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Kinetin and indole-3-butyric acid for effective *in vitro* propagation of the endangered *Plectranthus phuluangensis* (Lamiaceae) and successful ISSR genetic fidelity test

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ABSTRACT: *Plectranthus phuluangensis*, a critically endangered plant belonging to the Lamiaceae family, is characterized by its unique herbal aroma and is endemic to the Phu Langka Forest in Nakhon Phanom province, Thailand. This investigation aimed to establish an efficient *in vitro* micropropagation protocol for the rapid propagation of this imperiled species utilizing nodal segment explants. The research successfully devised a protocol for the multiplication of shoots, root initiation, and acclimatization. A half-strength Murashige and Skoog solid medium (½ MS) was used, along with different amounts of auxin (indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA)) and cytokinins (6-benzyladenine (BA), kinetin, and thidiazuron (TDZ)). The optimal medium for maximum shoot induction (9.01±1.10 shoots per explant) was at ½ MS supplemented with 4 mg/l of kinetin. Moreover, the ½ MS with 0.5 mg/l of kinetin produced the longest shoots with an average length of 2.69±0.21 mm per shoot. Additionally, the introduction of IBA and NAA led to the formation of adventitious roots. The maximum number of roots (7.35±0.63 roots per shoot) was observed in ½ MS media supplemented with 2.0 mg/l of IBA. After transferring to greenhouses, approximately 65% of the plantlets cultivated in vermiculite survived after 90 days. Furthermore, the ISSR molecular marker analysis revealed no genetic variation between the propagated and mother plants, confirming the genetic fidelity of the propagation method. This study represents the first account of *in vitro* propagation and genetic fidelity evaluation for *P. phuluangensis*, presenting a feasible strategy for the preservation of this endangered species.

KEYWORDS: *Coleus phulangkaensis*, conservation, Inter Simple Sequence Repeat, multiple shoots, root initiation

INTRODUCTION

Located in the northeastern part of Thailand, Phu Lanka National Park covers an area of 50 square kilometers, which represents only 9.74% of the nation's total land area. This park, managed collaboratively by the Ministry of Health and the Ministry of Natural Resources and Environment, serves as a crucial protected herbal zone, integral to the herb conservation strategy across 7 designated regions nationwide. However, despite conservation efforts, 37 plant species in the Phu Langka-Phu Wua National Parks are endangered, with some nearing extinction. *Plectranthus phulangkaensis*, with its homotypic synonyms: *Coleus phulangkaensis* (Suddee, Suphuntee & Saengrit), an endemic species identified in 2014, is one such critically endangered species unique to Phu Langka National Park [1, 2]. The status of this species is monitored by the Forest and Plants Reservation Research Office, a division of Thailand's Department of National Parks, Wildlife and Plants Conservation.

P. phulangkaensis, a perennial herbaceous plant from the Lamiaceae family, can grow up to 1.5 meters tall. This species has single leaves with lateral edges that are either perpendicular or both serrated and laterally edged, ranging from 1–3.5 centimeters wide and 3–10 centimeters long, with either pointed or rounded tips and a triangular base. The plant's flowering and fruiting period spans from September to November. The leaves of Plectranthus plants possess essential oil, cinnamic acid, flavonoids, and terpene derivatives, which have antineoplastic activities by suppressing antioxidants and impeding the division of bacterial and cancer cells [3–5]. *P. phulangkaensis* is threatened by habitat destruction due to illicit harvesting and smuggling, attributed to its alleged restorative properties such as enhancing the circulatory system and rejuvenating potency. This species is only found on inaccessible cliffs. The scarcity of the plant, its difficulty in accessing and harvesting, and its unsuccessful propagation through stem cutting all contribute to its decline.

In vitro propagation offers a promising strategy for rapidly augmenting plant populations to ensure their preservation, recovery, and conservation [6]. The organogenic response in plants is dependent on a variety of factors, including the basal culture medium, temperature, photoperiod, and plant growth regulators (PGRs). The specific types and concentrations of PGRs such as auxins and cytokinins have been shown to facilitate the advancement of bud and root growth in diverse species. *In vitro* propagation techniques have successfully boosted the populations of various *Plectranthus* species, including *P. amboinicus* [3],

P. bourneae [7], and *P. scutellarioides* [8].

However, *in vitro* propagation may lead to somaclonal variation due to a variety of factors, including plant species, the constituents of the artificial growth medium, the length of culture in test tubes, and the quantity and type of growth-regulating substances. To address this, different molecular markers such as protein or DNA markers are employed to detect the traits.

Presently, several DNA markers are utilized in evaluating the genetic fidelity of plant species. These include Inter Simple Sequence Repeats (ISSR), a reliable and efficient method that integrates the advantages of microsatellite markers, Amplified Fragment Length Polymorphisms (AFLP) markers, and Random Amplified Polymorphic DNA (RAPD) markers [9]. Due to its minimal data requirements, similarity to the nucleotide sequence of RAPD techniques, and superior reproducibility, polymorphism, and species discrimination capabilities, the ISSR method is widely employed. ISSR amplifies DNA using the polymerase chain reaction (PCR), utilizing primers with repetitive base sequences such as (AG)6, (TC)8, or (ACG)4. This technique is time-efficient and does not necessitate complex procedures [10]. *In vitro* propagation methods are extensively utilized for the mass multiplication of plant species and are crucial for performing genetic fidelity assessments to verify the true-to-type characteristics of regenerated plants such as *Anarrhinum pubescens* [11], *Bauhinia variegata* [12], and *P. bourneae* [7].

The development of an *in vitro* propagation system for *P. phulangkaensis* is crucial for facilitating uncontaminated propagation and ensuring the preservation and restoration of this critically endangered plant species. Thus, this research aims to devise an effective propagation system for *P. phulangkaensis* by employing the organogenesis approach via shoot multiplication promotion through nodal segment explants for the first time. Furthermore, the study also looks into the genetic fidelity of propagation-derived plantlets using ISSR marker analysis.

MATERIALS AND METHODS

Plant material and surface sterilization

Healthy young branch cuttings, featuring 3–4 internodes from 2–3-year-old *P. phulangkaensis*, were obtained from wild specimens growing in Phu Langka National Park. A representative voucher specimen (MPH-2141) has been preserved at the Phu Langka National Park Herbarium. Nodal segments, 3–4 cm in length, underwent a thorough cleaning process using water and dishwashing liquid wash, followed by a 30 min rinse under running tap water. The segments were then immersed in a 1% solution of commercial fungicide carbendazim (Cabendazim 50, Extra Agrochemical Co., Ltd., Thailand) for 1–3 h. Aseptic conditions were maintained during surface sterilization. Nodal segments were first dipped in 70% (v/v) ethanol for 1 min, then soaked in a 5% commercial sodium hypochlorite solution (6% active chlorine) and 2–3 drops of Tween 20 for 10 min, with twice repeating of the step. To remove residual sodium hypochlorite, segments were rinsed multiple times with sterile distilled water (dH_2O). Dissected the nodal segments about 0.7–1.0 cm in length were cultured on ½ MS medium [13] and augmented with 2% (w/v) sucrose and 0.7% (w/v) agar. pH of the medium was adjusted to a range of 5.0–5.8, prior to sterilization at 121 °C for 15 min.

Effect of cytokinin on *in vitro* shoot induction

To investigate the effect of cytokinins on lateral bud induction in *P. phulangkaensis*, 0.7 cm nodal segments were cultured on ½ MS medium (PhytoTech Labs®, USA) supplemented with different concentrations (0.1, 0.5, 1.0, and 4.0 mg/l) of BA, kinetin, and TDZ (all reagents purchased from PhytoTech Labs®), alongside a control group devoid of PGRs. Nodal explants were positioned in 8 oz bottles containing 40 ml of the prepared solid medium and sustained under a 16 h photoperiod, illuminated by cool-white LED lights at a photon flux density of 40 µmol/m^2/s and maintained at a temperature of 25±2 °C for 30 days. Following this initial period, the buds were subcultured onto the same composition. After 60 days in culture, various attributes such as the number of regenerated shoots, shoot length, callus formation, and root development were documented. The culture medium was replaced every 30 days using the same formulation.

Effect of auxin on *in vitro* root induction and acclimatization

Elongated shoots (2–3 cm) derived from multi-shoot cultures were initially cultivated on ½ MS medium without PGRs for 14 days prior to their transfer onto rooting media. Root induction was carried out on ½ MS medium supplemented with various concentrations (0.1, 0.5, 1.0, and 2.0 mg/l) of NAA and IBA (both from PhytoTech Labs®). In order to evaluate the impacts of NAA and IBA concentrations on rooted shoot percentages, root numbers, and root lengths, these parameters were documented after 30 days in culture.

Shoots exhibiting well-developed root systems were carefully selected and extracted from the cultures without causing damage to the roots. These shoots were subsequently rinsed thoroughly with water to eliminate any remaining traces of the culture medium. The shoots were then transplanted to transparent seedling trays filled with vermiculite and housed in an environmentally controlled room under a 16/8 h (light/dark) photoperiod at 25±2 °C with a light intensity of 40 µmol/m^2/s for 30 days. Sterile water was applied to irrigate the plantlets every 4 days over a 12-

week period. Following successful acclimatization, the well-established plantlets were transplanted to field conditions.

Experimental design and statistical analysis

The efficacy of various PGR treatments on shoot and root induction was assessed using a completely randomized design with a minimum of 10 independent replicates for each treatment (comprising 3 explants per unit). The significance of shoot induction and rooting data was ascertained through an analysis of variance (ANOVA), employing the SPSS Version 26 software (Chicago, IL, USA). To discern significant differences between means, Duncan's multiple range test was applied at a p-value < 0.05.

DNA extraction and evaluation of genetic fidelity through ISSR analysis

Total plant genomic DNA was extracted from 20 mg of fresh leaves using a modified CTAB-based protocol (100 mM Tris/HCl; 20 mM Na2EDTA; 1.4 M NaCl; and 2% (w/v) CTAB, pH 8.0 [14]). The concentration of the extracted DNA was ascertained using a nanophotometer (IMPLEN, Munich, Germany) and 1% agarose gel electrophoresis, respectively.

The genetic fidelity of the mother plant and *in vitro* plantlets were assessed through PCR-based ISSR analyses. An initial set of 13 ISSR primers was selected to evaluate genetic homogeneity. Each PCR reaction consisted of a 10 µl reaction mixture containing 2 µl of genomic DNA (10 ng/µl), 4 µl of 5x HOT FIREPol® Blend Master Mix (Solis Biodyne, Estonia), and 0.5 ng of ISSR Primer. The final volume of the reaction mixture was adjusted to 10 µl with DI water. Amplification was executed using a thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 95 °C for 20 s, annealing for 30 s at 50–60 °C of primer-specific temperature, extension at 72 °C for 30 s, and a final extension cycle at 72 °C for 5 min. The amplified DNA fragments were separated on a 2% agarose gel with 1X TBE buffer and stained with ethidium bromide. The gel was then examined under ultraviolet light with a standard 100 bp DNA ladder. The primers that were capable of amplifying DNA and providing a distinct and robust band were selected for further analysis.

RESULTS AND DISCUSSION

Effect of BA, kinetin, and TDZ on *in vitro* shoot multiplication of *P. phulangkaensis* axillary bud

Previous studies have demonstrated that ½ MS medium considerably improves *in vitro* growth results across different plant species. Investigations on *Mentha piperita* and *M. spicata* (Lamiaceae) revealed that ½ MS media produced the greatest number of shoots and roots per nodal explant as well as the longest average shoot length with no significant differences in comparison to full-strength MS medium [15, 16]. Additionally, lowering the ammonium ion concentration to half of the normal MS level successfully decreased hyperhydricity in *Tagetes patula*, *Castanea sativa*, and *Amelanchier arborea* [17, 18]. These findings reveal the efficacy of ½ MS medium in reducing physiological problems while promoting vigorous development. Therefore, ½ MS medium has multiple applications, making it suitable for examining the impact of specific PGRs on *P. phulangkaensis* nodal explants.

The cultivation nodal explants of *P. phulangkaensis* on ½ MS medium augmented with BA, kinetin, and TDZ at different concentrations (0.1, 0.5, 1.0, and 4.0 mg/l) revealed that ½ MS containing 4 mg/l kinetin yielded the highest average number of shoots, registering 9.01 shoots per explant. Kinetin at the concentration of 0.5 mg/l facilitated the maximum average shoot length of 2.69 mm. Conversely, 1.0 and 4.0 mg/l TDZ did not induce shoot development. A statistical assessment employing Duncan's multiple range test indicated significant disparities in both the average number of shoots per explant and average shoot length at a 95% confidence interval (Table 1 and Fig. 1). Furthermore, the explants propagated on ½ MS supplemented with 4 mg/l BA exhibited shorter internodes in microshoots and displayed hyperhydricity signs. Hyperhydricity of microshoots was also observed in explants grown in 0.1 and 0.5 mg/l TDZ (Fig. 1).

Cytokinin growth regulators such as BA, kinetin, and TDZ have the capacity to stimulate the generation of numerous lateral buds [19–21]. Specifically, kinetin can induce the greatest quantity of lateral buds in *P. phulangkaensis* across all concentration levels without exhibiting any abnormalities or signs of hyperhydricity. This finding aligns with observations in the Lamiaceae *Micromeria croatica* species, which reportedly produces an abundance of shoots when exposed to BA or kinetin at concentrations ranging from 0.022–6.678 mg/l.

The application of 0.225 mg/l kinetin resulted in the highest shoot production with an average of 10.34 shoots per explant [21]. This outcome contrasts with that of *P. bourneae*, which attained maximal shoot multiplication at 6.14 shoots per explant, when cultured in ½ MS medium supplemented with 0.5 mg/l TDZ [8]. Therefore, distinct responses to specific growth regulator types and concentrations have been observed for the induction of shoot multiplication across various plant species [22, 23].

Furthermore, a negative correlation was observed between increasing BA concentrations and shoot production. At 4.0 mg/l BA, developed shoots presented anomalies such as shortened internodes, densely aggregated shoots and nodes forming clusters, and hy-

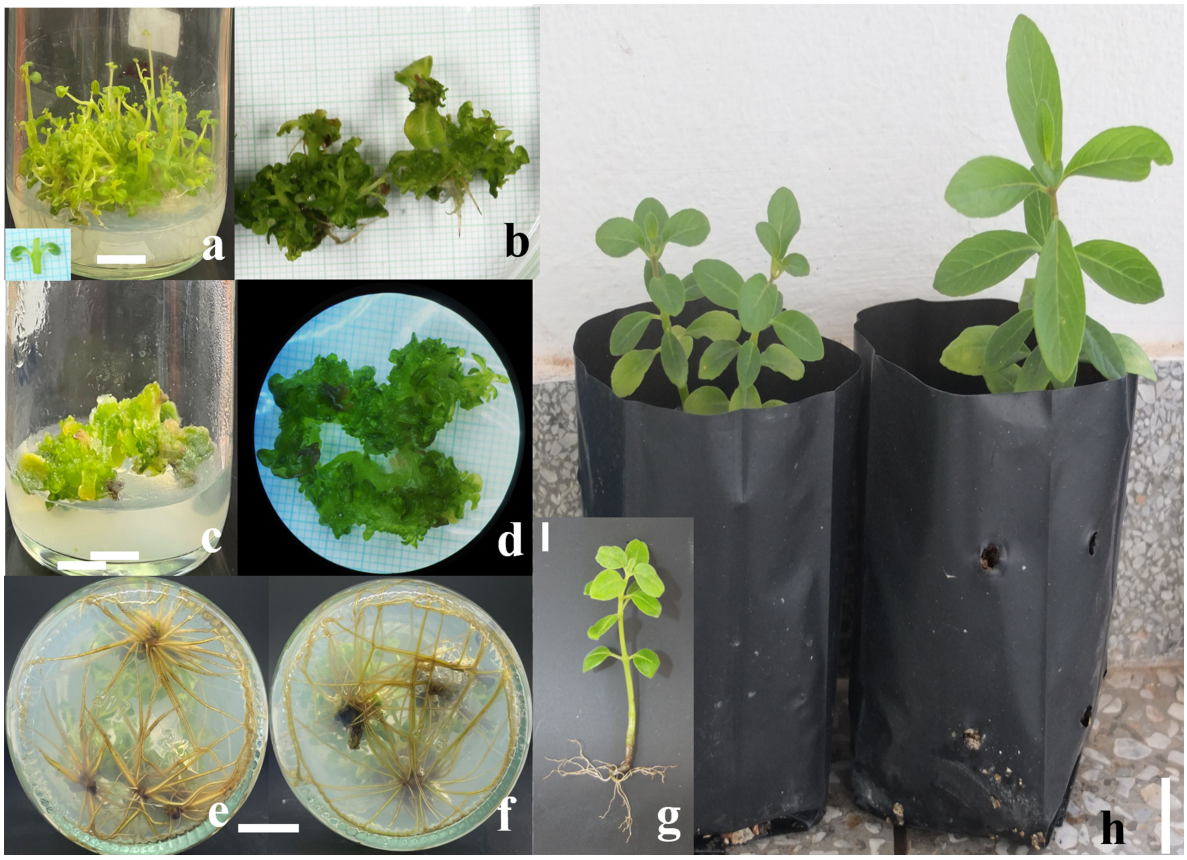

Fig. 1 Plant regeneration of *P. phulangkaensis* on ½ MS medium supplemented with cytokinin (0.1–4.0 mg/l BA, kinetin, and TDZ) and auxin (0.1–2.0 mg/l NAA and IBA). (a) Multiple shoots with a normal phenotype emerging from nodal segment explants on 4 mg/l kinetin after 8 weeks of culture; (b) multiple shoots with shortened internodes and signs of hyperhydricity on 4 mg/l BA; (c) leaf swelling and callus formation on 4 mg/LTDZ; (d) partial shoot development and signs of hyperhydricity on 0.1 mg/LTDZ; (e and f) root induction on ½ MS medium supplemented with 2.0 mg/l NAA and IBA, respectively, after 30 days of culture; (g) fully developed plantlet; and (h) plantlet grown in greenhouse conditions after 12 weeks. (bar = 1 cm).

Table 1 The influence of BA, kinetin, and TDZ on the number of shoots per nodal explant and the length of axillary bud growth in *P. phulangkaensis*.

Cytokinin concentration (mg/l)				No. of shoot/explant	Shoot length (mm)	Callus fresh weight (+/−)
control	BA	Kinetin	TDZ	Mean ± SE	Mean ± SE	
0				1.42 ± 0.12^{ef}	1.68 ± 0.10^{cd}	−
	0.1			2.67 ± 0.28^{de}	1.52 ± 0.09^{de}	−
	0.5			3.03 ± 0.20^{d}	1.51 ± 0.07^{de}	−
	1			4.54 ± 0.41^{c}	1.58 ± 0.08^{cde}	−
	4			1.24 ± 0.13^{ef}	1.52 ± 0.12^{de}	+
		0.1		6.67 ± 0.79^{b}	2.10 ± 0.15^{bc}	−
		0.5		6.67 ± 0.90^{b}	2.69 ± 0.21^{a}	−
		1		5.79 ± 1.07^{bc}	2.30 ± 0.12^{ab}	−
		4		9.01 ± 1.10^{a}	2.11 ± 0.11^{bc}	+
			0.1	1.13 ± 0.28^{ef}	1.27 ± 0.30^{de}	+
			0.5	1.17 ± 0.28^{ef}	1.04 ± 0.28^{de}	+
			1	0 ± 0^{f}	0 ± 0^{f}	+
			4	0 ± 0^{f}	0 ± 0^{f}	+

The data is presented as the mean value±standard error (SE). Mean values accompanied by distinct letters (a,b,c,d,e,f) within the same column exhibit significant differences, based on Duncan's multiple range test conducted at a 95% confidence level. −, No response and +, Response.

Table 2 Impact of auxins in ½ MS medium on the *in vitro* rooting of propagated shoots.

Auxin concentration (mg/l)			Mean number of root /shoot	Mean of root length/shoot (mm)
control	NAA	IBA	Mean ± SE	Mean ± SE
0			$4.35 \pm 0.42^{b,B}$	2.96 ± 0.26^{aA}
	0.1		6.52 ± 0.44^{a}	2.59 ± 0.16^{a}
	0.5		6.17 ± 0.43^{a}	2.44 ± 0.12^{a}
	1		6.55 ± 0.47^{a}	2.51 ± 0.19^{a}
	2		7.35 ± 0.63^{a}	2.56 ± 0.18^{a}
total	NAA		6.29 ± 0.24^{A}	2.51 ± 0.80^{A}
		0.1	4.57 ± 0.62^{b}	2.33 ± 0.25^{a}
		0.5	6.10 ± 0.53^{a}	3.02 ± 0.27^{a}
		1	6.72 ± 0.40^{a}	2.86 ± 0.17^{a}
		2	6.71 ± 0.45^{a}	2.61 ± 0.15^{a}
total		IBA	6.29 ± 0.26^{A}	2.69 ± 0.10^{A}

The data is presented as the mean value±standard error (SE). Mean values accompanied by different lowercase letters (a, b; auxin concentration) or capital letters (A, B; auxin type) signify significant variations identified by Duncan's multiple range test at a 95% confidence level.

perhydricity in the plant, attributable to excessive cytokinin levels [23, 24]. Plant development responses are influenced by the interplay among the concentration of growth-regulating substances in the nutrients assimilated by the plant, endogenously produced plant hormones, and environmental conditions during cultivation. This interaction impacts the emergence of new shoots, leading to abnormalities and manifestations of hyperhydricity. For example, *P. amboinicus* grown in MS medium supplemented with 0.5 mg/l benzylaminopurine (BAP) display stunted shoots and challenges in multiplication due to reduced survival rates upon nutrient medium exchange [3]. Hyperhydricity has been detected in other plants within the Lamiaceae family, cultivated in the presence of elevated concentrations of cytokinin growth regulators, including *Agastache mexicana* [25], *Teucrium scorodonia* [26], and *Thymus leucotrichus* [27]. The hyperhydricity problems can be mitigated by transferring the plant to a nutrient medium devoid of growth regulators [28, 29] and by modifying the cultivation technique to a temporary immersion system [29, 30].

Effect of auxin on *in vitro* root development and acclimatization in *P. phulangkaensis*

Vigorous and healthy plant shoots with a height of approximately 2–3 cm were chosen to examine the *in vitro* root formation. The experiment was carried out using ½ MS supplemented with varying concentrations of auxin-based growth regulators, specifically IBA and NAA, at 0, 0.1, 0.5, 1.0, and 2.0 mg/l. The results indicated a statistically significant difference in root initiation between the control group and the different concentrations of growth regulators. However, no significant variation was observed in the average number of roots at concentrations of 0.5–2 mg/l for both IBA and NAA. The ½ MS medium containing 2 mg/l NAA yielded the highest average root count at 7.35 roots per shoot, followed by the medium with 1 mg/l IBA, which produced an average of 6.72 roots per shoot. The ½ MS medium with 0.1 mg/l IBA resulted in the lowest average root count of 4.57 roots per shoot. In the absence of PGRs, the medium generated an average of 4.35 roots per shoot (Table 2 and Fig. 1). Subsequently, seedlings with a minimum shoot height of 3 cm and robust, healthy root systems were transferred to a vermiculite-based growing substrate. A survival rate of 65% was observed 12 weeks after the transition to greenhouse conditions. The *in vitro* regenerated plantlets exhibited normal morphological characteristics comparable to those of the mother plants.

Root initiation in growth media without supplemental growth regulators can be attributed to the sufficient accumulation of auxin hormones within the plant shoot, which subsequently stimulates root development [31]. Various plants belonging to the Lamiaceae family have demonstrated enhanced root formation in ½ MS medium, a medium containing half the nutrients compared to full-strength nutrient media. Such plants include *Zhumeria majdae* [32] and *Perovskia abrotanoides* [33]. The diminished nutrient content may correlate with the plants' reduced nitrogen needs, potentially impacting root generation [33]. Additionally, *C. forskohlii* displayed the most rapid root growth in ½ MS medium without growth regulators and a decreased sucrose content of 1.5% (W/V) [34]. Conversely, *P. phulangkaensis* shoots cultivated in ½ MS medium enriched with low concentrations of IBA and NAA (0.1 and 0.5 mg/l) yielded a greater number of roots than those grown in medium lacking growth regulators (Table 2), aligning with findings in *Z. majdae* [32]. Concerning root size, it was observed that NAA led to larger roots than IBA (Fig. 1). In addition, it was ascertained that alterations in the concentrations of IBA and NAA did not engender significant statistical variations in root length, a conclusion analogous to

Table 3 The list of primers, including their sequences, the number of amplified bands, and the lengths of the resulting amplified fragments generated by ISSR markers.

No.	Primer	Sequence 5′ – 3′	Annealing (°C)	Total number of amplified and monomorphic band	Approximate band length (bp)
1	ISSR 1	(AG)8T	50	11	400–1500
2	ISSR 2	(AG)8G	50	6	300–1000
3	ISSR 3	(GA)8T	55	10	400–1500
4	ISSR 4	(CA)8A	50	5	300–1500
5	ISSR 5	(CA)8G	55	10	400–1500
6	ISSR 6	(TC)8C	50	10	400–2500
7	ISSR 7	(AG)8YT	55	12	200–2500
8	ISSR 8	(AG)8YC	50	7	400–1500
9	ISSR 9	(AG)8YA	50	3	300–500
10	ISSR 10	(CA)8YG	50	–	–
11	ISSR 11	(CT)8RC	50	10	500–1500
12	ISSR 12	BDBC(AC)7	55	8	700–1500
13	ISSR 13	DBD(AG)7	55	8	500–1000
		total		100	

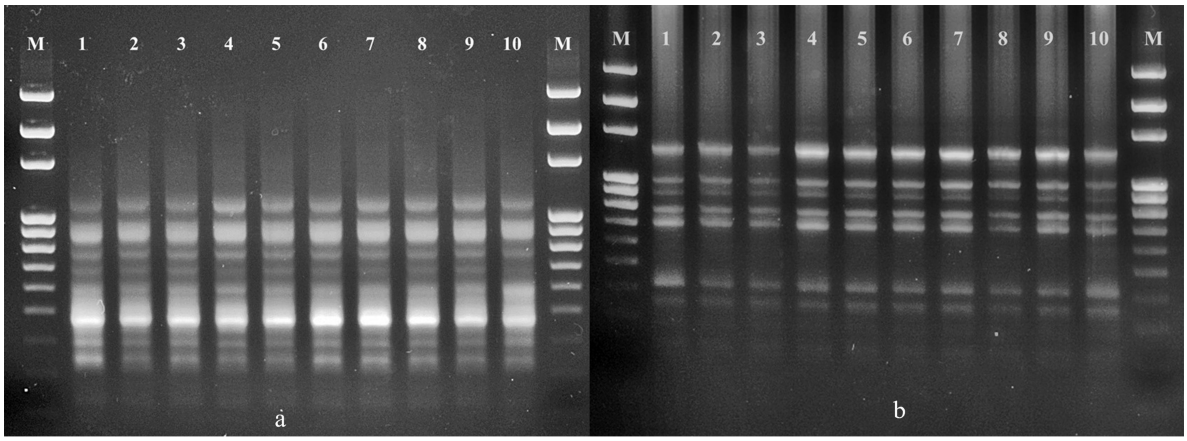

Fig. 2 Consistency of genetic profiles using ISSR markers in *in vitro* propagated and naturally plants of *P. phulangkaensis*. PCR amplified banding pattern obtained with ISSR11 (a) and ISSR13 (b). Lane M: standard DNA ladder, lanes 1–9: *in vitro* propagated *P. phulangkaensis* plantlets, and lane 10: wild plant of *P. phulangkaensis*.

outcomes in previous *Z. majdae* [32].

Assessing genetic fidelity using ISSR markers

The factors influencing *in vitro* propagation processes are manifold, encompassing the explant origin, fluctuations in growth conditions, the range of regeneration techniques, and the frequency of subculturing. These factors necessitate the application of growth regulators, which can potentially instigate somaclonal variation [35]. High concentrations of growth hormones may induce chromosomal irregularities, obstructing the generation of plants identical to the original [36, 37]. ISSR markers were employed to assess genetic uniformity among 9 randomly selected *P. phulangkaensis* specimens, comprising 1 control group sample and 8 samples from propagated *in vitro* and subsequently successfully acclimatized plants, comparing their genetic profiles to the mother plant. Of the 13 ISSR primers, 12 (ISSR1-9,11-13) produced distinctive and reproducible banding patterns, whereas 1 (ISSR 10) primer lacked specificity for *P. phulangkaensis*. The most effective primers produced amplified bands ranging between 200–2,500 bp in size. In total, there were 100 amplified bands; each primer yielded 3–10 bands with an average of 7.69 bands per primer (Table 3). The ISSR markers of 12 primers were determined to be monomorphic and similar. ISSR11 and ISSR13 exhibited amplicon profiles of 10 and 8, respectively, with separate DNA bands and high intensity (Fig. 2). This suggested that *P. phulangkaensis* plantlets derived from tissue culture techniques employing plant growth regulators maintained genetic fidelity without somaclonal variation. The findings indicate that *P. phulangkaensis* plantlets produced via tissue culture methods preserve genetic fidelity and do not exhibit somaclonal variation. Furthermore, the

results of ISSR markers here were also in line with those of the previous genetic consistency evaluations in several plants in the Lamiaceae family, including *Origanum majorana* [38], *Orthosiphon stamineus* [39], and *P. bourneae* [7].

CONCLUSION

The present study successfully identified the optimal conditions for auxillary bud propagation via *in vitro* techniques in *P. phulangkaensis*. This approach facilitates shoot formation through organogenesis on ½ MS medium supplemented with cytokinins (BA, kinetin, and TDZ), with 4 mg/l kinetin eliciting the greatest number of shoots while also exhibiting a positive response to root induction. It was also observed that increased concentrations of BA and TDZ resulted in callus formation and the emergence of abnormal physical traits. Furthermore, shoot development was responsive to root induction on ½ MS medium in combination with auxins (IBA and NAA), wherein 2 mg/l IBA produced the highest root count. All *in vitro* regenerated plantlets exhibited typical morphological traits identical to the parent plants and successfully acclimatized in a vermiculite-based substrate. ISSR analysis revealed no somatic variation between *in vitro* plantlets and their mother plants under natural conditions. Consequently, this study demonstrates the efficacy of this method in rapidly augmenting the *P. phulangkaensis* seedling population, ensuring genetic uniformity, and providing significant advantages for conservation and restoration efforts. This study is the first to apply *in vitro* propagation and evaluate the genetic consistency of *P. phulangkaensis* propagated via tissue culture techniques.

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