrans*, Vegetative propagation, Gibberellic acid, Micropropagation, Conservation

1 Introduction

The Kashmir Himalaya, located in the north-western part of the Indian Himalayan Region, is recognized as a biodiversity hotspot. A recent study of its plant diversity has identified 1123 plant species that are primarily utilized for medicinal purposes [1]. A substantial increase in global interest regarding plant-derived therapeutic systems has been observed in recent years. In both developed and developing regions, these traditional methods are increasingly being viewed as viable substitutes for standard allopathic



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medicine. The growing demand for raw materials has led to a rise in the depletion of natural medicinal plant populations, with nearly 15,000 species now facing the risk of extinction [2]. Additionally, factors such as unregulated development, improper collection methods, unsustainable harvesting, habitat destruction, uncontrolled grazing, and the rapid effects of climate change are all posing significant threats to the global supply of medicinal plants [3, 4]. Additionally, due to the slow and lengthy regeneration process of many plant species, conservation efforts are crucial for their survival [5]. Two main approaches exist for protecting plant genetic diversity: *in situ*, where plants are safeguarded in their natural habitats, and *ex situ*, where they're preserved outside these environments. Acknowledging the pressing need to protect these species, *ex situ* conservation methods are seen as a practical and effective way to preserve and sustainably use endangered plant species [6].

Vegetative propagation serves as a significant and advantageous technique for the large-scale cultivation of economically valuable species, particularly those that are challenging to grow from seeds. Utilizing rhizomes for propagation is a practical and efficient approach for quickly increasing elite plant populations while preserving their vital genetic traits [7, 8]. This technique is simple, economical, and effective for the large-scale cultivation, preservation, and restoration of endangered plant species. Although seed germination can yield many individuals from one mother parent plant, its effectiveness is limited by the necessity for intricate stratification procedures and the typically slow growth of seedlings in most forest understory plants [9, 10]. Therefore, it is thought that vegetative propagation is especially in alpine species is more significant in plants than sexual reproduction [11]. Vegetative propagation, unlike sexual reproduction, allows plants to reach maturity within a year of cultivation. This makes it a powerful tool for conserving commercially valuable species that have been overharvested, promoting the development of improved cultivation methods, and enabling large-scale production for commercial purposes.

Currently, Micropropagation has been utilised for conservation of large variety of endangered MAPs including *Eryngium viviparum* [12], *Dyckia brevifolia* [13] and *Bistorta amplexicaulis* [14]. Micropropagation enables the rapid production of healthy, disease-free planting material for medicinal, ornamental, and nutritional applications [14]. Micropropagation is used for faster multiplication of true-to-type genotypes in the minimum possible time [15].

Aquilegia fragrans Benth., an endangered plant, of family Ranunculaceae has been traditionally utilized for medicinal purposes [16], is commonly known by various names in Kashmir, such as Jangli kuth, Kalumb, and Zaoneel. It is often known as sweet-scented columbine [17]. Naturally, the plant thrives at high altitudes in the Kashmir Valley in India [16, 18]. This herb has long been used to treat a variety of inflammatory conditions and wounds [16]. Moreover, rhizome of this plant is used to treat different ailments like psoriasis, eczema, kidney stones, gout, blood sugar, cystitis, and bovine mastitis [19, 20]. Dried flower paste is frequently used to cure headaches, while traditional therapies for jaundice and pneumonia entail the use of seeds and the entire plant, respectively [20]. The root has lately been revealed to have ethnomedicinal properties as an antipyretic and analgesic [21]. A paste made from plant flowers and refined butter is prepared to treat boils and is applied daily for ten to fifteen days [22]. Despite its medicinal importance, *A. fragrans* faces propagation constraints, as although the species is capable of

both sexual and asexual reproduction, slow seed germination and the low efficiency of vegetative propagation under natural conditions significantly limit the availability of well-established seedlings [23]. Consequently, the poor regenerative capacity of *A. fragrans* poses a serious threat to its natural populations, increasing the risk of decline and possible extinction due to inadequate natural regeneration and insufficient in vitro conservation strategies.

To address this research gap, the present study was designed with the following primary objectives: (1) To develop a reliable propagation protocol by assessing the influence of different plant growth regulators on the vegetative propagation potential of *A. fragrans* (2) to determine how different soil compositions affect the rate of sprouting and other growth factors; and (3) to establish a reliable and reproducible micropropagation protocol using rhizome nodal explants.

2 Materials and methods

2.1 Chemicals

All the chemicals utilized in this study were procured from Himedia, India.

2.2 Plant material

In May 2022, we collected fresh *Aquilegia fragrans* rhizomes from wild populations in Gulmarg (34° 02' 20.64" N; 74° 22' 43.72" E) and Aharbal (33° 39' 00" N; 74° 47' 15" E), Jammu and Kashmir, India (Fig. 1). To prevent bud growth before experimentation, the collected rhizomes were thoroughly washed with running tap water and stored in a cool, dark place room at 4 °C. The plant material was verified by the curator at the Centre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir, Srinagar, and the specimen (KASH-8306/KASH-8307) has been deposited in the Herbarium. The experiments were conducted at the Department of Botany, University of Kashmir in Srinagar, India. This location has a humid temperate climate with an average elevation of

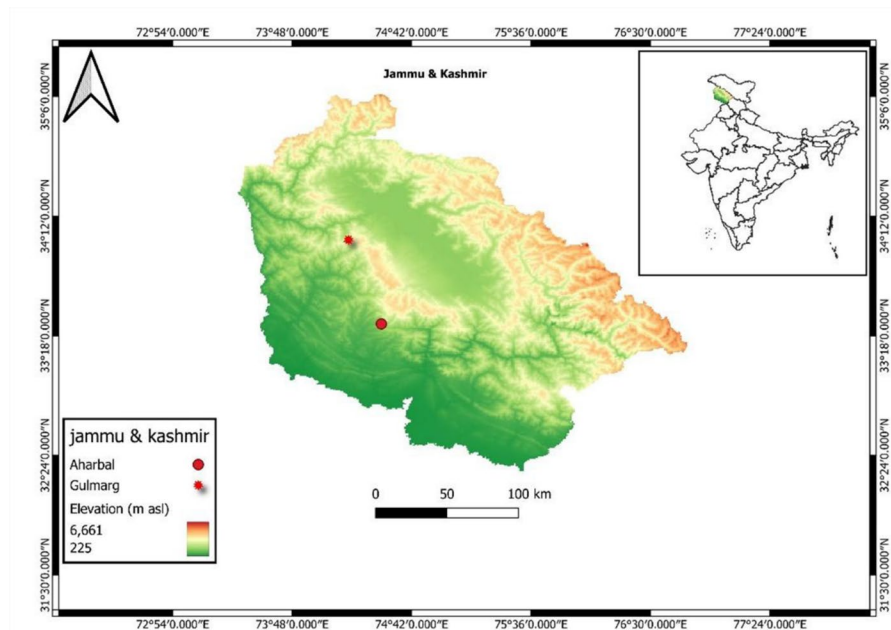


Fig. 1 Study site map

1590 m above sea level and coordinates of 34° 07' 40.7" N, 74° 50' 11.5" E. The area also receives a high amount of annual rainfall, averaging 1005.5 mm [24]. The plants collected for micropropagation were maintained in a polyhouse under controlled environmental conditions to obtain healthy explants. The average temperature was maintained at 24 ± 2 °C, with relative humidity ranging between 70 and 80%. The plants were exposed to natural photoperiod supplemented with a light intensity of approximately $40\text{--}60 \mu\text{mol m}^{-2} \text{s}^{-1}$. These conditions ensured optimal growth and development of the mother plants for explant collection.

2.3 Hormone experiment

To investigate how various plant hormones influence the clonal reproduction of *A. fragrans*, rhizomes were sectioned lengthwise into 2–4 pieces using a sterilized, sharp blade (Supplementary Fig. 1). The size and bud count of the parent rhizome determined the number of sections. The rhizome segments were first disinfected by submerging them in a 2% Bavistin solution for surface sterilization. They then received a 48-h treatment with various growth hormones 6-Benzylaminopurine (BAP), Indole-3-butyric acid (IBA), Meta-topolin, and Gibberellic Acid (GA_3) at concentrations of 50, 100, 150, and 200 parts per million (ppm). Distilled water served as the control. Following the hormone treatment, the rhizome sections were planted in pots filled with a 3:1 mixture of soil and sand. These pots were kept inside a growth chamber and watered daily for the first ten days. Following this period, the watering frequency was decreased to once every three days. The experiment involved 13 different treatments (Supplementary Table 1), each replicated four times in a randomized block design with three sections per treatment. Throughout the experiment, close monitoring was conducted. Plant growth parameters like root and shoot length, number of leaves, sprouting time (in days), and sprouting percentage were all documented. The average number of leaves per plant was determined manually, while root and shoot lengths (in cm) were measured with a ruler [25].

2.4 Effect of different soil compositions

To initiate the experiment, freshly collected rhizomes were sectioned longitudinally. These prepared sections were then disinfected by submerging them in a 2% solution of Bavistin, a systemic fungicide, to ensure surface sterilization. Next, the disinfected rhizome cuttings were planted in pots containing various pre-mixed soil compositions. These compositions were created by combining specific weights of soil, sand, vermicompost (VC), and peat moss. Each soil composition was replicated five times, with each replicate containing three rhizome cuttings. The planted pots were placed in a growth chamber under controlled conditions. Watering was initially done daily for the first week to establish the plants. Subsequently, watering was adjusted based on the moisture level of the topsoil, with irrigation only occurring when the surface soil began to dry. The experiment monitored the overall sprouting success (sprouting percentage) across all treatment groups. At the conclusion of the experiment, plants were harvested destructively for detailed analysis of various growth parameters (Supplementary Table 2).

2.5 Micropropagation explant sterilization

Healthy nodal segments were harvested as explants from donor plants maintained under polyhouse conditions. The explants were initially subjected to a thorough rinse

under running tap water for 10–15 min to remove surface debris. This was followed by a 10-min treatment with 0.2% (w/v) Bavistin (a systemic fungicide) and a subsequent 5-min wash with a 0.1% (v/v) aqueous solution of Tween-20 (Sigma-Aldrich, India). Residual surfactants and fungicides were removed by rinsing the segments multiple times with sterile distilled water.

Surface sterilization was performed under aseptic conditions within a laminar air-flow hood. To determine the most effective decontamination protocol, various chemical sterilants and exposure durations were evaluated (Supplementary Table 3). The sterilized nodal segments were then inoculated into culture tubes containing MS media. The medium was supplemented with varying concentrations and combinations of plant growth regulators such as Thidiazuron (TDZ), Indole-3-acetic acid (IAA), Naphthalene acetic acid (NAA) and IBA to evaluate their effect on morphogenesis.

2.6 Culture medium and conditions

The study utilized MS medium containing diverse growth regulator treatments. The media, which included 0.8% agar and 3% sucrose, was pH-balanced to 5.6 and autoclaved (121 °C for 15 min at 15 psi). Surface-disinfected nodal segments were then introduced to the media and incubated in a controlled environment (25 ± 2 °C) with a 16/8-h light/dark cycle. Experimental data, including shoot count, length, and rhizome growth, were recorded through consistent monitoring of the specimens.

2.7 Hardening of plantlets

The *in vitro* grown plantlets having well developed shoots and rhizome were carefully taken out from the phyta jars and rinsed with autoclaved distilled water to remove the traces of agar associated with them. For primary hardening these washed plantlets were transferred to plastic pots containing vermiculite and kept in growth chamber having temperature 25 °C under 16 h light and 8 h dark and relative humidity 70–80% for 3 weeks before being transferred to the green house. After 3 weeks in the culture room, plantlets were shifted to green house in pots containing soil: sand: manure (1:1:1) for acclimatization and subsequent field transfer.

2.8 Statistical analysis

To assess the influence of various soil compositions and growth hormones on plant growth and morphology, we employed ANOVA using Minitab software version 21.1.0. This statistical method allowed us to investigate the significance of these factors on different growth parameters and morphological characteristics. We further utilized Tukey's honestly significant difference (HSD) test (at a significance level of $\alpha = 0.05$) to pinpoint specific pairwise mean differences between the treatment groups. For *in vitro* studies, the trials were achieved in triplicates with minimum 12 explants per treatment. Observations were recorded and data was analysed by means of SPSS 16.0 software (SPSS Inc., Chicago, USA) and mean values are expressed as mean $\pm$ SE and significance level at 5% for each trial was done by Duncan's Multiple Range Test (DMRT).

3 Results

Growth parameters of rhizome cuttings exhibited significant variation after treatment with different phytohormones. Rhizome sections treated with 150 ppm GA₃ displayed the highest sprouting rate (83.34%), followed by those treated with 100 ppm GA₃ (75%). In contrast, the control group only exhibited a 25% sprouting rate (Table 1). The synthetic cytokinin (Meta-topolin) was unsuccessful in stimulating the sprouting of rhizome cuttings. The sprouting time varied among different treatments, ranging from 20.75 days to 35.50 days. Among the GA₃ concentrations applied, 150 ppm resulted in the shortest sprouting time for rhizome cuttings. Rhizome cuttings treated with 150 ppm and 100 ppm GA₃ displayed significantly higher sprouting rates (83.34% and 75%, respectively) and faster sprouting times (20.75 and 23.50 days) compared to the control group, which exhibited a lower sprouting rate (25%) and a longer sprouting time 35.50 days (Table 1). Applying GA₃ to rhizome cuttings significantly enhanced various growth parameters, including shoot length, root length, and leaf number. Notably, rhizome sections treated with 150 ppm GA₃ exhibited the highest number of leaves (44.75 ± 1.49), followed by those treated with 100 ppm GA₃ (32 ± 1.87). Similarly, 150 ppm GA₃ resulted in the longest rhizome length (9.5 ± 0.65 cm) and shoot length (16.6 ± 0.49 cm) (Fig. 2).

So far, the effect of soil composition is considered it significantly affected the sprouting percentage among the treatments. The sprouting rate of rhizome cuttings ranged from 41.66 to 100%, with the highest sprouting percentages observed in T5 and T9 (Table 2). The time taken by rhizome section to sprout within different soil composition treatments revealed a significant variation. The minimum time taken for sprouting was demonstrated by T9 (19.75 ± 0.85 days) followed by T5 (21.50 ± 0.64 days). Among the different treatments; morphological parameters such as plant height, below ground fresh and dry weight, rhizome length and leaf number also indicated significant variations (Table 2). Plant height and rhizome length varied between 18.35 ± 0.37 cm to 35.07 ± 0.65 cm and 5.72 ± 0.24 cm to 13.27 ± 0.39 cm respectively in different soil

Table 1 The impact of various growth hormones on vegetative propagation of *A. fragrans*

Treatment	Concentration	PS	RL	SL	LN	TTS
BAP	50	41.67 ± 8.34^{cde}	4.98 ± 0.3^{cd}	6.68 ± 0.32^{de}	9 ± 0.82^{def}	30.25 ± 0.48^{abc}
BAP	100	50 ± 9.62^{bcde}	6.78 ± 0.17^{bc}	7.1 ± 0.32^d	12.5 ± 1.44^{de}	29 ± 1.08^{abc}
BAP	150	58.34 ± 8.34^{abcd}	7.8 ± 0.26^{ab}	8.18 ± 0.4^{cd}	19.5 ± 1.19^c	30.25 ± 0.63^{abc}
BAP	200	33.33 ± 0^{de}	5.05 ± 0.38^{cd}	5.7 ± 0.26^{de}	8 ± 0.82^{ef}	31.5 ± 0.65^{ab}
IBA	50	25 ± 8.33^{ef}	3.63 ± 1.22^d	3.85 ± 1.33^e	7.5 ± 2.53^{ef}	21.5 ± 7.22^c
IBA	100	33.33 ± 0^{de}	7.7 ± 0.19^{ab}	7.68 ± 0.64^{cd}	14.75 ± 2.10^{cd}	35.25 ± 0.85^a
IBA	150	41.67 ± 8.34^{cde}	6.68 ± 0.32^{bc}	5.38 ± 0.38^{de}	8.25 ± 0.48^{ef}	34.75 ± 2.32^a
IBA	200	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Meta-topolin	50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Meta-topolin	100	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Meta-topolin	150	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Meta-topolin	200	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1GA ₃	50	66.67 ± 0^{aab}	6.88 ± 0.43^{bc}	13 ± 0.41^b	29 ± 1.08^b	24.5 ± 0.87^{bc}
GA ₃	100	75 ± 8.33^{ab}	7.75 ± 0.48^{ab}	12.9 ± 1.33^b	32 ± 1.87^b	23.5 ± 0.65^{bc}
GA ₃	150	83.34 ± 9.62^a	9.5 ± 0.65^a	16.6 ± 0.49^a	44.75 ± 1.49^a	20.75 ± 0.63^c
GA ₃	200	50 ± 9.62^{bcde}	6.25 ± 0.48^{bc}	10.5 ± 0.65^{bc}	27.25 ± 1.93^b	26.5 ± 0.87^{abc}
Control	–	25 ± 8.33^{ef}	5 ± 0.41^{cd}	8 ± 0.71^{cd}	5.5 ± 0.29^g	35.5 ± 0.65^a

PS Percentage sprouting, RL Root Length, SL shoot length, LN Number of leaves, TTS Time taken for sprouting (days)

*Mean $\pm$ SE, Means with the same superscript letters in the same column are not significantly different while those with different superscript letters are significantly different at $p \leq 0.05$



Fig. 2 (A–M) Effect of Various phytohormones on the different growth parameters of *A. fragrans*. (A and B) Plant growth from rhizome cuttings treated with GA₃ (150 ppm); (C–E) Plant growth from rhizome cuttings treated with GA₃ (100 ppm) ; (F–H) Plant growth from rhizome cuttings treated with TDZ (150 ppm) ; (I–K) Plant growth from rhizome cuttings treated with TDZ (100 ppm); (L and M) Plant growth from rhizome cuttings treated with IBA (50 ppm and 100 ppm) respectively; (N and O) Record of morphological parameters. Scale bars represent 1 cm (A–M) and 5 cm (N–O)

Table 2 The effect of various soil compositions on the growth and morphological characteristics of rhizome cuttings

Treatment	SP	BGDW	BGFW	Days	RL	PH
T1	50 ± 9.62 ^{ab}	22.50 ± 0.44 ^c	67.42 ± 0.59 ^{bc}	35.00 ± 0.40 ^h	5.72 ± 0.24 ^a	21.92 ± 0.47 ^b
T2	66.67 ± 0.00 ^{bcd}	24.55 ± 0.42 ^{cd}	71.87 ± 0.53 ^{bcd}	29.75 ± 0.47 ^e	8.02 ± 0.70 ^{cd}	27.50 ± 28 ^{ef}
T3	58.35 ± 8.33 ^{abc}	25.42 ± 0.88 ^d	73.77 ± 1.17 ^{bcd}	32.25 ± 0.47 ^{fg}	6.35 ± 0.48 ^{ab}	24.25 ± 0.47 ^{cd}
T4	41.66 ± 8.33 ^a	23.57 ± 1.73 ^{cd}	68.02 ± 0.86 ^{bc}	31.50 ± 0.86 ^f	6.02 ± 0.35 ^a	18.35 ± 0.37 ^a
T5	100 ± 0.00 ^f	34.87 ± 0.44 ^f	96.05 ± 0.51 ^e	21.50 ± 0.64 ^b	13.27 ± 0.39 ^g	35.07 ± 0.65 ^h
T6	75.00 ± 8.33 ^{cde}	28.20 ± 0.52 ^e	82.60 ± 0.81 ^{cde}	33.25 ± 0.47 ^{gh}	7.42 ± 0.60 ^{bc}	26.90 ± 0.70 ^e
T7	75.00 ± 8.33 ^{cde}	29.42 ± 1.05 ^e	89.55 ± 0.47 ^{de}	32.25 ± 0.62 ^{fg}	6.17 ± 0.14 ^{ab}	28.40 ± 0.83 ^{ef}
T8	50.00 ± 9.62 ^{ab}	28.10 ± 0.28 ^e	63.87 ± 21.30 ^{bc}	31.75 ± 0.25 ^f	6.52 ± 0.25 ^{ab}	24.92 ± 0.93 ^d
T9	100 ± 0.00 ^f	35.65 ± 0.51 ^f	98.05 ± 0.32 ^e	19.75 ± 0.85 ^a	12.15 ± 0.17 ^g	33.05 ± 0.56 ^g
T10	83.33 ± 9.62 ^{def}	30.00 ± 0.52 ^e	90.60 ± 0.65 ^{de}	23.50 ± 0.64 ^c	10.30 ± 0.31 ^f	27.70 ± 0.47 ^{ef}
T11	66.67 ± 0.00 ^{bcd}	19.12 ± 0.43 ^b	57.12 ± 0.74 ^{ab}	35.00 ± 0.40 ^h	8.92 ± 0.33 ^{de}	23.05 ± 0.26 ^{bc}
T12	58.33 ± 8.33 ^{abc}	15.27 ± 0.41 ^a	45.82 ± 1.06 ^a	34.00 ± 0.40 ^{gh}	9.70 ± 0.34 ^{ef}	25.05 ± 0.76 ^d
T13	91.66 ± 8.32 ^{ef}	33.66 ± 0.43 ^f	89.95 ± 0.62 ^{de}	25.75 ± 0.47 ^d	10.12 ± 0.63 ^{ef}	29.22 ± 0.48 ^f

SP sprouting percentage, BGDW belowground dry weight, BGFW belowground fresh weight, RL rhizome length, PH plant height

*Mean ± SE, Means with the same superscript letters in the same column are not significantly different while those with different superscript letters are significantly different at *p* ≤ 0.05

compositions (Fig. 3). Maximum plant height (35.07 ± 0.65 cm) and rhizome length (12.15 ± 0.17 cm) was shown by treatment T5 and T9 respectively. The variance analysis revealed a significant difference in both below ground fresh and dry weight across all treatments. Below ground fresh weight and dry weight varied between 45.82 ± 1.06 g to 98.05 ± 0.51 g and 15.27 ± 0.41 g to 35.65 ± 0.51 g. Maximum below ground fresh and dry weight was observed in T9 followed by T5.



Fig. 3 Effect of different soil composition on the growth parameters of *A. fragrans*. Pictures (A–O) represents plants grown from rhizome cuttings upon different treatments of phytohormones. (A–C). Treatment T5 (Soil: sand: vermicompost); (D–G) T9- Soil: pebbles: vermicompost (H–J); (L, M) T13-Sand: soil: peat: vermicompost; (K) Plant growth from rhizome cuttings in treatment T1; (L and M); Plant growth from rhizome cuttings in treatment T2 and T3 respectively; (N and O). Plant growth from rhizome in treatment T11 and T12 respectively. *All scale bars represent 1 cm

3.1 Micropropagation

3.1.1 Sterilisation optimisation

The present study aimed to develop an efficient *in vitro* propagation protocol for *A. fragrans* using node as explant which were cultured on Murashige and Skoog (MS) medium augmented with varying concentrations of plant hormones (2, 4-D, BAP, IBA, IAA, TDZ meta-Toplin and NAA, either individually or in combination). Initially, to standardize the explant surface sterilization protocol, explants were subjected to six different chemical sterilization treatments (*ST-1* to *ST-6*) varying in sterilant concentration and exposure duration. The efficacy of each treatment was assessed based on contamination rate, percentage of necrotic tissue, and explant survival. The results demonstrated significant variation among treatments. Amongst all protocols, *ST-3* proved to be the most effective, exhibiting the lowest contamination ($8.46 \pm 0.16\%$) and necrosis ($2.84 \pm 0.08\%$) along with the highest survival rate ($93.14 \pm 0.16\%$). In contrast, *ST-2* was the least effective, recording maximum contamination ($76.11 \pm 0.12\%$) and minimal survival ($4.10 \pm 0.11\%$). Moderate responses were observed under *ST-4*, while *ST-5* and *ST-6* showed comparatively higher contamination and necrosis, resulting in reduced explant survival (Table 3). Overall, the differences among treatments were statistically significant ($p \leq 0.05$), confirming *ST-3* as the optimal surface sterilization protocol for achieving maximum explant viability with minimal tissue damage.

3.1.2 Effect of plant growth regulators (meta-Topolin and TDZ) on shoot bud initiation

The data presented in Table 4 indicates that shoot regeneration was significantly influenced by cytokinin type and concentration. No shoot induction was observed in the control medium. Both meta-Topolin (mT) and TDZ exhibited a concentration-dependent

Table 3 Effect of different chemical sterilant treatments for standardisation of surface sterilisation of explants

Sterilization treatments (ST)	Contamination of explants (%)	Necrosis of explants (%)	Survival of explants (%)
ST-1	38.96 ± 0.10 ^e	10.99 ± 0.01 ^f	62.96 ± 0.09 ^d
ST-2	76.11 ± 0.12 ^f	9.08 ± 0.05 ^e	4.10 ± 0.11 ^a
ST-3	8.46 ± 0.16 ^a	2.84 ± 0.08 ^a	93.14 ± 0.16 ^f
ST-4	12.26 ± 0.14 ^b	6.02 ± 0.03 ^b	68.07 ± 0.78 ^e
ST-5	19.28 ± 0.27 ^c	8.06 ± 0.04 ^c	54.07 ± 0.05 ^c
ST-6	21.89 ± 0.10 ^d	8.49 ± 0.03 ^d	44.71 ± 0.29 ^b

*Mean ± SE

Different letters in the same column indicate means that are significantly different among different treatments (DMRT: $p \leq 0.05$)**Table 4** Effect of meta Topolin (mT) and TDZ on direct shooting from nodal explants of *A. fragrans*

Growth hormone	Concentrations	Response (%) ± SE	Number of shoots (mean ± SE)	Shoot length (mean ± SE)	No. of days (mean ± SE)
Meta-Topolin (mTP) (mg L ⁻¹)	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	36.52 ± 0.19 ^a	3.03 ± 0.03 ^a	2.95 ± 0.03 ^{ab}	26.60 ± 0.12 ^f
	1.0	45.33 ± 0.27 ^b	4.09 ± 0.01 ^b	2.59 ± 0.15 ^a	25.59 ± 0.24 ^e
	1.5	54.07 ± 0.07 ^c	4.88 ± 0.05 ^d	3.55 ± 0.23 ^c	24.45 ± 0.21 ^d
	2.0	60.93 ± 0.24 ^d	5.25 ± 0.14 ^e	3.35 ± 0.16 ^{bc}	24.64 ± 0.16 ^d
	2.5	63.06 ± 0.07 ^e	5.38 ± 0.05 ^e	4.15 ± 0.07 ^d	23.68 ± 0.08 ^c
	3.0	68.06 ± 0.04 ^f	5.88 ± 0.05 ^f	4.51 ± 0.24 ^{de}	22.86 ± 0.01 ^b
	3.5	72.25 ± 0.19 ^g	6.00 ± 0.07 ^f	5.22 ± 0.10 ^f	21.70 ± 0.08 ^a
TDZ (mg L ⁻¹)	4.0	63.52 ± 0.18 ^e	4.37 ± 0.10 ^c	4.74 ± 0.12 ^e	24.80 ± 0.04 ^d
	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	41.67 ± 1.67 ^a	1.50 ± 0.33 ^a	1.33 ± 0.33 ^a	28.33 ± 0.88 ^d
	1.0	46.67 ± 3.33 ^{ab}	2.00 ± 0.58 ^{ab}	1.67 ± 0.33 ^{ab}	25.67 ± 1.86 ^{cd}
	1.5	52.67 ± 2.67 ^{bc}	3.00 ± 0.58 ^{abc}	2.33 ± 0.89 ^{abc}	24.00 ± 1.15 ^{bc}
	2.0	60.00 ± 2.89 ^{cd}	3.67 ± 0.89 ^{abcd}	3.00 ± 0.58 ^{abcd}	23.33 ± 0.88 ^c
	2.5	63.33 ± 4.40 ^d	4.33 ± 1.20 ^{cd}	3.33 ± 0.33 ^{bcd}	22.67 ± 1.45 ^b
	3.0	68.33 ± 4.40 ^{de}	4.67 ± 0.89 ^{cd}	3.67 ± 0.33 ^{cd}	21.00 ± 0.58 ^{ab}
	3.5	75.00 ± 2.89 ^e	5.33 ± 0.33 ^d	4.33 ± 0.88 ^d	18.33 ± 0.88 ^a
	4.0	65.33 ± 2.60 ^{de}	4.00 ± 0.58 ^{bcd}	3.00 ± 0.58 ^{abcd}	22.00 ± 0.58 ^{bc}

*Mean ± SE

*Mean ± SE

response, with regeneration frequency, shoot number, and shoot length increasing progressively up to 3.5 mg L⁻¹ (Fig. 4). At this concentration, TDZ produced the highest regeneration response (75.00%), with a maximum of 5.33 shoots per explant, longest shoots (4.33 cm), and the shortest induction period (18.33 days), while mT resulted in a comparable response (72.25%) with higher shoot proliferation (6.00 shoots per explant), longer shoots (5.22 cm), and shoot induction within 21.70 days. A further increase to 4.0 mg L⁻¹ of either cytokinin led to a decline in regeneration efficiency (Table 4). Overall, 3.5 mg L⁻¹ of both TDZ and mT was optimal for shoot regeneration.

3.1.3 Combined effect of meta-Topolin (mT) and TDZ in association with different auxins (IAA, IBA, and NAA) on shoot

The interaction of cytokinins (TDZ or meta-Topolin at 3.5 mg L⁻¹) with auxins (IAA, IBA, and NAA) significantly influenced shoot regeneration in a concentration-dependent

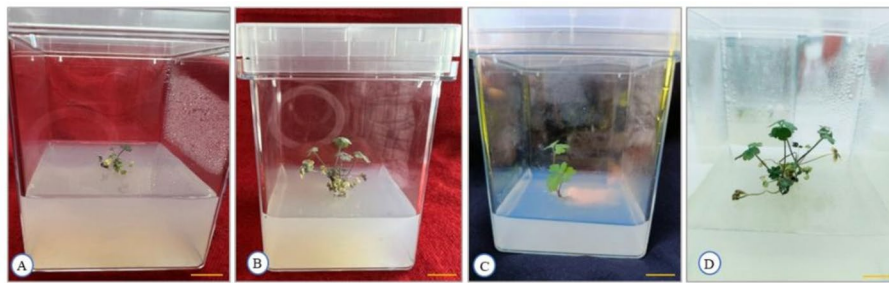


Fig. 4 Effect of different PGRs on shooting (A) 0.5 mg L⁻¹ mTP (B) 3.5 mg L⁻¹ mTP (C) 0.5 mg L⁻¹ TDZ (D) 3.5 mg L⁻¹ TDZ. *All scale bars represent 1 cm

manner. In both cytokinin backgrounds, IBA proved to be the most effective auxin, producing the highest regeneration response, enhanced shoot elongation, and the shortest induction period (Table 5). The optimal response was recorded with TDZ + IBA at 1.25 mg L⁻¹, yielding 82.00% regeneration, 6.00 shoots per explant, longest shoots (6.41 cm), and minimum (19.33 days) induction time (Fig. 5), followed closely by the meta-Topolin + IBA combination at 1.75 mg L⁻¹, producing the highest regeneration response (80.00%), maximum shoot number (6.00 shoots per explant), longest shoots (6.07 cm), and minimum time (20.00 days) for shoot induction (Fig. 6). The IAA treatments showed moderate to high regeneration efficiency, with optimal performance at 1.50 mg L⁻¹ in both systems, whereas NAA elicited comparatively lower responses, although its best results were observed at 1.25–1.50 mg L⁻¹. In all treatments, auxin concentrations beyond the optimum led to a decline in regeneration efficiency (Table 5).

3.1.4 Effect of auxin (NAA) on rhizome formation

Presently, after two weeks of incubation, rhizome induction became noticeable at the base of the cultured shoots. The culture response rate for rhizome formation ranged between 34.4 ± 0.88 and $71.45 \pm 0.00\%$ across the different treatments (Table 6). NAA significantly affected rhizome induction in a concentration-dependent manner. No response was observed in the control and at the highest concentration (2.0 mg L⁻¹). Rhizome induction frequency, rhizome length, and reduction in induction time increased progressively with increasing NAA concentration up to 1.50 mg L⁻¹, which resulted in the maximum response (71.45%), longest rhizomes (6.02 cm), and the shortest induction period (35.73 days). A further increase to 1.75 mg L⁻¹ caused a decline in rhizome formation efficiency, indicating an inhibitory effect at supra-optimal levels. Overall, 1.50 mg L⁻¹ NAA (Fig. 7) was identified as the optimal concentration for efficient rhizome induction and growth under the present experimental conditions.

3.1.5 Hardening of plantlets

Presently, plantlets bearing well-developed micro-rhizomes were transferred to plastic pots containing a soil: sand: vermicompost mixture (1:1:1) and maintained under greenhouse conditions, resulting in a 75% survival rate. Moreover, no phenotypic variations were observed between the micropropagated plants and their wild counterparts (Fig. 8).

Table 5 Effect of various doses of auxins with optimal meta-Topolin and TDZ (3.5 mg L⁻¹) on shooting from nodal explants

PGR(s)	Concentration (mg L ⁻¹)	Response (% ± SE)	Number of shoots (mean ± SE)	Shoot length (mean ± SE)	No. of days (mean ± SE)
Meta-Topolin 3.5 + IAA	0.25	58.33 ± 1.67 ^a	2.66 ± 0.33 ^a	3.92 ± 0.07 ^{ab}	27.33 ± 0.88 ^d
	0.5	63.29 ± 1.64 ^{ab}	4.00 ± 0.57 ^{abc}	4.33 ± 0.33 ^b	25.33 ± 0.33 ^{cd}
	0.75	67.67 ± 0.33 ^{bc}	4.00 ± 0.57 ^{abc}	4.89 ± 0.48 ^b	23.67 ± 0.33 ^{bc}
	1.0	70.67 ± 1.76 ^{cd}	5.00 ± 0.57 ^c	5.17 ± 0.09 ^b	22.00 ± 1.00 ^{ab}
	1.25	74.33 ± 2.91 ^{de}	5.33 ± 0.33 ^c	5.33 ± 0.89 ^b	21.00 ± 1.00 ^{ab}
	1.50	77.00 ± 1.15 ^e	5.66 ± 0.88 ^c	5.54 ± 0.74 ^b	20.33 ± 0.88 ^a
	1.75	70.00 ± 1.15 ^{cd}	4.66 ± 0.33 ^{bc}	4.00 ± 0.58 ^{ab}	21.33 ± 0.88 ^{ab}
	2.0	60.67 ± 2.33 ^a	3.00 ± 0.75 ^{ab}	2.67 ± 0.33 ^a	22.33 ± 0.88 ^{ab}
Meta-Topolin 3.5 + IBA	0.25	60.00 ± 0.58 ^a	3.33 ± 0.33 ^a	4.43 ± 0.35 ^{ab}	26.33 ± 0.33 ^d
	0.5	64.00 ± 0.58 ^b	4.00 ± 0.57 ^{ab}	4.83 ± 0.16 ^b	25.00 ± 0.58 ^c
	0.75	68.33 ± 0.88 ^c	5.00 ± 0.57 ^{bc}	5.39 ± 0.20 ^b	24.00 ± 0.58 ^{bc}
	1.0	71.33 ± 1.33 ^c	5.23 ± 0.57 ^b	5.67 ± 0.20 ^b	23.00 ± 1.00 ^{bc}
	1.25	75.67 ± 1.76 ^d	5.33 ± 0.33 ^{bc}	5.83 ± 1.09 ^b	22.00 ± 1.15 ^{ab}
	1.50	70.33 ± 1.20 ^c	4.66 ± 0.33 ^{ab}	4.50 ± 0.29 ^a	21.67 ± 0.67 ^{ab}
	1.75	80.00 ± 1.00 ^e	6.00 ± 0.57 ^c	6.07 ± 0.51 ^b	20.00 ± 1.00 ^a
	2.0	62.67 ± 1.45 ^{ab}	3.33 ± 0.33 ^a	3.17 ± 0.44 ^a	23.33 ± 0.33 ^{bc}
Meta-Topolin 3.5 + NAA	0.25	56.67 ± 0.88 ^a	2.91 ± 0.07 ^a	3.76 ± 0.14 ^{ab}	27.00 ± 0.58 ^e
	0.5	59.67 ± 1.67 ^{ab}	3.67 ± 0.12 ^b	4.17 ± 0.44 ^{bc}	25.67 ± 0.33 ^{de}
	0.75	64.00 ± 0.58 ^{bc}	4.03 ± 0.57 ^{bc}	4.72 ± 0.43 ^b	24.67 ± 1.20 ^{cd}
	1.0	68.67 ± 0.88 ^{de}	4.17 ± 0.07 ^{bc}	5.00 ± 0.17 ^{bc}	23.67 ± 0.33 ^{bcd}
	1.25	74.67 ± 1.20 ^f	5.58 ± 0.15 ^e	5.41 ± 0.30 ^c	20.67 ± 0.88 ^a
	1.50	70.00 ± 1.73 ^e	5.07 ± 0.19 ^{de}	5.17 ± 0.93 ^b	22.67 ± 0.67 ^{abc}
	1.75	65.33 ± 1.45 ^{cd}	4.46 ± 0.16 ^{cd}	3.83 ± 0.17 ^{ab}	22.33 ± 0.67 ^{ab}
	2.0	58.00 ± 2.52 ^a	3.94 ± 0.03 ^{bc}	2.50 ± 0.29 ^a	24.00 ± 0.58 ^{bcd}
TDZ 3.5 + IAA	0.25	57.33 ± 0.88 ^a	3.78 ± 0.55 ^{ab}	4.09 ± 0.21 ^b	26.67 ± 0.67 ^d
	0.5	61.33 ± 1.76 ^{ab}	3.83 ± 0.73 ^{ab}	4.50 ± 0.29 ^{bc}	25.33 ± 0.33 ^{cd}
	0.75	66.00 ± 1.53 ^c	4.32 ± 0.45 ^{ab}	5.05 ± 0.53 ^{bc}	24.33 ± 0.88 ^{bcd}
	1.0	69.00 ± 0.58 ^{cd}	4.47 ± 0.32 ^{abc}	5.33 ± 0.20 ^{bc}	23.33 ± 0.67 ^{bc}
	1.25	73.33 ± 0.88 ^e	5.10 ± 0.49 ^{bc}	5.50 ± 0.50 ^{bc}	22.33 ± 1.45 ^{ab}
	1.50	78.67 ± 1.76 ^f	5.83 ± 0.17 ^c	5.84 ± 0.58 ^c	20.33 ± 1.33 ^a
	1.75	72.00 ± 1.15 ^{de}	4.17 ± 0.17 ^{ab}	4.17 ± 0.60 ^b	22.00 ± 0.58 ^{ab}
	2.0	61.67 ± 1.67 ^b	3.50 ± 0.50 ^a	2.67 ± 0.44 ^a	23.67 ± 0.33 ^{bc}
TDZ 3.5 + IBA	0.25	59.00 ± 1.15 ^a	3.33 ± 0.03 ^{ab}	4.76 ± 0.65 ^{ab}	25.67 ± 0.33 ^d
	0.5	63.00 ± 0.58 ^b	4.33 ± 0.33 ^b	5.17 ± 0.44 ^{ab}	24.33 ± 1.20 ^{cd}
	0.75	67.67 ± 0.33 ^c	2.33 ± 0.66 ^a	5.72 ± 0.15 ^b	23.33 ± 0.33 ^{bcd}
	1.0	70.67 ± 1.76 ^{cd}	4.00 ± 0.57 ^b	6.00 ± 0.51 ^b	22.33 ± 1.67 ^{abc}
	1.25	82.00 ± 0.58 ^f	6.00 ± 0.57 ^c	6.41 ± 0.59 ^b	19.33 ± 0.33 ^a
	1.50	75.00 ± 1.73 ^e	5.00 ± 0.57 ^{bc}	6.17 ± 1.01 ^b	21.33 ± 0.67 ^{abc}
	1.75	73.33 ± 1.76 ^{de}	4.00 ± 0.52 ^b	4.83 ± 0.17 ^{ab}	21.00 ± 1.15 ^{ab}
	2.0	63.33 ± 1.67 ^b	4.66 ± 0.33 ^{bc}	3.50 ± 0.50 ^a	22.67 ± 0.88 ^{bcd}
TDZ 3.5 + NAA	0.25	55.00 ± 1.00 ^a	3.11 ± 0.39 ^a	3.42 ± 0.23 ^{ab}	29.33 ± 0.67 ^e
	0.5	60.00 ± 1.15 ^b	3.17 ± 0.44 ^a	3.83 ± 0.44 ^b	28.00 ± 1.15 ^{de}
	0.75	64.33 ± 1.33 ^{cd}	3.65 ± 0.35 ^{ab}	4.39 ± 0.77 ^b	27.00 ± 1.53 ^{cde}
	1.0	67.00 ± 0.58 ^{de}	3.80 ± 0.47 ^{ab}	4.67 ± 0.38 ^b	25.00 ± 0.58 ^{bcd}
	1.25	73.00 ± 0.58 ^f	4.43 ± 0.30 ^{bc}	4.83 ± 0.17 ^b	24.00 ± 0.58 ^{abc}
	1.50	76.67 ± 0.67 ^g	5.17 ± 0.44 ^c	5.17 ± 0.49 ^b	21.33 ± 1.20 ^a
	1.75	69.67 ± 0.33 ^e	3.50 ± 0.29 ^{ab}	3.50 ± 0.76 ^{ab}	23.33 ± 0.88 ^{ab}
	2.0	63.33 ± 1.67 ^c	2.83 ± 0.17 ^a	2.00 ± 0.76 ^a	25.33 ± 0.88 ^{bcd}

*Mean ± SE, Means with the same superscript letters in the same column are not significantly different while those with different superscript letters are significantly different at $p \leq 0.05$

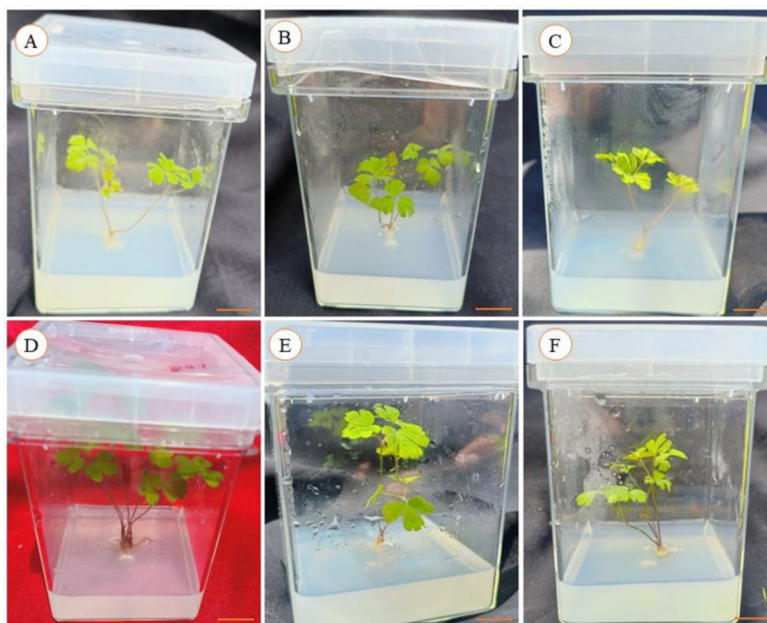


Fig. 5 Effect of different PGRs on shoot induction and multiplication (A) TDZ + 0.25 mg L⁻¹ IAA (B) TDZ + 1.50 mg L⁻¹ IAA (C) TDZ + 0.25 mg L⁻¹ IBA (D) TDZ + 1.25 mg L⁻¹ IBA (E) TDZ + 0.25 mg L⁻¹ NAA (F) TDZ + 1.50 mg L⁻¹ NAA. All scale bars represent 1 cm. *All scale bars represent 1 cm

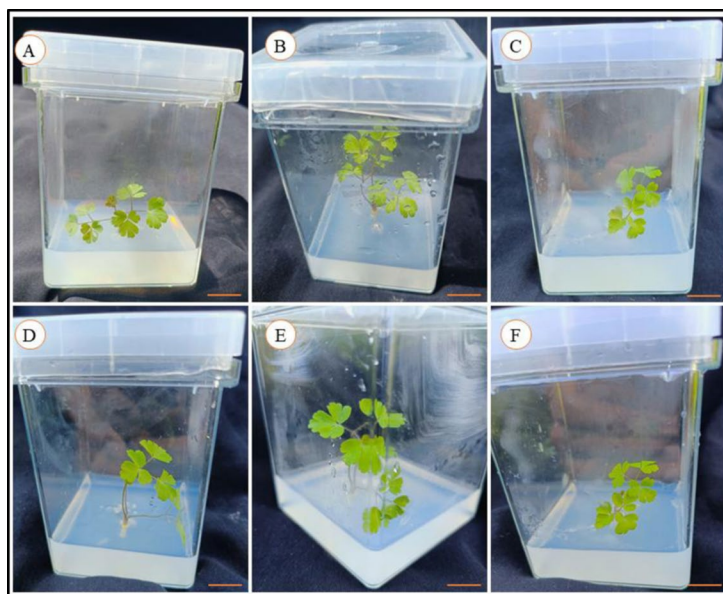


Fig. 6 Effect of different PGRs on shoot induction and multiplication (A) mTP + 0.25 mg L⁻¹ IAA (B) mTP + 1.50 mg L⁻¹ IAA (C) mTP + 0.25 mg L⁻¹ IBA (D) mTP + 1.75 mg L⁻¹ IBA (E) mTP + 1.25 mg L⁻¹ NAA (F) mTP + 0.25 mg L⁻¹ NAA. *All scale bars represent 1 cm

4 Discussion

4.1 Vegetative propagation

For plant species that struggle to reproduce effectively through seeds, vegetative propagation provides a valuable method for large-scale germplasm multiplication and cultivation [26]. The erratic weather fluctuations experienced at high altitudes can disrupt plant reproduction at different stages of their development [27]. *A. fragrans*, a plant commonly

Table 6 Effect of auxin (NAA) on rhizome formation

Auxin conc. (mg L ⁻¹)	Response (% ± SD)	Rhizome length (cm) (mean ± SD)	No. of days (mean ± SD)
Control			
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Naphthalene acetic acid (NAA)			
0.5	34.41 ± 0.88 ^a	2.13 ± 0.01 ^a	46.74 ± 0.58 ^f
0.75	48.37 ± 0.58 ^b	3.39 ± 0.09 ^b	43.47 ± 1.67 ^e
1.0	56.86 ± 1.53 ^d	4.72 ± 0.09 ^c	41.17 ± 1.00 ^d
1.25	63.56 ± 3.79 ^e	5.12 ± 0.04 ^d	38.65 ± 1.15 ^c
1.50	71.45 ± 0.00 ^f	6.02 ± 0.06 ^e	35.73 ± 0.00 ^a
1.75	54.72 ± 0.00 ^c	4.00 ± 0.07 ^c	37.84 ± 0.00 ^b
2.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

*Mean ± SE

Different letters in the same column indicate means that are significantly different among different treatments (DMRT: $p \leq 0.05$)**Fig. 7** Effect of NAA on rhizome formation (A) Rhizome in phytajar (B) 1.25 mg L⁻¹ (C, D) 1.50 mg L⁻¹**Fig. 8** (A–I) Hardening of *in vitro* grown plantlets; J Field transfer of plants after hardening, K One week old plantlets, L Three weeks old plantlet. *All scale bars represent 1 cm

found in sub-alpine regions, shows poor seed germination in its natural environment [23]. Our study showcased multiple methods for propagating *A. fragrans* via rhizome cuttings to overcome this reproductive limitation. In vegetative propagation, the use of plant growth regulators and various chemicals have been widely utilized to improve rooting and subsequent growth of rhizome cuttings [28, 29]. Our research revealed that intermediate concentrations of the applied hormones resulted in the most vigorous vegetative growth and the highest percentage of sprouts emerging from the rhizome

cuttings (Table 1). The most favourable outcomes were observed with GA₃ at 150 ppm and BAP at 150 ppm. Notably, Gibberellic Acid plays a crucial role in plant growth and development, acting as a key phytohormone that influences numerous physiological processes. Rhizome cuttings treated with GA₃ exhibited notable enhancements in root and shoot development [30]. Studies have reported that GA₃ application enhances sprouting percentage and improve growth and survival of vegetatively propagated plants under controlled conditions [31]. Additionally, Zang et al. [32] observed that GA₃ induces plant growth and enhances leaf development in blueberries compared to untreated controls. In line with previous findings [33, 34], the application of GA₃ improved several plant growth traits in *A. fragrans*, including leaf number, root length, and shoot length. Our findings align with those of Shabir et al. [35], who similarly reported significant improvements in growth-related parameters in *Inula racemosa* through the application of GA₃ to rhizomes. Gibberellins can affect plant growth by speeding up the transport of cytokinins to developing buds and by modifying carbohydrate metabolism [36]. Multiple studies have demonstrated that treating rhizome explants with kinetin and GA₃ effectively enhances sprouting and organogenesis. For instance, research conducted on *Pongamia pinnata* [37], *Euphorbia wallichii* [31], *Jurinea dolomiaea* [38] and *Picrorhiza kurrooa* [39] all of which confirm the positive impact of these growth regulators on shoot and root formation. Research by Nautiyal and Nautiyal [40] documented that for *Nardostachys jatamansi*, utilizing rhizome splitting as a vegetative propagation technique outperforms traditional seedling cultivation. This method was not only found to be more effective but also offered a significantly faster rate of multiplication and overall higher yield.

The current study demonstrated that various soil mixtures had a significant impact on the sprouting percentage and growth performance of *A. fragrans*. The highest sprouting percentage was observed in treatments T5 and T9, both achieving a 100% sprouting rate comprising of soil, sand, vermicompost (1:1:1) and soil, gravel, vermicompost (1:1:1) respectively. This was followed by T13 which consisted of soil, sand, peat and vermicompost in the ratio of 1:1:1:1 (Table 2). This plant species is found in small, patchy populations, typically growing in exposed alpine meadows among rocks or rocky slopes and wet shaded soils [16]. Porous sandy soil is ideal for successful large-scale cultivation, as it enables the plant to establish a thick and deep root system [41]. It has been documented that porous sandy soil provides sufficient aeration besides allowing easy penetration of roots, making it ideal for propagating and developing strong rootstock in *Jurinea dolomiaea* [38]. Sandy loam soils have also been reported by many researchers [31] as the best for maximizing the growth and yield of several economically important medicinal plant species. In *Paris polyphylla* sandy soils were observed to have maximum sprouting percentage [42]. Sand particles retain more oxygen and less water due to faster drainage owing to the fact of their larger size than soil particles, which leads to better aeration compared to densely packed particles of soil [43].

The inclusion of vermicompost in the growth media notably enhanced the development of rhizome cuttings in this study. Specifically, the SC10 treatment—consisting of a 1:1:1:1 ratio of soil, sand, pebbles, and vermicompost—resulted in a marked expansion of both aerial and subterranean biomass. This substrate combination also led to superior morphological outcomes, including increased plant height, leaf count, and rhizome dimensions, reinforcing the role of vermicompost in maximizing total yield.

In *Angelica glauca* and *Heracleum candicans*, similar to our findings, sandy loam soil mixed with vermicompost has demonstrated a significant effect on the cultivation and growth parameters of these species [28]. Mixing of soil with vermicompost increased the rate of germination, biomass of roots and pod number in pea crops [44]. In *Polygonatum cirrhifolium*, adding vermicompost to the soil results in increased biomass production [45]. The growth of plants is greatly influenced by availability of resources in soil, especially nutrients [46]. Numerous researchers [47] reported enhancement in plant biomass as a result of vermicompost application. Composted organic matter provides essential nutrients such as nitrogen and phosphorus, which are crucial for the healthy growth and development of plants [48]. Adding organic materials like compost, manure, and animal dung to the soil not only enhances crop yields but also helps sustain soil fertility over extended periods. Moreover, organic fertiliser improves soil retention and water holding capacity [49]. These fertilizers encourage the growth of microorganisms, which play a vital role in breaking down soil nutrients and making them available to plants [50]. While vegetative propagation remains the standard for cost-effective large-scale production, tissue culture was integrated into this study to exploit its superior propagation rate and its ability to generate disease-free starting material. However, the application of micropropagation is tempered by higher operational costs and the inherent risk of somaclonal variation. This genetic polymorphism, often triggered by the stress of *in vitro* regeneration, necessitates careful screening to ensure clonal fidelity—a challenge less prevalent in traditional vegetative methods.

4.2 *In vitro* studies

4.2.1 Surface sterilisation

Contamination arising from endophytic microorganisms, the culture environment, and the nutrient medium represents one of the major constraints in plant tissue culture, as it can severely inhibit the initiation and subsequent growth of *in vitro* cultures. To mitigate contamination and ensure successful culture establishment, efficient surface sterilization of explants is indispensable and represents a critical step in *in vitro* micropropagation protocols. Sterilization can be achieved with a range of chemical agents, including fungicides, antibiotics, sodium hypochlorite, calcium hypochlorite, chlorine dioxide, ethanol, mercuric chloride, hydrogen peroxide, and silver nitrate [51]. The success of various sterilization methods is influenced by parameters such as the type and concentration of sterilizing agents, duration of exposure, and the inherent characteristics of the explant tissue, necessitating careful optimization of sterilization protocols for each plant species [52]. In the present investigation, explants showed differential responses to the various sterilization treatments applied. Among these, nodal explants subjected to ST3 (Table 3) proved to be the most effective sterilization treatment, resulting in the lowest contamination rate ($8.46 \pm 0.16\%$) and the highest survival percentage ($93.14 \pm 0.16\%$). A similar observation was documented by Kumar et al. [53] who observed that a combined treatment of HgCl_2 (0.1%) and NaOCl (4%) yielded the highest survival rate (73.42%) during sterilization protocol optimization for *Gerbera jamesonii*. The present findings are consistent with earlier reports showing 83.33% survival in *Momordica dioica* nodal explants treated with HgCl_2 and NaOCl [54] and 73.33% survival in *Punica granatum* using 4% sodium chlorite and 0.1% mercuric chloride [55]. Besides ST3 (Table 3) also showed minimum contamination rate ($8.46 \pm 0.16\%$). Similar findings were reported by Naderi

et al. [56] and Nadhira et al. [57] in studies on *Cyclamen persicum* and *Cyathea latebrosa* and , where they observed that employing a two-step sterilization process resulted in minimal contamination rates. The application of mercuric chloride in sterilisation process has been found to be very effective in numerous *in vitro* studies [58]. However, in the present study, using mercuric chloride alone was less effective in controlling contamination compared to its combination with sodium hypochlorite. These results were in sharp contrast with those of Gautam et al. [59], who found maximum survival rate and minimal contamination rate when using 0.1% mercuric chloride. Mercuric chloride (HgCl_2) acts as an effective sterilizing agent by disrupting enzymes and proteins within the cell membrane and cytoplasm [60]; however, due to its high toxicity, careful optimization of its concentration and exposure duration is essential to minimize explant necrosis and mortality [61].

4.2.2 Effect of single cytokinins (meta-Topolin and TDZ) on shoot initiation and multiplication

During the present study, nodal explants responded to direct organogenesis. Efficient regeneration protocol through nodal segment have already been developed in various plant species such as in *Ruta graveolens* [62], *Cinnamomum camphora* [63] and *Tylophora indica* [64]. Cytokinins are essential for de novo shoot organogenesis in plant species, as the selection of optimal and effective cytokinins in the tissue culture medium determines an explant's morphogenetic potential for shoot and root organogenesis. Our results demonstrated that the shoot induction response varies based on the concentration of meta-Topolin present in the regeneration medium. The explants did not exhibit morphogenesis in the control experiments (MS medium without growth regulators) even after 4 weeks of incubation, suggesting that the endogenous growth regulator levels in the mother tissues of *A. fragrans* were insufficient to induce regeneration (Table 4). In the present investigation, MS media augmented with 3.5 mg L^{-1} of meta Topolin showed maximum (72.25%) shoot induction from nodal explant and producing an average of 6.00 ± 0.07 shoots per explant. Similar findings were reported in *Carthamus tinctorius* by [65], where nodal explants exhibited 80% shoot induction when cultured with 3.5 mg L^{-1} of meta-Topolin. In *Dioscorea pentaphylla*, a concentration of 1.0 mg L^{-1} of meta-Topolin in MS media proved to be the most effective for shoot induction, yielding a 96.9% culture response and producing an average of 5.0 ± 0.39 shoots per explant [66]. Moreover, in *Santalum album* MS media supplemented with 1.0 mg L^{-1} meta-Topolin demonstrated a 100% success rate for direct organogenesis from nodal explants [67]. Meta-Topolin, a benzyladenine analog of N6-(3-hydroxybenzyl amino purine), is recognized as a highly active cytokinin [68]. Recently, its use in micropropagation systems has garnered attention due to numerous positive effects, including increased shoot multiplication, improved shoot quality, successful root formation, ease of acclimatization, reduction of shoot tip necrosis, and enhanced biomass content [69]. The possible reason might be the presence of hydroxyl group in the side chain of topolins which allows them to readily convert into O-glucoside conjugates, facilitating their translocation and enabling conversion back into free active forms when needed [70]. This characteristic makes meta-Topolin a favourable cytokinin choice in numerous micropropagation studies such as in *Salvia sclarea* [71] and *Allamanda cathartica* [72].

Studies indicate that TDZ promotes shoot regeneration more efficiently than other cytokinins [73]. TDZ triggers varying regeneration responses depending on the plant

genotype and the type of explant used [74]. Similar to our observations TDZ also induced direct organogenesis from nodal explants in *Salvia sclarea* [75] and *Gymnocladus assamicus* [76]. Considering the individual effect of TDZ on direct shoot induction from nodal explants of *A. fragrans*, it was observed that a concentration of 3.5 mg L^{-1} of TDZ was the most effective showing maximum culture response (75.00%) with highest mean number of shoots 5.33 ± 0.33 (Table 4). Similar to our findings, Wu et al. [77] observed the highest number of shoots per internode in *Caryopteris terniflora* when MS medium was supplemented with 3.0 mg L^{-1} TDZ. Gupta et al. [76] observed comparable findings in *Gymnocladus assamicus*, where the use of 0.75 mg L^{-1} TDZ resulted in a 100% culture response when cotyledonary nodal explants were inoculated on MS medium. Our results also align with those of Najar et al. [78] in *Tylophora indica*, where supplementing MS media with 1.5 mg L^{-1} TDZ proved to be highly efficient in promoting the highest number of shoots from nodal explants. In *Aerva lanata*, highest shoot organogenesis (23.6%) was observed on 1.0 mg L^{-1} TDZ after 21 days of culture incubation [79]. In addition, *Glycine max* cotyledonary nodes, when cultured on MS medium supplemented with 0.5 mg L^{-1} TDZ, resulted in the highest direct shoot organogenesis rate of 96.3% [80].

4.2.3 Combined effect of cytokinins (meta-Topolin and/or TDZ) in association with different auxins

Auxins are widely employed in plant tissue culture for inducing indirect organogenesis via callus formation and for promoting root initiation. Nevertheless, in combination with cytokinins, auxins have been reported to significantly enhance *in vitro* shoot proliferation, as demonstrated in *Erythrina variegata* [81]. Conversely, several studies have shown that cytokinins alone can effectively induce high shoot multiplication rates [82]. The interaction between auxins and cytokinins may be synergistic, additive, or antagonistic, depending on the species and developmental context, thereby modulating physiological responses that regulate optimal growth and morphogenesis [83].

In the present study, meta-Topolin and NAA were detected as the most suitable combination and showed a synergistic effect on culture response, number of shoots and shoot length; while combination of meta-Topolin with IAA and IBA were having almost similar response as shown by MS media supplemented with meta-Topolin alone (Table 5). Previous studies have shown the effectiveness of combining NAA with cytokinins in various plant species [84]. One possible explanation might be that the interaction between cytokinin and auxin supports the production of endogenous growth regulators within the tissues and reduces the need for external growth regulators [85]. In addition, the combined use of auxins and cytokinins regulates cell proliferation and differentiation [86]. This combination also controls the expression of genes involved in the development of shoots and root meristems [87]. Although the combination of meta-Topolin with auxin have shown synergistic effect in our study but the improvement in the rate of shoot multiplication was in the sharp contrast with numerous studies such as in, *Hedy-chium coronarium* [88] *Ribes grossularia* [89] and *Santalum album* [90].

The combination of an optimal TDZ concentration (3.5 mg L^{-1}) with different auxins (IAA, IBA, and NAA) at concentrations ranging from 0.25 mg L^{-1} to 2.0 mg L^{-1} revealed that the treatment with 3.5 mg L^{-1} TDZ in combination with 1.25 mg L^{-1} IBA resulted in the highest regeneration frequency of 82.00%. Additionally, an average shoot number of

6.00 ± 0.57 and an average shoot length of 6.41 ± 0.59 cm were observed after 4 weeks of incubation (Table 5). These findings closely resemble those of recent studies on *Ceropegia evansii* [91] and *Dioscorea bulbifera* [92] where the combined application of cytokinin and IAA significantly enhanced regeneration frequency. Our results akin with those reported in *Tylophora indica* where the highest average shoot length was observed in MS media supplemented with 1.5 mg L^{-1} TDZ + 0.25 mg L^{-1} IAA [78]. Research has demonstrated that the combination of TDZ and IBA effectively promotes shoot regeneration in strawberry tissue cultures [93]. Furthermore, TDZ paired with IBA has also been found to be successful in enhancing shoot multiplication in *Fragaria viridis* [94]. TDZ played a vital role in the organogenesis of the *A. fragrans*, aligning with the findings of Hassan et al. [95] who reported that the highest percentage of direct shoot bud formation was achieved on MS medium supplemented with 1.0 mg L^{-1} TDZ in combination with auxin.

4.2.4 Rhizome development

During plant propagation, *in vitro* rhizome induction represents an efficient strategy for promoting early field establishment. Rhizomes formed under *in vitro* conditions are capable of producing both shoots and roots upon germination, thereby developing into complete plantlets and functioning as seed-like propagules for the large-scale multiplication of rhizomatous species [96]. Plant growth regulators are recognized for their influence on the *in vitro* rhizome induction in various rhizomatous plants including *Kaempferia galanga* [97], *Aconitum chasmanthum* [98] and *Cnidium officinale* [99]. Presently, our results indicated that half strength MS media without any PGRs didn't exhibit any microrhizome formation (Table 6). Similarly, Rafiq et al. [98] in *Aconitum chasmanthum* didn't get any response on MS basal medium. Moreover, significant difference was observed in the percent response culture, length of rhizome and mean number of days across various treatments (Table 6). Half-strength MS medium combined with 1.50 mg L^{-1} NAA was found to be the most effective concentration of plant growth regulator, exhibiting the highest culture response ($71.45 \pm 0.0\%$) for rhizome formation. The response varied significantly among treatments, ranging from $34.41 \pm 0.88\%$ to $71.45 \pm 0.00\%$ (Table 6). These results contrast with the findings of Rout et al. [100], where neither NAA nor IAA alone was effective in promoting rhizome formation. In *Bambusa bambos* var. *gigantea* rhizome induction from shoot tips was obtained on $50 \text{ }\mu\text{M}$ NAA [96]. Similarly, in *Curcuma longa* shoot cultures, rhizome formation was observed when transferred to MS medium augmented with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP [101]. In *cymbidium goeringli*, rhizome development was seen on MS medium supplemented with 10 mg L^{-1} NAA + 0.1 mg L^{-1} BA [102].

Since the therapeutic value of this species is concentrated majorly in its rhizomes, wild harvesting is inherently destructive and threatens the survival of natural populations. The vegetative and micropropagation protocols established in this study provide a critical framework for conservation by offering an alternative to the exploitation of wild habitats. Furthermore, this method facilitates sustainable production by ensuring a genetically uniform and disease-free supply of raw materials, which is essential for the standardized extraction of medicinal compounds required by the pharmaceutical industry.

4.2.5 Hardening of *in vitro* raised plantlets

Acclimatization of *in vitro* raised cultures to external environmental conditions remains a major bottleneck in plant micropropagation [103]. Although plantlets develop well under aseptic conditions, their establishment *ex vitro* requires careful acclimatization and hardening. Successful transition depends on optimizing key factors such as substrate and growth conditions. An integrated approach is essential to improve survival rates of micropropagated plants. In the present study, a survival rate of 75% was achieved when plantlets were acclimatized in a potting mixture of soil, sand, and vermicompost in a 1:1:1 ratio. Comparable observations have been reported in earlier studies, highlighting the influence of substrate composition on acclimatization success. Behera et al. [104] recorded a 90% survival rate in micropropagated *Dioscorea oppositifolia* using soil: sand: manure (1:1:1), while Jova et al. [105] successfully acclimatized *in vitro* raised plants using soil rite composed of equal proportions of coir and peat moss. Kadota and Niimi [106] reported 80% survival in *Dioscorea japonica* using a vermiculite and soil mixture (1:1), and Indravathi and Babu [107] achieved 82% survival in *Albizia amara* using a complex medium comprising soil, peat, vermicompost, and perlite (1:1:1:1) supplemented with bacterial inoculants (*Pseudomonas fluorescens* and *Trichoderma viride*). The regenerated plants were transferred in June, corresponding to the summer season in Kashmir, to a prepared 5 × 3 m plot at the Kashmir University Botanical Garden under gardener-managed soil conditions.

Although no phenotypic variation was observed among the propagated plants, however, somaclonal variation may remain undetected at the genetic level. As molecular marker-based validation (e.g., ISSR, RAPD) was not performed due to resource constraints, genetic stability could not be confirmed and this is acknowledged as a limitation of the study.

5 Conclusion

The present study successfully optimized propagation protocols for the endangered medicinal species *A. fragrans* through both vegetative propagation and micropropagation techniques. Treatment of rhizome cuttings with 150 ppm GA₃ yielded the highest sprouting percentage (83.34%) and significantly improved growth parameters, including shoot length (16.6 cm), root length (9.5 cm), and leaf number (44.75 leaves per plant). Additionally, a soil mixture of soil, sand, and vermicompost (1:1:1) produced the highest sprouting rate and favorable morphological traits, demonstrating its potential for large-scale cultivation. In the micropropagation experiments, the combination of TDZ (3.5 mg L⁻¹) and IBA (1.25 mg L⁻¹) induced the highest regeneration frequency (82%) and produced up to 6 shoots per explant, with shoots measuring up to 6.41 cm in length. The acclimatization of *in vitro* derived plantlets under greenhouse conditions resulted in a 75% survival rate, confirming the robustness of the established protocols for *ex situ* conservation and sustainable cultivation. These findings underscore the potential for large-scale cultivation and conservation of *A. fragrans* using optimized vegetative and micropropagation methods, which could be applied to other endangered medicinal plant species.

The current lack of comprehensive phytochemical and pharmacological data for *A. fragrans* highlights a critical research gap that necessitates further investigation. Future studies should leverage the established *in vitro* protocols to scale up root biomass and

utilize elicitation strategies for enhanced secondary metabolite production. Consequently, this micropropagation framework serves as a vital foundation for the standardized extraction and clinical validation of the species' therapeutic compounds.

Supplementary Information

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Supplementary Material 1.

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Author contributions

Irshad Ahmad Bhat: conceptualization, experimentation, data analysis, methodology, writing—original draft preparation. Khushboo Guleria: data collection, visualization, manuscript review. Anamika Kumari: analysis, original draft preparation, visualization. Sajida Banoo: original draft preparation, manuscript review. Zahoor A. Kaloo: investigation, manuscript review, methodology and supervision. Mudassir Fayaz: experimentation, review, original draft preparation, manuscript review.

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Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate

The plant material (*Aquilegia fragrans* (Benth.) used in the present study was collected from wild populations in Kashmir, India. All collections were carried out in accordance with applicable local and national guidelines for plant collection and biodiversity conservation. Institutional (Department of Botany, University of Kashmir) approval for the collection and use of plant material for research purposes was obtained from the concerned institutional authority.

Consent for publication

Not applicable.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve the readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the research article.

Competing interests

The authors declare no competing interests.

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