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MICROPROPAGATION OF GENETICALLY STABLE *JASMINUM LEPTOPHYLLUM* —A NARROW ENDEMIC SHRUB— FOR CONSERVATION PURPOSE

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Abstract

Endangered endemic plants face critical survival challenges, often hindered by reproductive barriers such as deep seed dormancy. Current study provides a meaningful contribution to conservation –by optimization of micropropagation protocol for *Jasminum leptophyllum* Rafiq, a critically endangered species– overcoming its major propagation constraint. A dual pre-treatment of sulfuric acid scarification followed by culture on a simplified, sucrose-free half-strength MS medium increased germination from 26.9% to 38.5%. Shoot multiplication was achieved with 0.4 mg/L BAP for multiple bud induction, while a previously unreported response was observed using 3 mg/L BAP + 4 mg/L Kinetin combination significantly enhanced shoot elongation. Rooting was most efficient with 1.6 mg/L NAA or IAA, producing robust roots within 16 days. Crucially, genetic fidelity of regenerated plants was rigorously validated using RAPD and SCoT markers, confirming true-to-type propagation and ensuring preservation of the species genetic integrity. This robust verification underlines the protocol's reliability for ex situ conservation and potential reintroduction programs. By integrating an effective regeneration system with comprehensive genetic fidelity assessment, this study not only safeguards *J. leptophyllum* but also establishes a model framework for conserving other rare, recalcitrant taxa. This work represents a significant advancement in plant conservation biotechnology, demonstrating that high-efficiency micropropagation can be combined with molecular verification to secure the future of endangered plants.

Key words: Micropropagation; Endemic; Endangered; Biotechnology; Ex situ conservation

Introduction

Jasminum leptophyllum Rafiq, belonging to family Oleaceae is a relatively newly discovered species of the genus, reported by Rubina Rafiq during her visit to Palas valley in 1993 (Rafiq, 1996; Sinnott *et al.*, 2000). This taxon is facing extreme conservation crisis due to synergistic effects of intensifying threats. The most prominent threat is widespread habitat degradation caused by soil erosion attributed to the overgrazing, vast deforestation and effects of climate change. Further anthropogenic pressures—including pollution, tourism, the introduction of invasive species, and recreational activities—worsen the risk to this species in its fragile, endemic-rich environment (Sinnott *et al.*, 2000; Cahill *et al.*, 2013). Occupying only one known locality with least occupancy, *J. Leptophyllum* qualifies under the IUCN Red List of Endangered Species as Critically Endangered (CR), the riskiest category of the International Union for Conservation of Nature (Sinnott *et al.*, 2000; Coelho *et al.*, 2020; Majid *et al.*, 2020). Due to area inaccessibility and new discovery, there is not much scientific knowledge available for this taxon. Ethnically it is used as insect repellant and as fire wood, however, this underexplored ornamental shrub has potential for use in horticulture due to its shrubby appearance and striking yellow blossoms. While it shows great promise for landscaping and ecological use, its phytochemical and pharmaceutical potential remains largely unknown, warranting detailed scientific investigation (Majid *et al.*, 2020).

Endemic plants represent distinct evolutionary lineages that have adapted to the unique ecological conditions of their native habitats and *J. leptophyllum* is one of them and extremely narrow endemic (Lavergne *et al.*, 2004). Because of their restricted ranges and often specialized habitat requirements, these species are exceptionally vulnerable to extinction pressures such as habitat fragmentation, climate change, and human disturbance (Lavergne *et al.*, 2004; Işik, 2011; Cahill *et al.*, 2013; Salles *et al.*, 2019). As a result, they are among the highest priorities for conservation at both local and regional levels (Burlakova *et al.*, 2011). According to a recent study, 93.8% of the total extinct species are narrow endemics (Kraus *et al.*, 2023). Protecting endemic flora is not only about preserving regional biodiversity, it also safeguards irreplaceable evolutionary heritage and strengthens the resilience of natural ecosystems. Prioritizing endemic and threatened species, along with the specific habitats that sustain them, is a central focus of effective conservation strategies – especially in regions characterized by high levels of plant endemism (Bacchetta *et al.*, 2012; Coelho *et al.*, 2020).

Propagation/cultivation of *J. leptophyllum* has not been reported previously and no young plantlets has been observed in wild population (Majid *et al.*, 2020). This can be a result of changing climatic conditions and shift in rain

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pattern (Ullah *et al.*, 2018). Therefore, it is crucial to intervene and conserve this taxon through ex situ conservation techniques. Plant propagation through tissue culture has been established as an efficient and reliable method for clonal propagation for conservation of endangered species (Corral *et al.*, 2011; Deb *et al.*, 2018; Kausar *et al.*, 2019). Some other species of Jasminum as well as other endemic woody plants have been successfully propagated *In vitro* including *Jasminum sambac*, *J. officinale* and *J. azoricum* (Salim, 2016; Borah *et al.*, 2017; Palai *et al.*, 2017). Thus, *In vitro* propagation can be utilized as useful tool for conservation of *Jasminum leptophyllum* germplasm with promising results.

Compared to other propagation methods, bud induction offers a lower risk of genetic instability rendering it an efficient as well as safe approach for propagation of plants. Conservationists and botanical gardens have embraced tissue culture techniques, due to this advantage, for protection of threatened plant species (Khan *et al.*, 2021; Srinivasan *et al.*, 2021; Chandran *et al.*, 2023). However, various factors such as composition of culture media, physical factors of growth room, duration and number of subcultures etc., can lead to genetic variations in newly propagated plantlets (Bairu *et al.*, 2011). Consequently, it is crucial to certify the genetic fidelity of regenerated plants and by that, promote efficient propagation and conservation of endangered rare and endemic taxa. Random amplified polymorphic DNA (RAPD), Start Codon Targeted (SCoT), inter-retrotransposon amplified polymorphism (IRAPS) and Inter-simple sequence repeats (ISSR) are polymerase chain reaction (PCR) techniques used to evaluate genetic diversity in regenerated plantlets (Vemula *et al.*, 2020; Srinivasan *et al.*, 2021; Tikendra *et al.*, 2021a; Kumar *et al.*, 2022; Khan *et al.*, 2023; Luo *et al.*, 2023).

J. leptophyllum is an understudied species with almost no reports most probably due to area inaccessibility. No study has reported to date about conservation-oriented mass propagation of *J. leptophyllum* through tissue culture. Therefore, this study was designed with the objectives to develop a protocol for mass propagation –using *In vitro* germinated seedlings– and acclimatization to support eventual reintroduction. Although ex situ germination of seeds of *J. leptophyllum* is relatively easy, number of plants produced directly through seeds is limited and seedlings mortality is notably high. By using nodal culture and rooting of resulting shoots, number of propagated plants can be amplified which is a requirement for conservation process. Genetic stability is important factor to be considered for germplasm conservation. therefore, genetic fidelity assessment was carried out by using RAPD and SCoT markers. The protocol developed can be used for mass production for ex situ conservation reintroduction of this critically endangered species.

Materials and Methods

Explant collection and sterilization: Seeds of *J. leptophyllum* were collected from Palas Valley Kohistan Pakistan (35° 05.528' N, 73° 13.606' E, Altitude 1742m, and 35° 02.092' N, 73° 13.892' E, Altitude 1730m Fig. 1. For sterilization, seeds were washed with running tap water for 15 minutes. After that these seeds were soaked in 20% v/v solution of 0.5% sodium hypochlorite for 20 minutes than washed by sterile distilled water. Afterwards seeds were soaked in 0.1 % w/v solution of mercuric chloride for 1 minute. Although mercuric chloride is highly toxic and hazardous chemical however, its use is unavoidable due to ineffectiveness of various other sterilants e.g., H_2O_2, ethanol or sodium hypochlorite alone. These sterile seeds were washed 4 times with sterile distilled water shaking vigorously and then cultured on different media for growth. Data for germination and seedling mortality was collected after 30 days.

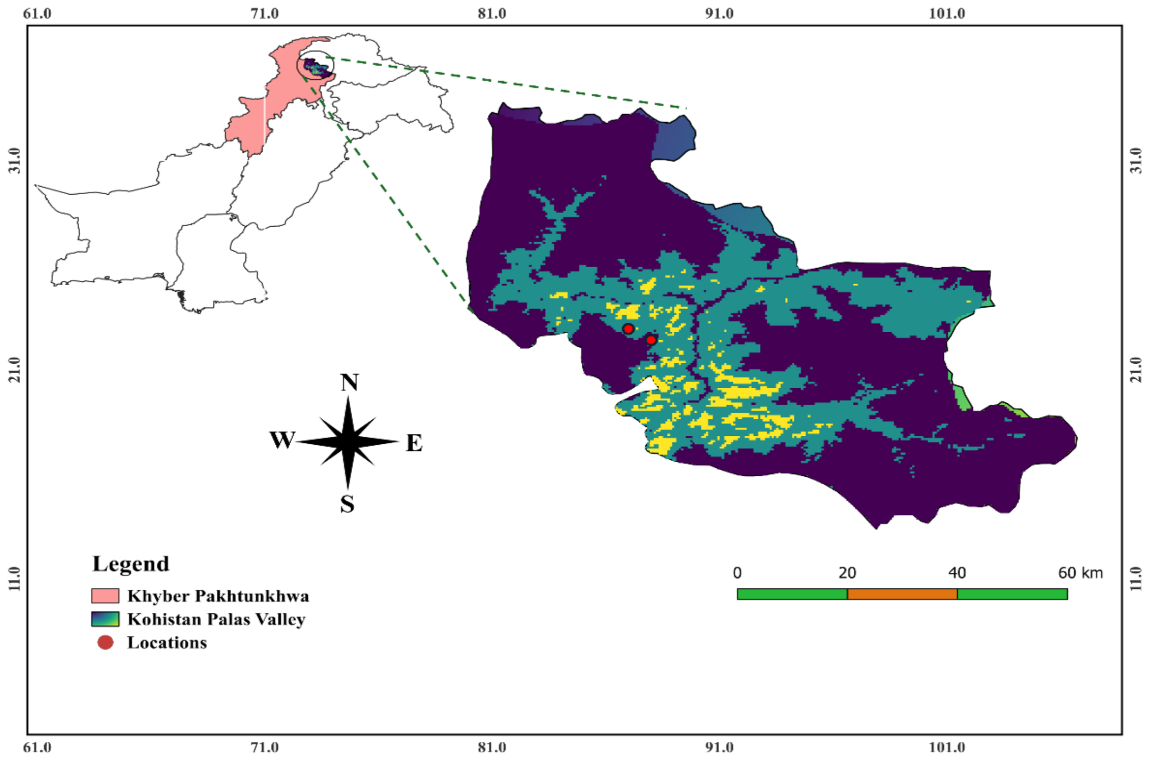

Fig. 1. Map describing location of study area.

Table 1. Effect of media composition and H2SO4 treatment on germination of seeds of *Jasminum leptophyllum* (SGM=seed germination media)

Treatment	Media composition	Sucrose	PGRs	H_2SO_4 treatment for 10 minutes	Germination percentage	Mean days to germination	Standard deviation
SGM 1	MH–MS*	-	-	+	38.5%	3.60	2.51
SGM 2	MH–MS	-	-	-	30.8%	3.25	0.50
SGM 3	MS	+	-	+	23.1%	4.67	2.89
SGM 4	MS	+	-	-	15.4%	3.00	0.00

*MH–MS is modified half strength MS media without vitamins and sucrose

Table 2. Various media used for shoot multiplication, with mean values, standard error and significance groups (SPM= shoot promoting media).

Treatment	BAP mg/L	Kinetin mg/L	TDZ mg/L	PNGS-I ml/L	Days to shoot induction	No. of shoot/ plant	No. of nodes/ plant	Average shoot length in cm
SPM 1	0.4	–	–	–	4.92 ± 0.38 ab	2.48 ± 0.19 A	3.79 ± 0.27 A	1.404 ± 0.24 abc
SPM 2	3	4	–	–	4.24 ± 0.38 Bc	2.21 ± 0.20 A	2.24 ± 0.27 ab	2.020 ± 0.28 ab
SPM 3	–	4	–	–	5.92 ± 0.38 A	1.23 ± 0.21 B	0.88 ± 0.27 B	0.212 ± 0.04 D
SPM 4	2	–	2	–	3.80 ± 0.38 cd	1.80 ± 0.19 ab	2.23 ± 0.27 B	0.972 ± 0.14 cd
SPM 5	2	2	–	–	3.40 ± 0.38 D	1.32 ± 0.19 B	2.46 ± 0.27 b	0.828 ± 0.13 cd
SPM 6	–	2	2	–	5.08 ± 0.38 ab	2.28 ± 0.19 A	1.99 ± 0.27 B	0.536 ± 0.11 cd
SPM 7	2.5	–	–	–	4.20 ± 0.38 bc	1.80 ± 0.19 ab	2.26 ± 0.27 bc	1.020 ± 0.20 bcd
SPM 8	–	–	–	12	3.40 ± 0.38 D	1.96 ± 0.19 ab	2.98 ± 0.27 C	2.292 ± 0.50 a

Values correspond to mean data derived from five (5) replications; mean ± SE is given. Means with different letters are significantly different according to HSD (Tukey) test ($p \leq 0.05$)

Table 3. various media used for root induction and growth, with mean values, standard error and significance groups (RPM= root promoting media).

Treatment	NAA mg/L	IAA mg/L	IBA mg/L	PNGS-I ml/L	PNGS-II ml/L	Days to root induction	No. of root/plant	Average root length in cm
RPM 1	–	–	–	–	4	48.3 ± 11.6 a	0.33 ± 0.33 b	0.30 ± 0.30 b
RPM 2	–	–	–	7	–	51.3 ± 8.7 A	0.33 ± 0.33 b	0.33 ± 0.33 B
RPM 3	–	–	1.6	–	–	33.0 ± 13.6 A	0.67 ± 0.33 ab	0.90 ± 0.46 b
RPM 4	1.6	–	–	–	–	15.7 ± 1.8 A	3.67 ± 1.45 a	4.03 ± 0.41 a
RPM 5	–	1.6	–	–	–	28.7 ± 2.6 A	3.33 ± 0.33 ab	3.57 ± 0.32 a

PNGS II–natural growth substances as reported by Shah (Shah & Jan, 2025). Values correspond to mean data derived from three (3) replications; mean ± SE is given. Means with different letters are significantly different according to HSD (Tukey) test ($p \leq 0.05$)

Media preparation and growth conditions: Basal MS media (Murashige & Skoog, 1962) was used for shoot multiplication with various concentrations of PNGS-I (Plant Natural Growth Substances) (Shah & Jan, 2025), NAA, IAA, IBA, 2,4-D, Kinetin, BAP and TDZ. Every media treatment contained 30 g/L sucrose and was adjusted to a pH of 5.8, coagulated using 7.5 g/L agar, and sterilized by autoclaving at 121˚C for 15–20 minutes. The cultures were maintained in the laboratory at a temperature of 27 ± 2˚C under a 16/8-hour (light/dark) photoperiod, illuminated with cool white fluorescent light at an intensity of 1500–3000 lux (Zhang *et al.*, 2015; Anjusha *et al.*, 2016).

Seed germination: Half and full-strength MS media without growth regulators and sucrose as well as effect of H_2SO_4 (98%) on seed germination for 10 minutes (Table 1) was tested and germination percentage was recorded (germination percentage % = Seeds germinated/ total number of seed s×100).

Shoot bud initiation and proliferation: *In vitro* germinated sterile seedlings with single node were used as explant for shoot proliferation on media– selected form pilot experiment– containing BAP (0.4, 2, 2.5 and 3mg/L) kinetin (2 and 4mg/L), TDZ (2 mg/L) and PNGS I (12ml/L) singly or in combination (Table 2). Number of shoots initiated, number of nodes and shoot length was recorded for 30 days of incubation in 5 passages of 6 days each. Every treatment had 5 replications and experiment was repeated 3 times.

Root induction: PNGS-I, PNGS II, IBA, NAA, IAA and GA_3 of various concentration in MS media were used for root induction. A total of 5 treatments were used with 5 replications each and data (number of roots and root length) recorded for 60 days Table 3.

Acclimatization: Plantlets with well-developed roots were selected for acclimatization. At first caps of tubes were removed and kept in growth chamber for 1 week. After that plantlets were removed from vessels and washed off with water to remove media from roots and planted in sand and soil mix in 1:1 ratio. For first week plants were covered with clear plastic cups to retain moisture. Survival rate was recorded for 6 months.

Genetic stability assessment: 6 samples of *J. leptophyllum* were used for analysis of genetic stability—using 15 RAPD and 5 SCoT markers—–out of which 1st sample was taken from wild population collected from Palas valley and 5 samples were from *In vitro* propagated plants growing for over 6 months at time of sample collection. SCoT markers have a clear edge over ISSRs for checking the genetic health of lab-grown endangered plants. They scan areas right next to important genes, so if they spot a change, it's more likely to affect the plant's survival in the wild. This functional insight, plus the fact that the same primers work for almost any plant, makes them the smarter, safer choice before sending precious plants back to their natural homes.

Genomic DNA extraction and PCR amplification: Genomic DNA was extracted from dried leaf material of randomly chosen samples following the Cetyltrimethyl Ammonium Bromide (CTAB) method described by Quiñones *et al*., (2024). Random amplified polymorphic DNA (RAPD) and Start Codon Targeted (SCoT) markers were subsequently analyzed through polymerase chain reaction (PCR) (Verma *et al.*, 2017; Kumar *et al.*, 2018; Shahwar *et al.*, 2022; Joshi *et al.*, 2023; Slameto, 2023). Each reaction mixture (20 µL total volume) included 2 µL of DNA template, 10 µL of 2× Master Mix with loading dye (Thermo-Fisher Scientific, USA), 1 µL of each primer, and 7 µL of nuclease-free water. PCR amplification was performed in a Kyratec Supercycler Trinity Gradient Thermal Cycler (Model: SC300G-R2) using the following program: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 32°C for RAPD and 5°C less than melting temperature of each SCoT primer for 45 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 7 minutes.

Statistical analysis

Data was organized using MS excel 2016 and statistical analysis was carried out using R studio version 2025.05.1+513. Results were presented as mean ± standard error, analyzed by using one-way ANOVA, and Tukey HSD test, considering results statistically significant at $p<0.01$. To assess genetic relationships between the propagated and wild *J. leptophyllum* plants, we carried out a statistically validated cluster analysis. RAPD and SCoT banding data were converted into a binary presence–absence matrix and analyzed in R using the pvclust package. A hierarchical dendrogram was generated based on Jaccard distance and the UPGMA method. Cluster stability was tested using 10,000 multiscale bootstrap resamplings, yielding Approximately Unbiased (AU) p-values; clusters with AU ≥ 0.95 were considered well supported.

Results

Explants sterilization: The applied surface sterilization protocol for seeds achieved a 0% contamination rate after 30 days of incubation. The two-step process—a 10-minute treatment with 20% (v/v) sodium hypochlorite followed by a 1-minute treatment with 0.1% mercury chloride—was entirely effective at suppressing all fungal and bacterial contaminants *In vitro*.

Seed germination: Germination percentage was recorded after 60 days of culture. Out of all cultured seeds 26.9% seed germinated within 10 days while rest failed to germinate even after 60 days of culture. Our results showed that half strength MS media without sucrose and growth regulators (both SMG 1 and SMG 2) is best suited for germination of seeds compared to full strength MS media with added sucrose (SMG 3and SMG 4) and H2SO4 treatment enhanced germination. Highest germination 38.5% was achieved on SGM 1 with H2SO4 treated seeds followed by 30.8% on SMG 2 (Table 1, Fig. 2).

Bud induction: Bud initiation mostly occurred within 3-7 days of culture. The rate and quality of bud formation did not differ significantly among the various types and concentrations of plant growth regulators (PGRs) used. Among the eight treatments tested, all resulted in bud induction. Bud initiation was earliest on treatment SPM 5 (MS medium fortified with 2 mg/L each of BAP and kinetin), occurring after just one day, followed by SPM 6 (MS with 2 mg/L each of TDZ and kinetin) at two days. Treatments SPM 5 and SPM 8 (MS supplemented with 1.2 ml PNGS-I) both resulted in the lowest average time to shoot initiation. In contrast, initiation was delayed on SPM 3 i.e., MS medium containing 4mg/L kinetin. The elevated concentration of kinetin not only delayed initiation but also negatively impacted the survival of the buds; however, this negative effect was slightly mitigated when used in combination with BAP (SPM 2). Conversely, a much lower-concentration of PGRs (0.4mg/L BAP) also resulted in a delayed induction, slower development of buds, and eventual mortality after 20 days (Table 1, Fig. 3).

Number of shoots per plant: The use of various treatments had a great impact on the number of shoots developed per plant. The highest number of shoot development was recorded in response to SPM 1 giving the highest average of 2.48 shoots per plant. This performance was also statistically equal to that of SPM 2 and SPM 6 which also promoted good shoot formation (2.21 and 2.28 shoots per plant, respectively). On the other hand, treatments SPM 3 and SPM 5 produced significantly fewer shoots (1.23 and 1.32, respectively) compared with the best-performing treatments. The SPM 4, SPM 7 and SPM 8 effects were intermediate, and yielded moderate shoot numbers that lacked statistical significance with the highest or the lowest-performing groups. These findings indicate a distinctive difference in the effects of the treatments on the shoot multiplication where SPM 1, SPM 2 and SPM 6 are the most effective regimens with respect to promoting shoot multiplication (Table 2, Fig. 4).

Number of nodes per plant: SPM 1 was the most successful treatment which yielded the highest average number of nodes (3.79). The rest of the treatments SPM 2, SPM 4, SPM 5, SPM 7 and SPM 8 had moderate outcomes with an average output ranging between 2.24 and 2.98. SPM 8 was also better with a mean of 2.98 producing more consistent results compared to SPM 2. SPM 3 was found to be the least efficient producing least number of nodes (mean of 0.88) when compared to the rest. SPM 6 treatment (mean of 1.99) was closer to the lower side of the intermediate group. The highest standard deviation (std) was observed in SPM 1 and SPM 5, meaning that there were the most changes in the response of individual plants in these treatments. Conversely, the most consistent (not so much variable) results were obtained with SPM 3 and SPM 4 between plants. The comparison demonstrates that Treatment SPM 1 developed better nodes in plants than all other treatments. The majority of alternative treatments produced moderate levels of nodal counts which statistically did not differ among each other forming a middle-performance group. SPM 3 was always the lowest treatment and it had a substantial inhibition of node formation as opposed to all others (Table 2, Figs. 5 and 7).

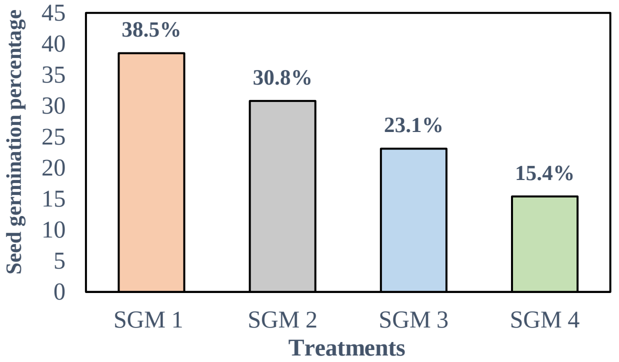

Fig. 2. Effect of media composition and H_2SO_4 treatment on seed germination of *Jasminum leptophyllum*, values represent percent germination.

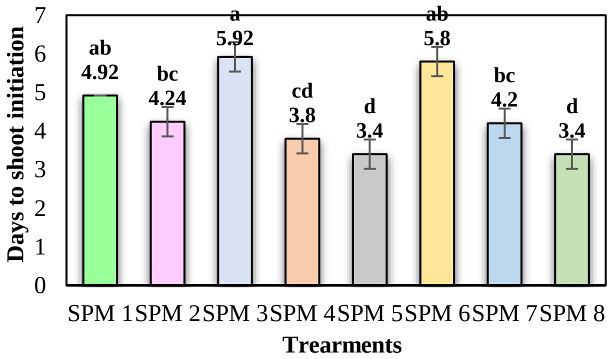

Fig. 3. Effect of media composition on bud induction of *Jasminum leptophyllum*, presenting mean values, ±SE and significance group letters ($p<0.05$).

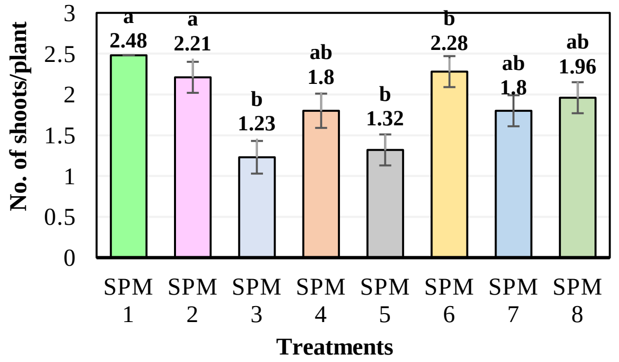

Fig. 4. Effect of treatments on number of shoots induced per plant, presenting mean values, ±SE and significance group letters ($p<0.05$).

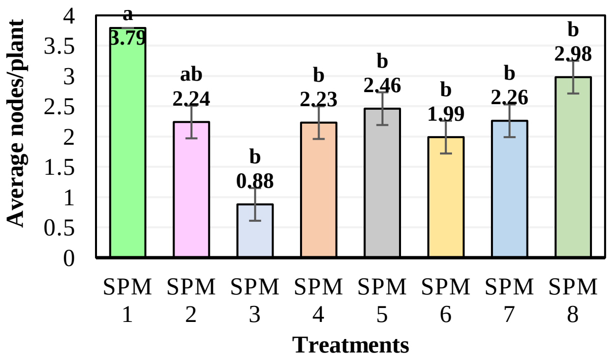

Fig. 5. Effect of various treatments on average number of nodes per explant presenting mean values, ±SE and significance group letters ($p<0.05$).

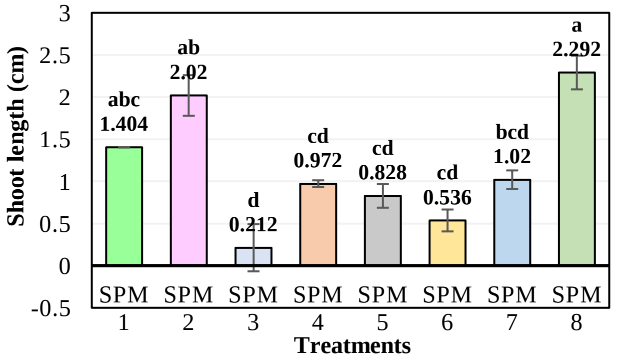

Fig. 6. Effect of treatments on shoot length, presenting mean values, ±SE and significance group letters ($p<0.05$).

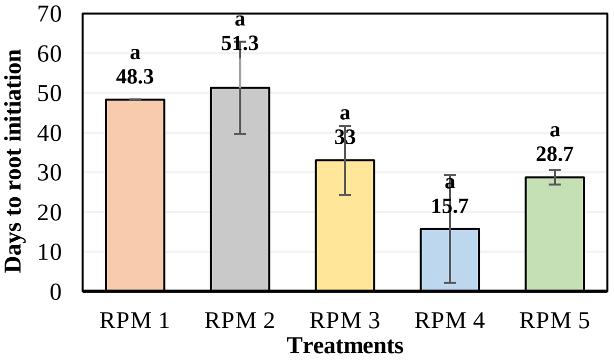

Fig. 7. Effect of various treatments on root induction, presenting mean values, ±SE and significance group letters ($p<0.05$).

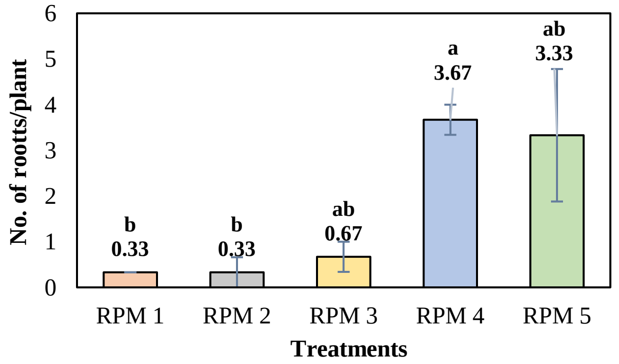

Fig. 8. Effect of treatments on number of roots induced per plant, presenting mean values, ±SE and significance group letters ($p<0.05$).

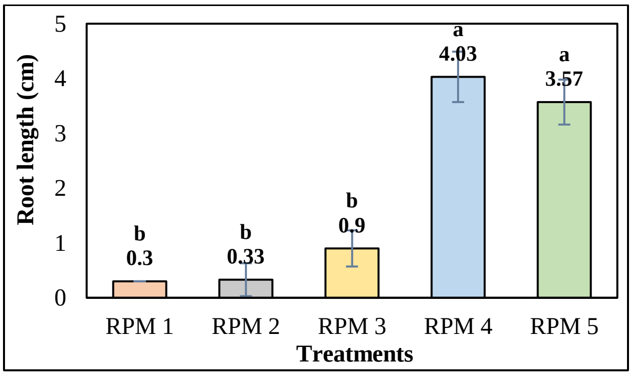

Fig. 9. Effect of treatments on root length, presenting mean values, ±SE and significance group letters ($p<0.05$).

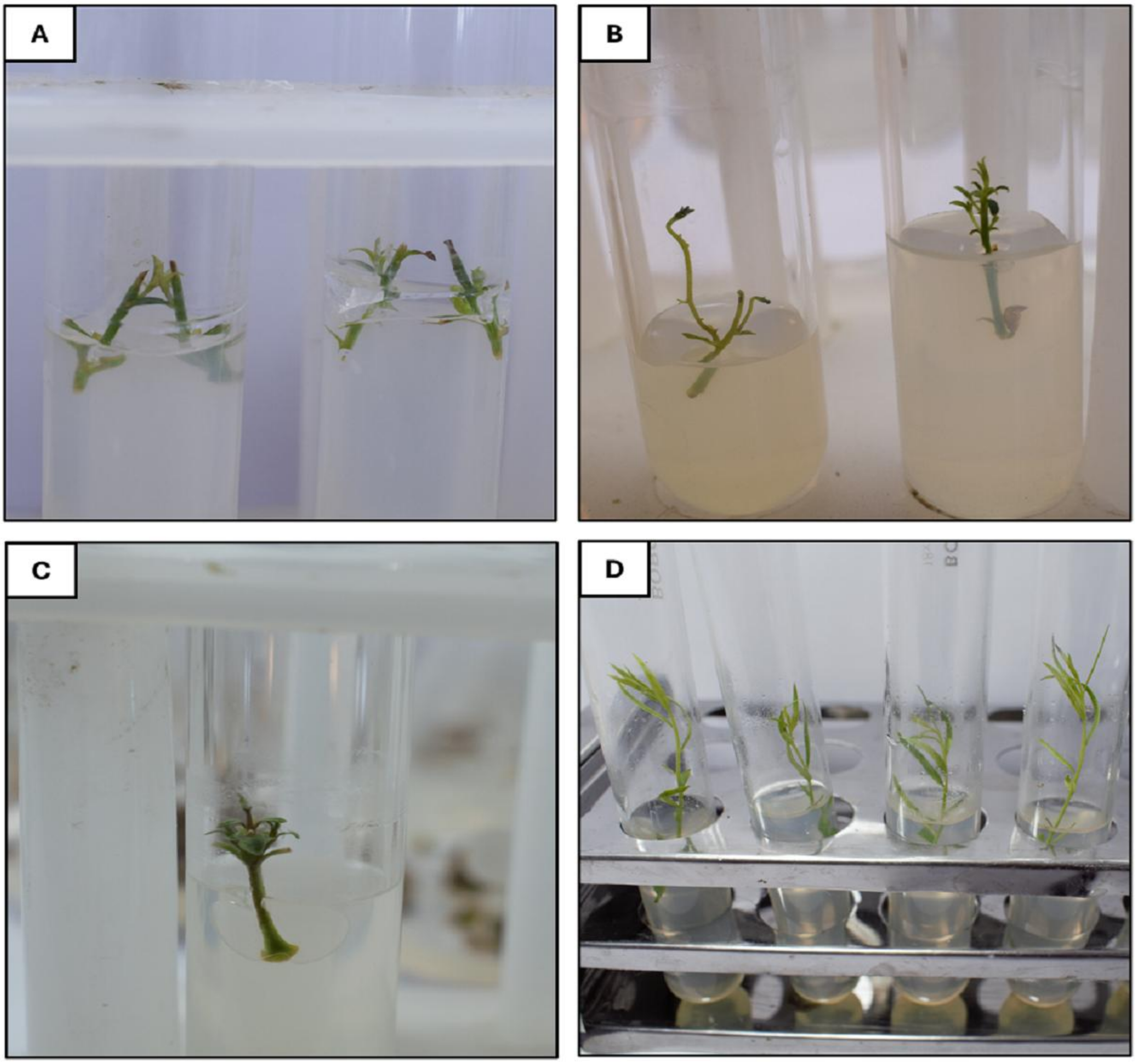

Fig. 10. Different stages of shoot proliferation in *Jasminum leptophyllum*, A: initiation of buds, B and D: multiple shoot induction, D: increasing shoot length.

Shoot length: A significant effect of culture medium on shoot elongation was observed. The highest mean shoot length 2.29 cm was recorded in treatment SPM 8 MS supplemented with PNGS-I, which demonstrated a statistically significant advantage over every other treatment. This was followed by 2.02 cm on treatment SPM 2—3mg/L BAP and 4mg/L kinetin—which also showed vigorous growth. The poorest performance was observed in treatment SPM 3, which resulted in the minimum average shoot length 0.21cm among the experimental groups (Table 2, Figs. 6 and 10**Error! Reference source not found.**).

Days to root initiation: The time required for root initiation showed clear differences across the treatments. The longest period was recorded in RPM2 (7ml/L PNGS II), where root formation took an average of 51.33 days, followed closely by RPM1 (4ml/L PNGS-I) with 48.33 days. An intermediate response was noted in RPM3 (33.00 days), while RPM5 required 28.67 days for root emergence. The most rapid root initiation occurred in RPM4 (1.6mg/L NAA), where roots developed after only 15.67 days on average. Although the treatments differed in numerical values, statistical analysis indicated that they did not vary significantly (Table 3, Fig. 7).

Number of roots: ANOVA results revealed that there was a significant difference between the number of roots among treatments. The highest number of roots (3.67) was produced in treatment RPM4 (1.6mg/L NAA) which was significantly higher than RPM1 (4ml/L Tang NGS) and RPM2 (PNGS II 7ml/L) 0.33 each. Treatments RPM3 (1.6mg/L IBA) and RPM5 (1.6mg/L IAA) –0.67 and 3.33 average number of roots respectively– exhibited intermediate outcomes, which were not significantly different to either of the best or worst performing treatments. These results proved that the type of treatment had a considerable impact on root development, RPM4 being the most effective one. (Table 3, Fig. 8).

Root length: Statistical analysis revealed significant effects of treatments on root elongation ($F = 24.48$, $p<0.001$). Post-hoc Tukey's HSD comparisons demonstrated that treatments

RPM4 and RPM5 (1.6mg/L NAA and 1.6mg/L IAA respectively) produced substantially longer roots than other treatments ($p<0.001$), with mean differences exceeding 3.2 cm. The two superior treatments showed statistically equivalent performance ($p = 0.892$), indicating comparable efficacy in promoting root development. Rest of the treatments RPM1, RPM2 and RPM3 did not differ significantly from each other. These results clearly identify RPM4 and RPM5 as optimal treatments for enhancing root growth (Table 3, Figs. 9 and 11).

Acclimatization: As shown in Fig. 12, eight of the nine *In vitro*–propagated plants successfully acclimatized in first experiment, reflecting a high survival rate 89 and confirming the effectiveness of the hardening protocol. The single loss likely resulted from individual stress sensitivity, a common occurrence in tissue culture–derived plants. Overall, the results demonstrate the reliability of the method for large-scale propagation and conservation applications.

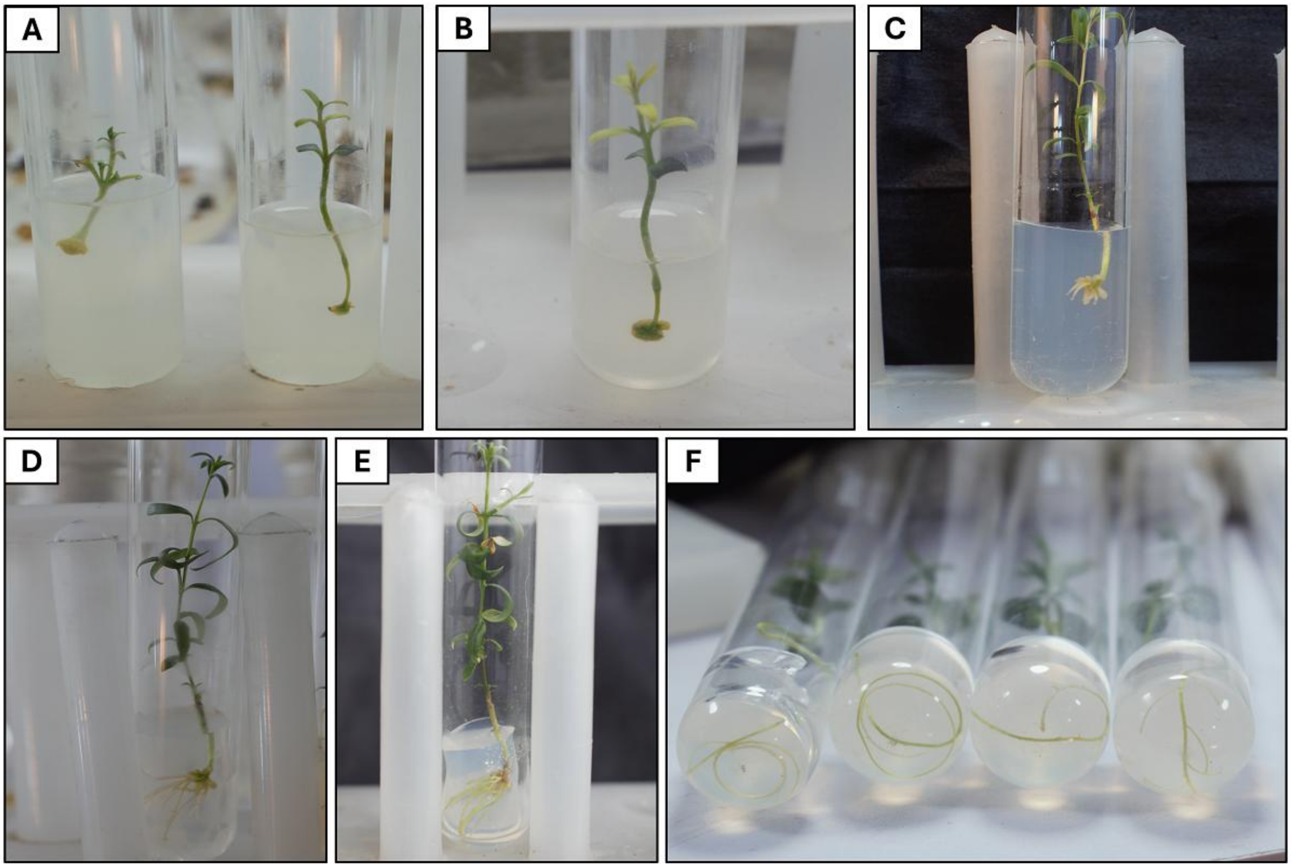

Fig. 11. Stages of root induction and growth in *Jasminum leptophyllum*, A: shoot inoculated for rooting, B and C: multiple roots initiation, D-F: healthy root system.

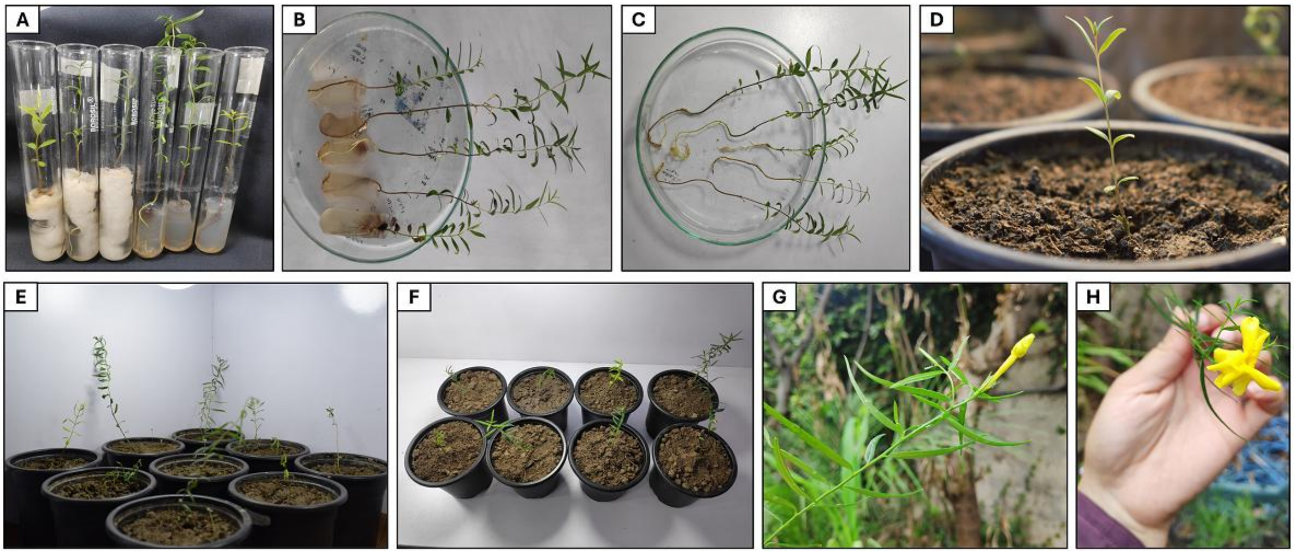

Fig. 12. Acclimatization of *In vitro* propagated *Jasminum leptophyllum*, A, B and C: preparation for shifting to pots, D: single healthy plant, E and F: plants growing for 2 months, G and H: flowering after six months.

Genetic uniformity assessment: PCR amplification with the 15 RAPD and 5 SCoT primers generated clear and reproducible banding patterns in all six samples of *J. leptophyllum* (Table 4, Fig. 13). A total of 58 bands ranging from ~1000 bp to ~100 bp produced throughout with slight deviation in OPB-10 where sample three produced single faint band and sample six failed to produce any band compared to three clear and distinct bands produced in all other samples. The amplification profile was consistent across samples, with major bands observed around 1000bp, 750 bp, 500 bp, 400 bp, and 250 bp approximately. SCoT primers mostly produced clear bands in similar size range, with no polymorphic bands detected. No amplification was detected in the negative control (NC), confirming the absence of contamination and ensuring the validity of the assay.

Pvclust dendrogram: The combined pvclust dendrogram RAPD and SCoT clearly separated the samples into distinct genetic groups with strong statistical support. Well-defined clusters with AU values above 95% indicated high reliability, showing that closely related samples grouped together at low linkage distances, while more divergent ones joined at higher levels. Overall, combining RAPD and SCoT markers improved the accuracy and depth of genetic diversity analysis compared to using either marker alone (Fig. 14).

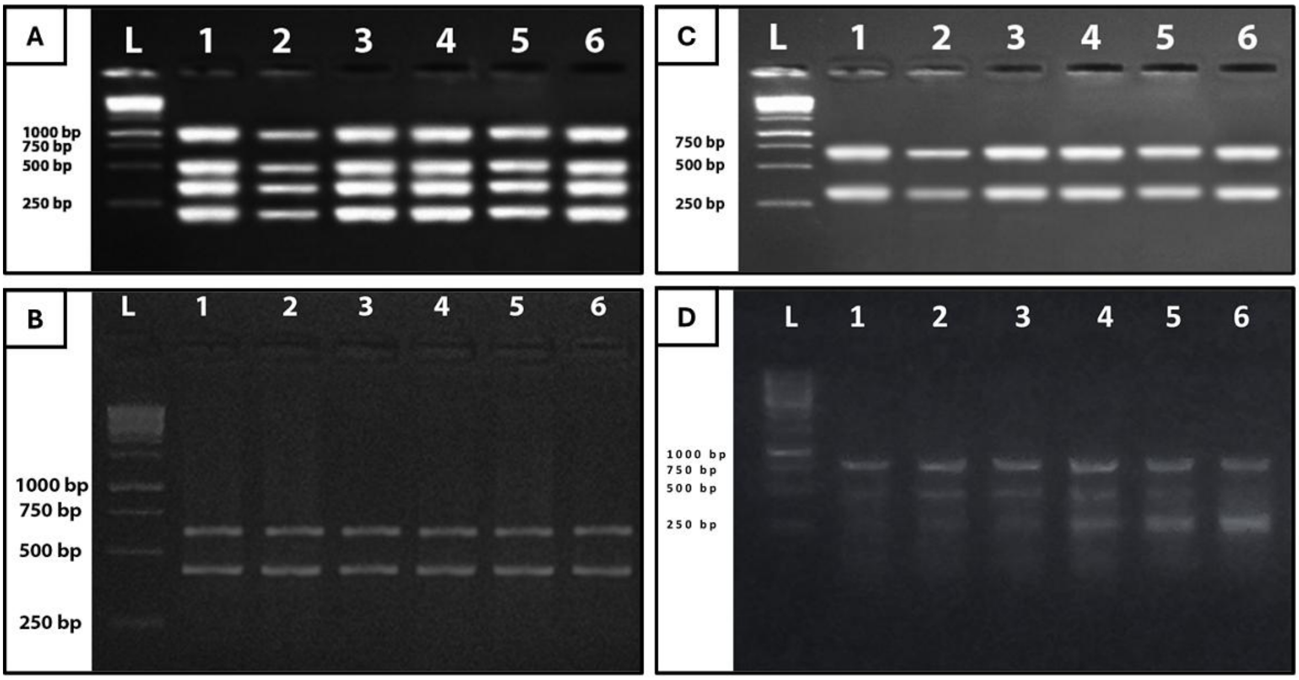

Fig. 13. RAPD and SCoT profile generated by PCR amplification by primers A. OPC-05, B. OPA-09, C. OPC-02 and. D. S25 Lane L is DNA marker of 1kb, lane 1 is wild plant sample, 2-6 are acclimatized *In vitro* propagated plants.

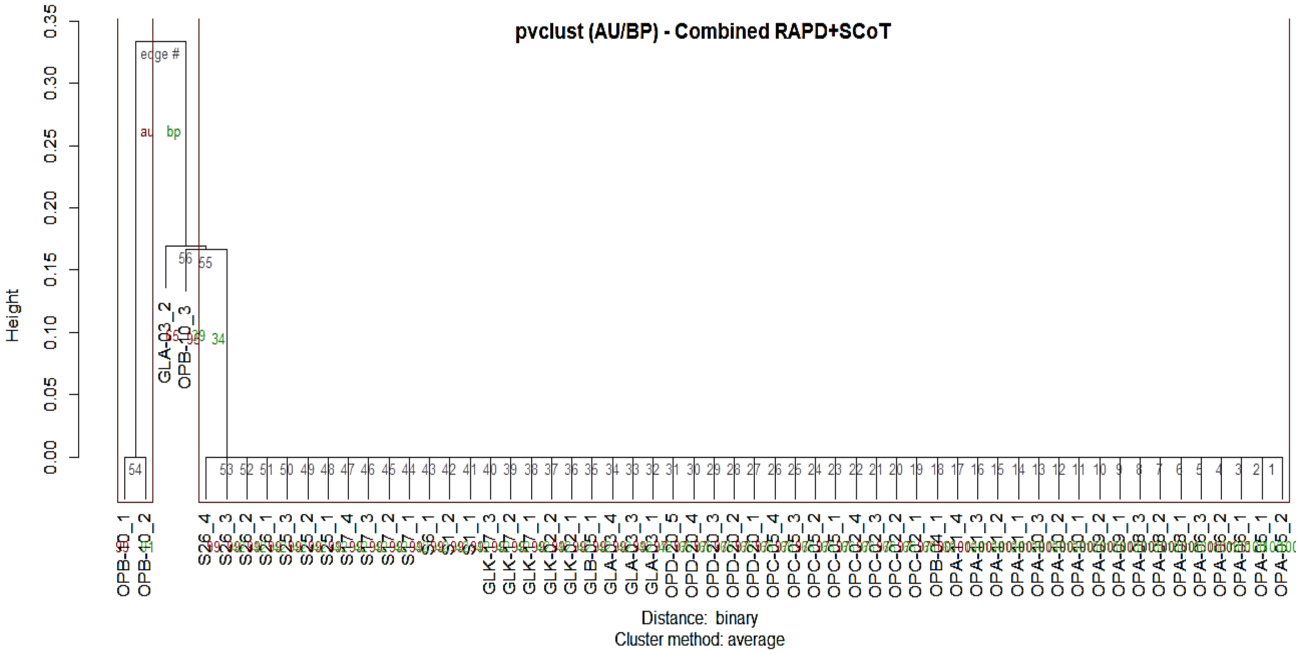

Fig. 14. Pvclust dendrogram of RAPD and SCoT primers used against 6 samples of *J. leptophyllum*.

Table 4. Primers used for genetic uniformity assessment along with sequence, number of bands per sample and range of amplification.

S. No.	Primers	Sequence	Tm ˚C	Annealing temperature ˚C	No. of bands	Polymorphic bands	Monomorphism %	Range band size
					RAPD Primers			
1.	OPA-05	AGGGGTCTTG	32	32	2	-	100	400-700
2.	OPA-06	GGTCCCTGAC	34	32	3	-	100	200-900
3.	OPA-08	GTGACGTAGG	32	32	3	-	100	150–400
4.	OPA-09	GGGTAACGCC	34	32	2	-	100	450–600
5.	OPA-10	GTGATCGCAG	34	32	3	-	100	150–300
6.	OPA-11	CAATCGCCGT	32	32	4	-	100	250–800
7.	OPB-04	GGACTGGAGT	32	32	1	-	100	~700
8.	OPB-10	CTGCTGGGAC	34	32	0-3	-	100	200–1000
9.	OPC-02	GTGAGGCGTC	34	32	4	-	100	250–750
10.	OPC-05	GATGACCGCC	34	32	4	-	100	180-1000
11.	OPD-20	ACCCGGTCAC	34	32	5	-	100	150–1000
12.	GLA-03	AGTCAGCCAC	32	32	4	-	100	100–750
13.	GLB-05	TGCGCCCTTC	32	32	1	-	100	~600
14.	GLK-02	GTCTCCGCAA	32	32	2	-	100	100-400
15.	GLK-17	CCCAGCTGTC	32	32	3	-	100	200-700
					SCoT Primers			
16.	S1	ECCA	48	43	2	-	100	200–1000
17.	S6	ECGC	50	44	1	-	100	~450
18.	S17	FGAG	53	48	4	-	100	180-1000
19.	S25	FGGG	55	50	3	-	100	100–750
20.	S26	FGTC	53	48	4	-	100	150–1000
		Total			**58**	**-**	**-**	**-**

E = CAACAATGGCTACCA; F = ACCATGGCTACCACC

Discussion

Seed germination and early seedling establishment represent the most vulnerable phases in the life cycle of endangered plants, often constrained by physiological and physical dormancy barriers (Thangjam *et al.*, 2017). These constraints are especially pronounced in *Jasminum leptophyllum*, and limiting its natural regeneration capacity and a significant challenge to ex situ conservation. The current study showed that dual pre-treatment with the use of sulfuric acid scarification and then culture on a simplified, sucrose free half-strength MS medium successfully increased germination through 26.9% to 38.5%. This result highlights the critical role of scarification in breaking the non-porous seed coat and promoting the imbibition of water, and the lowered salt level of the basal medium alleviated osmotic stress against frail embryos. On the contrary, media that contained an increased concentration of sucrose also inhibited germination, which was probably caused by osmotic stress or suppression of enzymes essential to germination (Xu *et al.*, 2010).

Regardless of all these improvements, the percentage of seed germination was still poor, even under the most conducive conditions, which indicates the possibility of other physiological dormancy determinants (Zhang *et al.*, 2023). However, the induced germination is an important milestone on the path to the understanding of a reliable seed-based regeneration mechanism of this endangered species and serves as a sound foundation of an *In vitro* optimization and regeneration experiment.

The balance of plant growth regulators (PGRs) was found to be a strong determinant of response of *J. leptophyllum* to *In vitro* culture with bud induction, shoot proliferation, and shoot elongation having distinct regulatory necessities. Even though the bud initiation was observed in all the media examined during the first week, further survival and viability was impaired in extreme levels of cytokinins; whether excessive or minimal. Our findings are consistent with the earlier literature that has found positive effectiveness of a balanced composition, specifically the association of BAP and kinetin, and natural growth-promoting substances (PNGS) supplementation in early bud induction and sustaining bud development, which supports the notion that intermediate PGR levels are essential in the establishment of morphogenesis in a stable manner (Swamy *et al.*, 2014; Chauhan *et al.*, 2018; Karimpour *et al.*, 2020; Bhat *et al.*, 2022; Shah & Jan, 2025).

Shoot multiplication was boosted by low levels of BAP, and this confirmed the potency of the compound in stimulating axillary bud break. BAP with kinetin, and other combinations of cytokinins also enhanced the growth shoots, but there were also inconsistent results with some formulations. Media amalgamated with unbalance PGR suppressed organized development in agreement with earlier research results, which point out the effectiveness of low-dose BAP and synergistic cytokinins interactions in increasing the growth of the shoot (Rahman *et al.*, 2018; Bhat *et al.*, 2022; Joshi *et al.*, 2023; Sharde *et al.*, 2024).

The elongation of shoots, however, was divergent. Maximum elongation was obtained on the media supplemented with natural growth-promoting substances i.e., PNGS (Shah & Jan, 2025), as well as combined BAP and kinetin also supported better elongation. Conversely, the costs of unequal concentrations of cytokinins were significant limitations to growth, highlighting the necessity to separate growth and elongation in protocol optimization. These findings reflected those that were previously published in that, elongation was dependent on recipes that were more likely to support cellular growth as opposed to recipes that were optimal to promote proliferation (Khan *et al.*, 2015; Sompornpailin & Khunchuay, 2016; Bhat *et al.*, 2022).

All these results indicate that effective micropropagation of *J. leptophyllum* needs stage-specific optimization: balanced cytokinins activation for bud formation, low-dose BAP for multiplication, and shoot elongating formulations. This sequential PGRs adjustment offers a powerful model to enhance propagation efficacy and demonstrate potential implementation of targeted cytokinins techniques in woody ornamentals. Along with synthetic PGRs, natural plant growth promotors also produced encouraging results that could be employed in terms of cost effective micropropagation (Bhat *et al.*, 2022; Deshmukh *et al.*, 2022; Shah & Jan, 2025).

The rooting response of *J. leptophyllum* was clearly shaped by the type of auxin applied. Although statistical differences in days to initiation were not detected, the biological trend was evident: NAA (1.6mg/L) consistently promoted the earliest root emergence, with roots appearing within about 16 days, while IAA also stimulated comparatively rapid development, whereas IBA (1.6mg/L) showed an intermediate response –diverging from reports in *Jasminum sambac* and *J. grandiflorum* –where it is reported effective root inducer (Rahman *et al.*, 2018; Bhat *et al.*, 2022; Deshmukh *et al.*, 2022). Such a difference is probably a species-specific difference in the efficiency of auxin metabolism and transport. By contrast, the plant natural growth promoting substances showed a much slower response, often taking more than 48 days, which may reflect the slower release or lower bioavailability of active compounds in these complex mixtures. Taken together, these results reinforce the advantage of synthetic auxins, particularly NAA, in accelerating the onset of rhizogenesis. these results are in line with earlier reports of root induction in *J. carophyllum* and *Pogostemon cablin* (Johnson, 2006; Swamy *et al.*, 2014).

In addition to the time of initiation, the type of auxin had a significant effect on the root number and elongation. NAA generated the greatest number of roots and this fact highlights its ability to induce root primordia. formation, while IAA enhanced roots of similar lengths indicating an important part in post-initiation. elongation. In contrast, IBA and the commercial preparations were much less effective in both parameters, likely due to differences in molecular stability, uptake, or metabolism within plant tissues. Artificial auxins like NAA could be more chemically stable in the culture medium and be more effective in stimulating rhizogenic tissues over an extended period than naturally occurring auxins (Nakhooda *et al.*, 2011). The complementary use of NAA and IAA where the former promotes initiation and the latter promotes elongation therefore becomes a viable approach in enhancing root quality and uniformity in *J. leptophyllum*. This auxin-specific response provides a strong basis for optimization of rooting protocols and improving the efficiency of clonal propagation in this species as reported in earlier studies (Johnson, 2006; Rahman *et al.*, 2018; Bhat *et al.*, 2022; Deshmukh *et al.*, 2022).

Molecular validation of true to type plants is a pre-requisite for conservation programs, as somaclonal variation is a known risk factor with long-term *In vitro* culture, which usually develops due to hormonal stress and sub cultures (Tikendra *et al.*, 2021b). Genetic profiling with 15 RAPD and five SCoT primers produced clear and reproducible banding patterns across all *J. leptophyllum* samples, with consistent fragments observed at ~1000, 750, 500, 400 and 250 bp. The identical amplification profiles of *In vitro*–propagated plants and wild counterparts indicate a high degree of genetic stability, suggesting that the micropropagation protocol did not induce detectable somaclonal variation even after six months of ex situ growth. Compared to RAPD, SCoT markers are much more efficient due to length and high annealing temperatures (Collard & Mackill, 2009; Abd El-Moneim *et al.*, 2021). The absence of amplification in the negative control further validated the assay. Our findings agree with past investigations using these primers as genetic fidelity measures. These findings are of robust support for the genetic similarity of regenerated plants, and illustrating the dependability of the conservation and large-scale propagation protocol (Harirah *et al.*, 2006; Swamy *et al.*, 2014; Verma *et al.*, 2017; Darmayani *et al.*, 2018; Kumar *et al.*, 2018; Sharma *et al.*, 2018).

This study has further more general implications on conservation biotechnology, despite its technical contributions. The combination of optimized micropropagation and molecular validation not only foster the genetic integrity of *J. leptophyllum*, but also creates an appropriate system that could be used in the future to protect other recalcitrant or endangered taxa on a wider basis. This dual approach being a synthesis of physiological understanding and genomic certainty fills a significant gap in plant conservation strategies where propagation efficiency often comes at the cost of genetic fidelity.

Conclusions

To conclude, this study highlights the value of integrating precise hormonal regulation with molecular diagnostics to enhance the regenerative capacity of endangered plant species while maintaining their genetic fidelity. The discovery of the synergistic effect between BAP and kinetin, together with the verified genetic uniformity of regenerated plants, positions *J. leptophyllum* as a promising model for conservation-focused micropropagation. Future investigations should focus on extending this protocol to field acclimatization, metabolomic characterization, and cryopreservation to ensure the long-term preservation of genetic resources. Collectively, these integrated strategies contribute to global biodiversity restoration efforts and reaffirm the role of micropropagation as both a propagation technique and a vital tool for plant conservation.

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