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ORIGINAL ARTICLE

Genetic Clonal Fidelity Assessment of Rhizome-Derived Micropropagated *Acorus calamus* L. – A Medicinally Important Plant by Random Amplified Polymorphic DNA and Inter-Simple Sequence Repeat Markers

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ABSTRACT

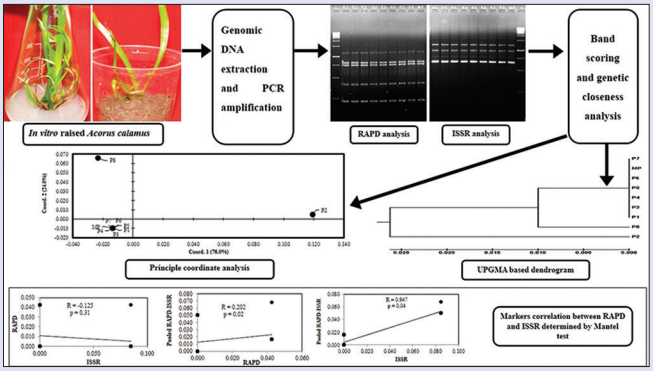
Background: *Acorus calamus* – a critical medicinal plant, is overexploited, leading to population reduction. Establishing an efficient *in vitro* protocol is essential for the large-scale production of genetically identical plants. **Objectives:** Development of fast and reliable *in vitro* regeneration protocol for *A. calamus* and clonal fidelity assessment of the regenerants using molecular markers. **Materials and Methods:** Plants were regenerated on Murashige and Skoog medium with different concentrations of growth regulators in two phases – shooting and rooting. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers were employed to evaluate the genetic stability of *in vitro* clones. **Results:** 6 Benzylaminopurine (BAP) at 1.6 and 2.4 mgL^{-1} was effective for shoot induction, while root induction was superior in indole-3-butyric acid-incorporated medium at 2.5 mgL^{-} . Thirteen RAPD and 16 ISSR primers produced 59 and 96 clear, unambiguous, and reproducible bands, respectively. Both the markers revealed a high monomorphism of 96.79% and 95.63% among the regenerants. Nei's genetic distance analysis disclosed a close genetic association (0.000–0.068) among the genotypes. **Conclusion:** ISSR was better than RAPD markers in clonal fidelity assessment of the regenerants. The *in vitro* protocol developed is reliable and suitable for the rapid propagation of true-to-type *A. calamus* plants.

Key words: *Acorus calamus*, dendrogram, DNA marker, genetic stability, genetic distance, micropropagation

SUMMARY

- BAP at 1.6 and 2.4 mgL^{-1} produced the best shooting response, while indole-3-butyric acid at 2.5 mgL^{-1} was most appropriate for root induction.
- Close genetic distances (0.000 to 0.068) were maintained between the mother plant and *in vitro* regenerants.
- Inter-simple sequence repeat markers were more effective than the random amplified polymorphic DNA in clonal fidelity assessment of micropropagated *Acorus calamus*.

Abbreviations used: %: Percentage; °C: Degree centigrade; µl: Microliter; AFLP: Amplified fragment length polymorphism; ANOVA: Analysis using analysis of variance; BAP: 6 Benzylaminopurine; bp: Base pair; cm: Centimeter; CNS: Central nervous system; CTAB: Cetyl-trimethyl-ammonium bromide; DMRT: Duncan's multiple range test; DNA: Deoxyribonucleic acid; dNTPs: Deoxyribonucleotide triphosphate; FRLHT: Foundation for Revitalization of Local Health Traditions; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; ISSR: Inter-simple sequence repeat; mgL-1: Milligram per liter; min: Minute; mM: Millimolar; MP: Mother plant; MS: Murashige and Skoog; ng: Nanogram; PCoA: Principal coordinate analysis; PCR: Polymerase chain reaction; PGRs: Plant growth regulators; RAPD: Random amplified polymorphic DNA; RFLP: Restriction fragment length polymorphism; SSR: Simple sequence repeat; Taq: *Thermus aquaticus*; TDZ: Thidiazuron; UPGMA: Unweighted pair group method for arithmetic averages.



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INTRODUCTION

Acorus calamus L. (Common name – Sweet flag), belonging to the family Acoraceae, is a littoral inhabitant, monocot plant with creeping rhizome. The plant typically exists in four different natural cytotypes with their geographical distribution based on the ploidy levels.[1] While the diploid and triploid plants are distributed in North America and Europe, and the temperate Asian regions, respectively, the tetraploid plants are widespread in the eastern and subtropical areas of Asia.[2,3] The triploid plants are mostly confined to the Indian subcontinent and are

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found cultivated mainly in Kashmir, Himachal Pradesh, Uttarakhand, Nagaland, Manipur, Tamil Nadu, Andhra Pradesh, and Maharashtra.[4,5] Morphologically, the plant grows up to 2–3 feet in length and bears branched rhizomes and sword-shaped leaves along with rarely grown yellowish or greenish miniature flowers which are long, cylindrical, and covered in a multitude of rounded spikes.[6] The main part of the plant is the rhizome which is pale to dark brown in coloration, horizontally placed, jointed, vertically compressed, and spongy with a thickness of 1.25–2.5cm.

A. calamus is widely popular for its high medicinal values. It possesses antispasmodic, antidiarrheic, antidepressant, antihelminthic, carminative, and central nervous system anxiolytic properties.[7] The rhizome is the most effective part of the plant utilized for formulating treatments of local ailments. The extract of the rhizome is used for the preparation of many general tonics and as a stimulant, laxative, expectorant, diuretic, and antitumor agent.[8,9] It is also applied as a traditional medicine in the management of insomnia, neurosis, cold, asthma, fever, epilepsy, hysteresis, memory loss, chest pain, and urinary tract infection.[10-12] While the dried roots are used as flavoring agents and appetizers, the essential oil extracted from the rhizomes and roots is reported to possess insecticidal and antimicrobial properties.[13,14] Asarone, palmitic, Heptanoic acid, choline, flavones, ethanol, zinc, methanol, camphor, eugenol, and many other medicinally beneficial bioactive compounds are also found existing in the plant extracts.[15-17]

There has been extensive exploitation of this highly valued medicinal plant from the natural habitats to meet the huge commercial demand. Traditional propagation of *A. calamus* through seeds is not possible as the triploid plants do not produce seeds. Vegetative propagation through rhizome cutting has limitations as plant production through this method is slow with the potential of depleting the natural genetic resources. There is an alarming decrease in the natural population of *A. calamus* due to indiscriminate collection and massive habitat destruction. The Foundation for Revitalization of Local Health Traditions (FRLHT), during an extensive survey, has perceived this plant as endangered in Kerala and vulnerable in Tamil Nadu and enlisted in the 100 red-listed medicinal plants of South India.[18] *In vitro* propagation through plant tissue culture technique offers an alternative to slow conventional methods by mass-producing genetically stable disease-free plants rapidly. Maintaining the genetic identity of the *in vitro* regenerants is important as somaclonal variation may appear in the plants due to high growth hormone concentration, long culture duration, nutrient stresses, and other adverse culture conditions.[19,20] Somaclonal variation may be beneficial, but the emergence of genetic variation is a major concern when the primary regenerants are the required end products for the commercialization and conservation of the elite genotypes.[21] Hence, it is crucial to assess the clonal fidelity of the *in vitro* regenerated plants by using molecular markers. Preserving the genetic uniformity of the regenerants is also highly essential to develop superior planting materials akin to the mother plants.

There are few reports on the micropropagation of *A. calamus,*[22-25] but no studies have been conducted to test the clonal fidelity of the regenerants using molecular markers. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers have been previously used successfully in the genetic fidelity assessment of many micropropagated plants.[26-30] However, it is more appropriate to use both the marker types than using a single marker system, as the more efficient ISSR markers can validate the results of the RAPD markers.[31] There are several reports of the combined use of RAPD and ISSR markers in the genetic homogeneity testing of different plants.[32-38] The present study was conducted to develop an efficient and fast *in vitro* regeneration protocol for *A. calamus* and assess for the first time the clonal fidelity of the micropropagated plants using molecular markers.

MATERIALS AND METHODS

Micropropagation of *Acorus calamus*

Source of explants and sterilization

Young rhizomes of *A. calamus* were collected during April–May from the natural populations of Manipur, India. The rhizome was washed thoroughly in the tap water and treated with 70% ethanol (v/v) for 1 min, followed by washing twice with sterilized distilled water. The rhizome was again treated with 0.2% $HgCl_2$ for 5 min followed by washing 3–5 times with sterilized distilled water to remove the traces of mercuric chloride from the explants.

Culture medium and conditions

The sterilized explants were inoculated under the aseptic conditions in the laminar air hood on the freshly prepared Murashige and Skoog (MS) medium.[39] The inorganic salts of MS medium were obtained from HiMedia, Mumbai. The medium was supplemented with 3% (w/v) sucrose (HiMedia, Mumbai) as the carbon source and was gelled using 9% (w/v) agar (HiMedia, Mumbai). The pH of the medium was adjusted at 5.6 using 1N NaOH and 1N HCl before autoclaving. Induction of shoot and roots from the rhizome was studied in different plant growth regulators' (PGRs) combinations and concentrations. Each PGR combination had 12 replicates, and the experiment was repeated thrice. Regular subculture was done every 3 weeks on a freshly prepared medium. After inoculation, the cultures were maintained at 25°C ± 2°C and illuminated by 3500 lux intensity for 16 h a day using fluorescent tubes.

Healthy and well-rooted plants were deflasked and treated with warm sterilized water containing an antifungal agent (5% Bavistin) to remove any agar residues and fungal contamination from the plants if any. The plants were acclimatized in the small plastic pots containing sterilized sand and soil mixture (1:1). The plantlets were sprayed with half-strength liquid MS medium without sugar alternate days for 3 weeks inside the laboratory before they were shifted to the glasshouse condition for further acclimatization for another 3 weeks.

Statistical analysis of culture data

In vitro response regarding the culture multiplication rate and shoot and root length growth was recorded every week. After successful shoot induction and growth, cultures with multiple shoots were transferred to the rooting medium containing different concentrations of auxins (indole-3-butyric acid [IBA] and indole-3-acetic acid [IAA]). The data were subjected to statistical analysis using analysis of variance (ANOVA, $P \leq 0.05$), and the mean values of the different treatments were compared using Duncan's multiple range test at $P \geq 0.05$. The statistical examination in the present study was accomplished using the SPSS (Version 16.0; SPSS Inc., Chicago, IL, USA).

Genetic stability assessment of *Acorus calamus*

DNA extraction

Genomic DNA was extracted from the leaves of the mother plant, and eight randomly selected *in vitro* raised *A. calamus* using a modified cetyl-trimethyl-ammonium bromide method.[40] The qualities and quantities of the isolated DNA samples were determined using a spectrophotometer (Perkin-Elmer Lambda 35) at 260 and 280 nm, respectively. The DNA samples were later checked for their purity and integrity by performing 0.8% agarose gel electrophoresis and comparing the intensity of the resultant bands with 1kb DNA ladder (HiMedia). The extracted DNA samples were finally stored at -20°C after performing dilution to 50 ng/µl.

Random amplified polymorphic DNA

Thirteen decamer RAPD primers (Eurofins) were selected after screening 25 different primers based on the production of clear, reproducible, and scorable bands. RAPD primer amplification was performed in 25 µl volume with 20 ng of genomic DNA, 2.5 µl of 10 × PCR buffer containing 15 mM $MgCl_2$, 0.02 mM dNTPs, 1 unit of Taq polymerase (Bangalore Genei, India), and 20 ng RAPD primer. The amplification reactions were executed with a program of initial DNA denaturation at 94°C for 4 min, followed by 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 30–32°C, and 1 min of extension at 72°C with the final extension at 72°C for 10 min.

Inter-simple sequence repeats

Sixteen ISSR primers that generated distinct and scorable bands were chosen after the initial screening of 27 ISSR primers obtained from integrated DNA technologies. PCR reactions were conducted in a 25 µl volume consisting of 20 ng of template DNA, 2.5 µl of 10 × PCR buffer with 15 mM $MgCl_2$, 0.02 mM dNTPs, 1unit of Taq polymerase (Bangalore Genei, India), and 20 ng of ISSR primer. The PCR amplification was performed with a program of initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 5°C less than the melting temperature (Tm) of the respective primer for 1 and 2 min extension at 72°C with a final extension at 72°C for 10 min.

Data analysis

The consistent and reproducible bands generated by the selected RAPD and ISSR primers were scored. The band intensity was not taken into account for the scoring. The data were pooled into a data binary matrix based on the presence (1) or absence (0) of the selected bands. Nei's similarity matrix values were determined by employing GenAlEx (Genetic Analysis in Excel) Version 6.5 software. The similarity matrix values were subjected to cluster analysis using UPGMA (unweighted pair group method for arithmetic averages), and dendrograms were generated using MEGA 5. The principal coordinate analysis (PCoA) was performed using GenAlEx 6.5 to define the spatial distribution of the *in vitro* regenerants and the mother. The correlation between the genetic distance matrices of RAPD and ISSR markers was analyzed using the Mantel test.[41]

RESULTS AND DISCUSSION

Variations in the growth response were observed when rhizome explants of *A. calamus* were cultured on MS medium supplemented with different growth hormones, namely BAP, TDZ (thidiazuron), IBA, and IBA IAA. However, high genetic stability was maintained among the genotypes of *in vitro* raised plants and mother plants, irrespective of the effect of different concentrations of PGRs.

In vitro rhizome culture

Successful shoot regeneration was observed in all the hormone combinations tested, with the initiation of shoot growth starting from the 3rd day of inoculation [Figure 1a and b]. Although the shoot regeneration did not differ much in the medium incorporated with BAP and TDZ, a lower shoot regeneration rate was evidenced in the medium when auxins were added along with BAP or TDZ in the medium. The low shoot regeneration could be ascribed to the inhibitory action of auxins on shoot development. However, the promotive effect of BAP partially reversed the inhibition induced by auxins.[42] Such an inhibitory effect of auxin on shoot induction via rhizome explant of *A. calamus* was also reported.[6] The highest percentage of shoot regeneration (83.33%) was observed in the medium fortified with 2.4 mgL^{-1} BAP. Rani *et al.*[22] similarly reported high shoot multiplication in BAP-incorporated medium. The effectiveness of BAP on shoot induction was also noticed on a rare medicinal plant, *Chlorophytum borivilianum*.[43] The least (44.72%) shoot formation was recorded in the medium augmented synergistically with equal concentration (0.8 mgL^{-1}) of TDZ and IBA [Figure 2]. Murch and Saxena[44] observed the accumulation and translocation of auxin in *Pelargonium* × *hortorum* Bailey when the plant tissues were exposed to TDZ, which led to its limited influence on shoot regeneration. As observed in the earlier work,[22] the shoot regeneration varied significantly when the concentration of BAP (0.8 mgL^{-1}) was increased to 1.6 mgL^{-1} and 2.4 mgL^{-1}. Among the six different combinations of BAP and auxins (IBA and IAA), the percentage of shoot regeneration was highest (67.78%) in the medium augmented with equal concentration (0.8 mgL^{-1}) of BAP and IAA [Figure 2]. Contrary to TDZ, the shoot induction increased when the concentration of BAP was enhanced from 0.8 mgL^{-1} to 2.4 mgL^{-1}. Verma and Singh[6] also reported a similar effect of higher BAP concentration on shoot induction. At equal PGR concentration, the rate of shoot development in 0.8 mgL^{-1} BAP + 0.8 mgL^{-1} IAA was superior than 0.8 mgL^{-1} TDZ + 0.8 mgL^{-1} IAA [Figure 2].

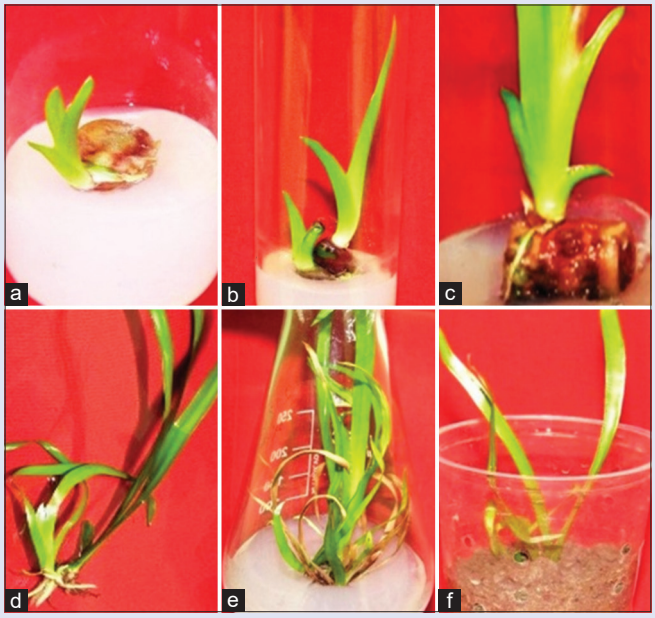

Figure 1: *In vitro* propagation of *Acorus calamus* from rhizome segment. (a) Shoot induction in Murashige and Skoog (MS) +0.8 mgL^{-1} 6 benzylaminopurine (BAP), (b) Shoot multiplication in MS + 2.4 mgL^{-1} BAP, (c) Initial root formation in MS + 0.8 mgL^{-1} thidiazuron, (d) Multiple root formation in plantlets grown in MS + 2.5 mgL^{-1} Indole-3-butyric acid, (e) Well-grown plants with complete leaf and root development appropriate for hardening, and (f) Hardening of the well-acclimatized *A. calamus*

The shoot number per explant was more in medium containing BAP than TDZ when present singly. The high efficiency of BAP on shoot induction was also witnessed in earlier studies.[6,45] Shoot formation was reduced from 2.69 ± 0.04 to 1.59 ± 0.24 when 1.6 mgL^{-1} BAP was incorporated into the medium with either 0.8 mgL^{-1} of IBA or IAA [Table 1]. Tikendra *et al.*[46] also witnessed the inhibitory effect of auxins on shoot development. Bhagat[47] made similar observations on shoot multiplication in a medium containing BAP and auxins. Development of stunted shoot length in BAP and IAA containing medium was also earlier found in *A. calamus*.[22] The highest shoot length

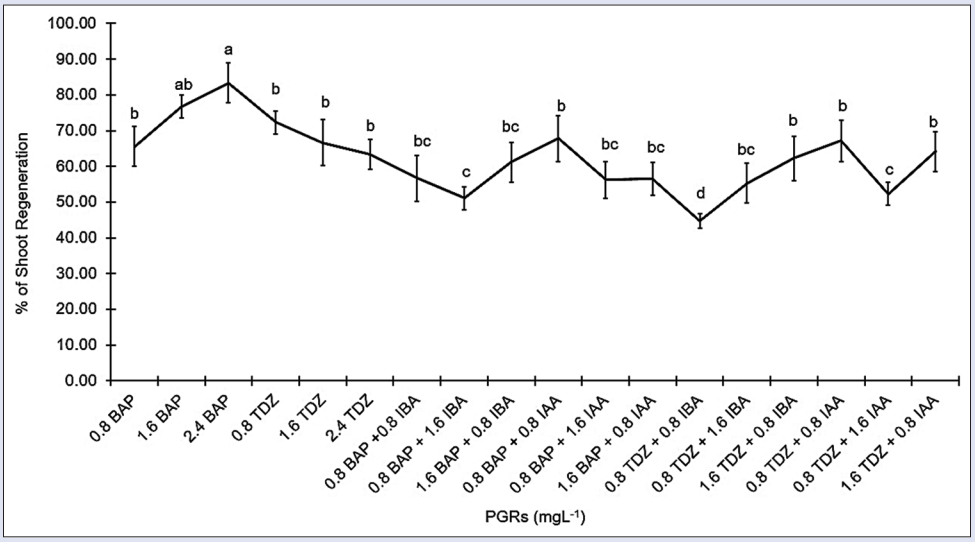

Figure 2: Effect of different plant growth regulators on the *in vitro* shoot regeneration of *Acorus calamus*

Table 1: Effect of various plant growth regulators on *in vitro* shoot development of *Acorus calamus*

PGRs (mgL^{-1})				After 3rd week of culture	
BAP	TDZ	IBA	IAA	Shoot number	Shoot length (cm)
0.8				1.97 ± 0.41^{abc}	2.58 ± 0.40^{efg}
1.6				2.69 ± 0.04^{a}	3.24 ± 1.02^{def}
2.4				2.76 ± 0.41^{a}	7.58 ± 0.39^{a}
	0.8			1.57 ± 0.16^{bc}	3.78 ± 0.43^{cde}
	1.6			1.21 ± 0.12^{bc}	2.11 ± 0.25^{fg}
	2.4			1.08 ± 0.13^{c}	2.91 ± 0.81^{defg}
0.8		0.8		0.90 ± 0.80^{c}	3.39 ± 0.63^{def}
0.8		1.6		1.17 ± 0.24^{bc}	2.88 ± 0.58^{defg}
1.6		0.8		1.59 ± 0.24^{bc}	6.57 ± 0.63^{ab}
0.8			0.8	1.12 ± 0.11^{bc}	2.32 ± 0.39^{efg}
0.8			1.6	1.15 ± 0.35^{bc}	1.59 ± 0.15^{g}
1.6			0.8	1.39 ± 0.55^{bc}	4.37 ± 0.66^{cd}
	0.8	0.8		2.17 ± 0.19^{ab}	3.40 ± 0.49^{def}
	0.8	1.6		1.23 ± 0.08^{bc}	2.53 ± 0.45^{efg}
	1.6	0.8		1.98 ± 0.48^{abc}	2.59 ± 0.58^{efg}
	0.8		0.8	1.79 ± 0.58^{abc}	5.97 ± 0.21^{b}
	0.8		1.6	1.09 ± 0.07^{bc}	1.60 ± 0.26^{g}
	1.6		0.8	1.54 ± 0.18^{bc}	5.06 ± 0.12^{bc}

Mean values (±SD) within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P\geq0.05$). Values are based on 12 replicates per treatment in three independent experiments. SD: Standard deviation; PGRs: Plant growth regulators; BAP: Benzylaminopurine; TDZ: Thidiazuron; IBA: Indole-3-butyric acid; IAA: Indole-3-acetic acid

was noticed in medium fortified with 2.4 mgL^{-1} BAP (7.58 ± 0.39 cm), followed by medium containing 1.6 mgL^{-1} BAP + 0.8 mgL^{-1} IBA (6.57 ± 0.63 cm), 0.8 mgL^{-1} TDZ + 0.8 mgL^{-1} IBA (5.97 ± 0.21 cm), and 1.6 mgL^{-1} TDZ + 0.8 mgL^{-1} IAA (5.06 ± 0.12 cm) [Table 1]. The earliest root development was observed at the 3rd week of culture in the medium enriched with 0.8 mgL^{-1} TDZ [Figure 1c]. In contrast, no such adventitious root formation was witnessed in BAP-incorporated medium. Bhagat[47] made a similar observation of the absence of root development in BAP containing medium in *A. calamus*, even after 3 weeks of culture. Following this observation, the plantlets were transferred to newly prepared rooting media incorporated singly with either IAA or IBA at different concentrations. Prominent rooting response with the highest root number (6.39 ± 0.78) and the most extended root length (4.58 ± 0.6 cm) was noticed in medium enriched with 2.5 mgL^{-1} IBA [Figures 1d and 3]. IBA was the most effective in inducing rooting compared to IAA in the present study. The earlier report also showed the effectiveness of IBA over IAA on *in vitro* root growth and development in *A. calamus*.[48] Well-grown healthy plants were selected and transferred to the small plastic cups containing sterilized sand and soil mixture (1:1) for proper acclimatization and successful hardening [Figure 1e and f].

Genetic homogeneity assessment

Although genetic variability among the crops, medicinal plants, and other rare species is important for the genetic improvement of the species, somatic variation among the *in vitro* clones is unwanted if one desires to conserve the elite genotype.[49,50] The loss of cellular regulation on the growth of *in vitro* cultured plants, somatic mutations associated with the explant tissues, the inappropriate concentration of PGRs, and prolonged culture duration are linked to the occurrence of somaclonal variation.[49,51-55] RAPD and ISSR were employed to assess the genetic stability among the genotypes of micropropagated *A. calamus* and mother plants. Unlike the morphological markers, these DNA markers are generally stable against the influences of various environmental factors and were widely used for determining the genetic homogeneity of several micropropagated plants.[56-59]

Random amplified polymorphic DNA and inter-simple sequence repeat banding profile analysis

Genetic homogeneity was analyzed among eight randomly selected *in vitro* raised plants and mother plants. Out of 25 RAPD primers screened, 13 oligonucleotide primers, which generated reproducible bands with sizes ranging between 250 and 2000 bp, were selected for analysis. A total of 61 amplified DNA fragments (loci) with an average of 4.69 loci per primer were detected. Fifty-nine loci were monomorphic, rendering a high monomorphism (96.79%) among the regenerants [Table 2]. Nei[60] estimated the minimum requirement of 50 different loci to evaluate the genetic distance between different species effectively. Different RAPD primers yielded variable numbers of informative amplified fragments, with OPE-07 generating the highest number of seven amplified

Table 2: Random amplified polymorphic DNA primer used, number of scorable bands produced, band size, and the percentage of monomorphism recorded among the mother plant and micropropagated *Acorus calamus*

RAPD Primer code	RAPD Primer sequence (5′→3′)	Number of scorable bands	Number of bands		Percentage of		Range of amplification (bp)
			Monomorphic	Polymorphic	Mono morphism	Polymorphism	
OPA-01	5'-CAGGC$_3$TTC-3'	5	5	-	100	-	2000-1000
OPA-03	5'AGTCAGCCAC-3'	3	3		100		1500-500
OPA-05	5'-AG$_4$TCT$_2$G-3'	3	3		100		1500-750
OPA-07	5'-GA$_3$CG$_3$TG-3'	6	5	1	83.3	16.7	1000-250
OPA-10	5'-GTGATCGCAG-3'	3	3	-	100	-	1500-750
OPA-11	CAATCGCCGT-3'	5	5		100		2000-750
OPA-13	5'-CAGCAC$_3$AC-3'	4	3	1	75	25.0	2000-750
OPB-02	5'-TGATC$_3$TGG-3'	6	6	-	100		2000-250
OPC-07	5'-GTC$_3$GACGA-3'	4	4	-	100	-	1500-250
OPC-08	5'-TG$_2$AC$_2$G$_2$TG-3'	5	5	-	100	-	2000-250
OPD-01	5'-ACCGCGAAGG-3'	6	6		100		2000-250
OPE-07	5'-AGATGCAGC$_2$-3'	7	7	-	100	-	1500-200
OPG-15	5'-ACTG$_3$ACTC-3'	4	4		100		1500-750
Total		61	59	02	96.79	3.21	-

RAPD: Random amplified polymorphic DNA

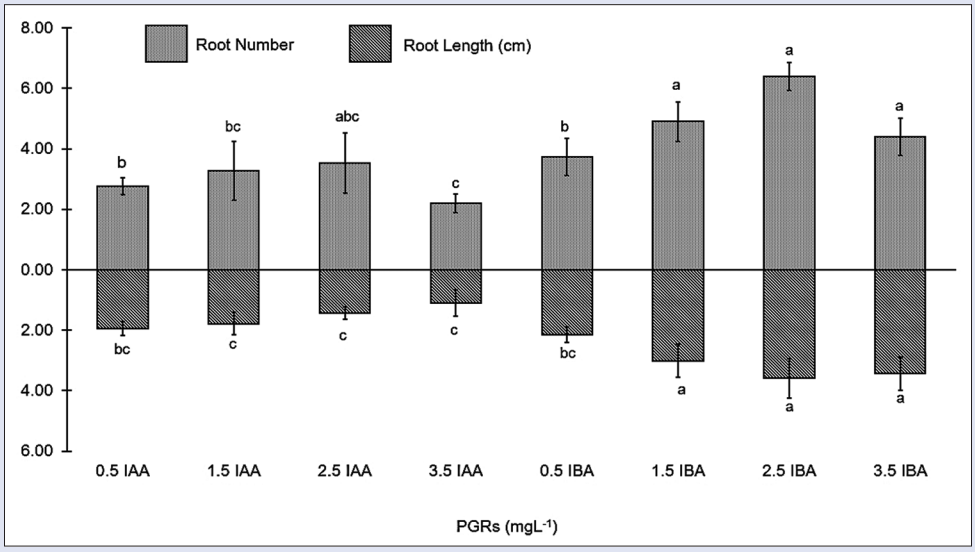

Figure 3: Effect of different concentrations of indole-3-acetic acid and indole-3-butyric acid on the *in vitro* root growth and development of *Acorus calamus*

fragments [Figure 4a]. At the same time, the least of three loci were observed for OPA-03, OPA-05, and OPA-10. Most primers showed monomorphic banding patterns except for one locus each of OPA-07 and OPA-13, which were polymorphic, accounting for 3.21% of observed polymorphism among the regenerants [Table 2]. Detection of low genetic polymorphism by RAPD analysis was also reported in genetic fidelity assessments of many *in vitro* propagated plants.[61,62] Although the RAPD markers have been used extensively in clonal fidelity assessment, in some instances, they failed to disclose the changes in the repetitive DNA sequences of some plants.[63] To affirm the outcome of RAPD analysis, the genetic homogeneity of *A. calamus* was further analyzed using the ISSR markers. The reason for selecting ISSR markers is their high variability, great potential to determine inter- and intra-genomic diversity, and the presence of high copy numbers in eukaryotic genomes.[64,65] Furthermore, technically, ISSR markers are simpler when compared to AFLP, RFLP, and SSR, as no prior sequence information for the genomic DNA is required for amplification.[66] The longer nucleotide units (15–30 mers) of ISSR than RAPD (10 mers) and their higher annealing temperature make them more stringent, reproducible, and informative.[67,68] The importance of two markers system in detecting the genetic stability was also demonstrated in almond,[69] *Ziziphora canescens, Ziziphora tenuior*,[70] and *Bacopa monnieri*.[37]

From a total of 25 ISSR primers screened, 16 primers were selected, which produced 96 clear and unambiguous bands generating six loci per primer. The size of amplified DNA fragments ranged from 250 to 2000 bp. Of the total amplified fragments, 91 loci were monomorphic, resulting in 95.63% monomorphism between the *in vitro* clones and the mother plant. UBC-868 produced the highest number of 10 amplified monomorphic loci, while UBC-807 and UBC-813 generated low amplified bands of 4 each [Figure 4b]. UBC-863, on the other hand, displayed the lowest number of three loci. The low polymorphism (4.37%) detected among the *in vitro* clones was due to the presence of four polymorphic loci (three loci for UBC-810 and one locus each for UBC-848 and UBC-871) [Table 3]. This observation showed higher discriminatory power of ISSR over RAPD markers in detecting polymorphism. Several workers have previously demonstrated the ISSR to be more effective than RAPD markers in genotyping and genetic diversity studies of plants.[37,70-72] Low polymorphism detection in the present investigation may also

be attributed to the absence of the transitional callus phase during *A. calamus* culture since callus formation may contribute to higher variability amongst the regenerants.[50,73]

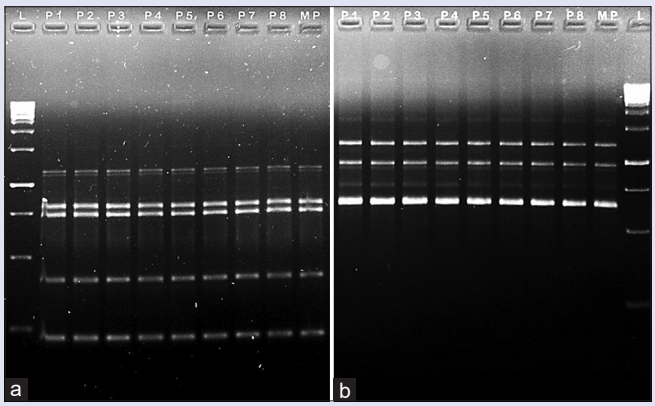

Figure 4: DNA banding patterns of the *in vitro* raised plantlets (P1-P8) and the mother plant of *Acorus calamus*. (a) Banding profile for random amplified polymorphic DNA primer (OPE-07); (b) Banding profile for inter-simple sequence repeat primer (UBC-807)

Genetic distance and cluster analysis

From the pooled RAPD-ISSR data, pairwise Nei's genetic distance matrices between the *in vitro* regenerants and the mother plant were estimated [Table 4]. The Nei's genetic distance matrix value close to or equal to 0 represents a high degree of genetic uniformity among the genotypes.[74] The recorded Nei's genetic distances were very low, ranging from 0.00 to 0.068, indicating a close genetic relationship between the regenerants (P1 to P8) and the mother plant (MP). The *in vitro* clones, except P2 and P8, were genetically identical (0.000) to the MP. P2 exhibited a genetic distance value of 0.068 with P8 and 0.050 with the remaining clones and MP. P8, on the other hand, showed a closer genetic identity than P2, with a genetic distance of 0.017 recorded with other regenerants and MP [Table 4]. Earlier works on *Dendrobium chrysotoxum* and *Bulbophyllum auricomum* also reported the detection of close genetic distance and low variability among the micropropagated plants.[35,75] The presence of low genetic distances due to differences in the observed loci can be attributed to the occurrence of genetic or epigenetic changes in the propagated plants, either by loss of certain loci or formation of new binding sites in the regenerants.[62] Since the culture condition such as salts composition of the medium, duration of photoperiod, and temperature are equally maintained, the variation detected could have arisen due to rapid disorganized growth induced by plant growth hormones.[49]

Table 3: Inter-simple sequence repeat primer used, number of scorable bands produced, band size, and the percentage of monomorphism recorded among the mother plant and micropropagated *Acorus calamus*

ISSR primer code	ISSR Primer sequence (5′→3′)	Number of scorable bands	Number of bands		Percentage of		Range of amplification (bp)
			Monomorphic	Polymorphic	Monomorphism	Polymorphism	
UBC-807	5'-$(AG)_8$T-3'	4	4	-	100	-	2000-750
UBC-810	5'-$(GA)_8$T-3'	9	6	03	66.7	33.3	1000-250
UBC-813	5'-$(CT)_8$T-3'	4	4	-	100	-	1500-500
UBC-814	5'-$(CT)_8$A-3'	5	5	-	100	-	1500-250
UBC-820	5'-$(GT)_8$C-3'	6	6	-	100	-	1500-250
UBC-822	5'-$(TC)_8$A-3'	6	6	-	100	-	1000-500
UBC-823	5'-$(TC)_8$C-3'	7	7	-	100	-	2000-500
UBC-824	5'-$(TC)_8$G-3'	6	6	-	100	-	2000-550
UBC-827	5'-$(AC)_8$G-3'	5	5	-	100	-	1500-250
UBC-830	5'-$(TG)_8$G-3'	8	8	-	100		2000-500
UBC-848	5'-$(CA)_7$CR*G-3'	5	4	1	80	20	2000-750
UBC-863	5'-$(AGT)_6$-3'	3	3	-	100	-	1000-500
UBC 868	5'-$(GAA)_6$-3'	10	10	-	100	-	2000-250
UBC-871	5'-$(TAT)_6$-3'	6	5	1	83.33	16.67	1500-250
UBC-875	5'-$(CTAG)_4$-3'	7	7	-	100		2000-500
UBC-877	5'-$(TGCA)_4$-3'	5	5	-	100		2000-750
Total		96	91	5	95.63	4.37	-

R*=A/G. ISSR: Inter-simple sequence repeat

Table 4: Genetic distance between the mother plant and the *in vitro* regenerants (P1–P8) of *Acorus calamus* based on Nei's coefficient of similarity obtained from pooled random amplified polymorphic DNA-inter-simple sequence repeat data

P1	P2	P3	P4	P5	P6	P7	P8	MP	
0.000									P1
0.050	0.000								P2
0.000	0.050	0.000							P3
0.000	0.050	0.000	0.000						P4
0.000	0.050	0.000	0.000	0.000					P5
0.000	0.050	0.000	0.000	0.000	0.000				P6
0.000	0.050	0.000	0.000	0.000	0.000	0.000			P7
0.017	0.068	0.017	0.017	0.017	0.017	0.017	0.000		P8
0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.017	0.000	MP

MP: Mother plant

The UPGMA dendrogram obtained from RAPD analysis consisted of two main clusters. One primary cluster comprised MP and P1, P2, P3, P4, P5, P6, and P7, while the lone P8 was positioned in another group [Figure 5a]. Similarly, the dendrogram from the ISSR analysis produced two clusters. The major cluster harbored MP, P1, P3, P4, P5, P6, P7, and P8, while P2 was only found in the minor cluster [Figure 5b]. The dendrogram obtained from the pooled RAPD-ISSR data showed a close similarity with the genotype clustering pattern of the ISSR marker. The main cluster consisted of two subclusters with P1, P3, P4, P5, P6, and P7 grouping in one subcluster and lone MP existing in another [Figure 5c]. The other minor cluster consisted of P2 only, indicating its genetic dissimilarity with the rest of the plants. Further, PCoA arranged the genotypes with respect to the two coordinates [Figure 6]. The first and second coordinates accounted for 76% and 24% of the total variation, respectively. The genotypes were spatially distributed in the first three quadrants. P2 was plotted in the first quadrant, P8 in the second quadrant, and the remaining P1, P3, P4, P5, P6, P7, and MP were located in the third quadrant. The distribution pattern in PCoA plot affirmed the genotype association as depicted by dendrogram analysis. A similar observation of consistency in genotype distribution as defined by UPGMA and PCoA was reported among micropropagated *Dendrobiums*.[72]

Correlation analysis of random amplified polymorphic DNA and inter-simple sequence repeat markers

The Mantel test was conducted to check the correlation between the genetic similarity matrices obtained from RAPD and ISSR analysis. Despite high genetic monomorphism revealed by both the markers, no significant correlation was found between the RAPD and ISSR markers (r = −0.125; P = 0.31) [Figure 7a]. The lack of correlation between the genetic matrices of RAPD and ISSR markers indicated that each marker system measured different aspects of genetic variability. Similar observations of noncorrelations between different marker types were also demonstrated in *Oleo europaea*[76] and *Dendrobium moschatum*.[62] The genetic correlation estimation between the genetic matrices based on RAPD and pooled RAPD-ISSR data was significant but relatively low (r = 0.202; P = 0.02) [Figure 7b]. However, the correlation test between the matrices of ISSR and pooled RAPD-ISSR was significantly high (r = 0.947; P = 0.04) [Figure 7c]. This could be due to higher band number (6) detected by ISSR than RAPD markers with low band numbers (4.69). Corroborating with the earlier reports,[33,77] the present analysis also revealed the effectiveness of ISSR over RAPD markers in determining the genetic polymorphism among the genotypes of micropropagated *A. calamus*. It further manifested the importance of ISSR markers as the main component of the two marker systems for validating the results of RAPD markers.

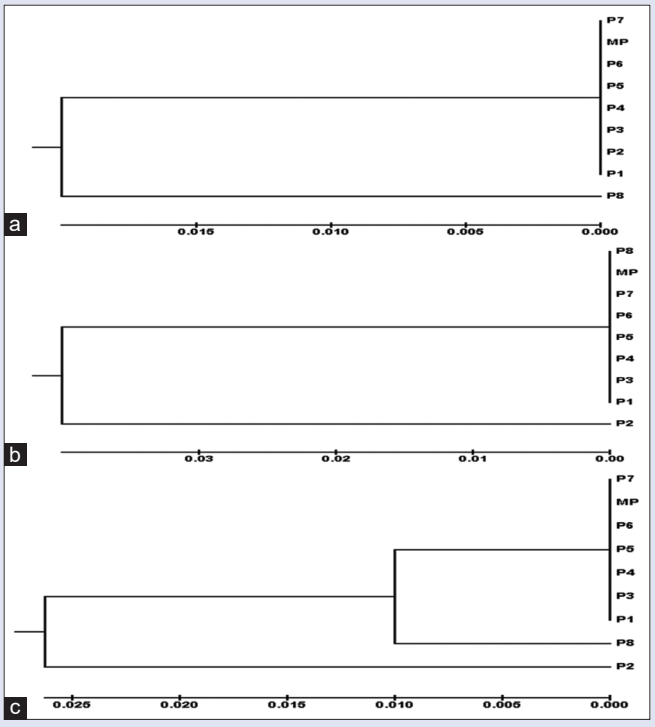

Figure 5: Unweighted pair group method for arithmetic averages dendrograms obtained from (a) Random amplified polymorphic DNA (RAPD) marker analysis, (b) ISSR marker analysis, and (c) Pooled RAPD-ISSR data analysis showing the genetic relationship between the mother plant and randomly selected *in vitro* regenerants (P1 to P8) of *Acorus calamus*

CONCLUSION

The high monomorphism disclosed through RAPD (96.79%) and ISSR (95.63%) marker analysis indicated the maintenance of genetic uniformity among the regenerants. The present investigation confirmed the potential application of RAPD and ISSR markers in effectively detecting genetic homogeneity among the regenerants and mother plants. This study can be considered a primary step towards propagating genetically stable *A. calamus* plants via rhizome explant using two marker systems. The use of molecular markers ensured the production of genetically identical *A. calamus* through the established *in vitro* protocols and detection of genomic variability, if any, at the early growth stage of this medicinally important plant.

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Conflicts of interest

There are no conflicts of interest.

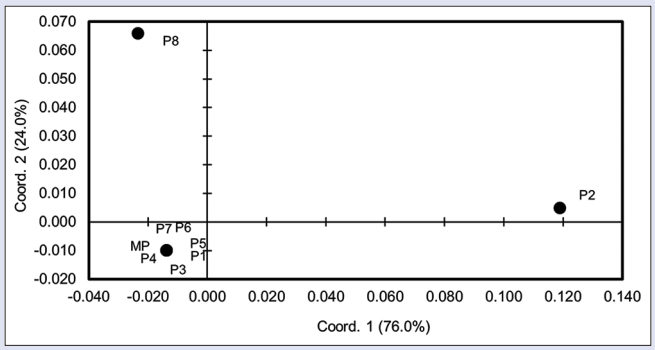

Figure 6: Principal coordinate analysis plot showing the distribution of the mother plant and the *in vitro* regenerated plants (P1 to P8) of *Acorus calamus*

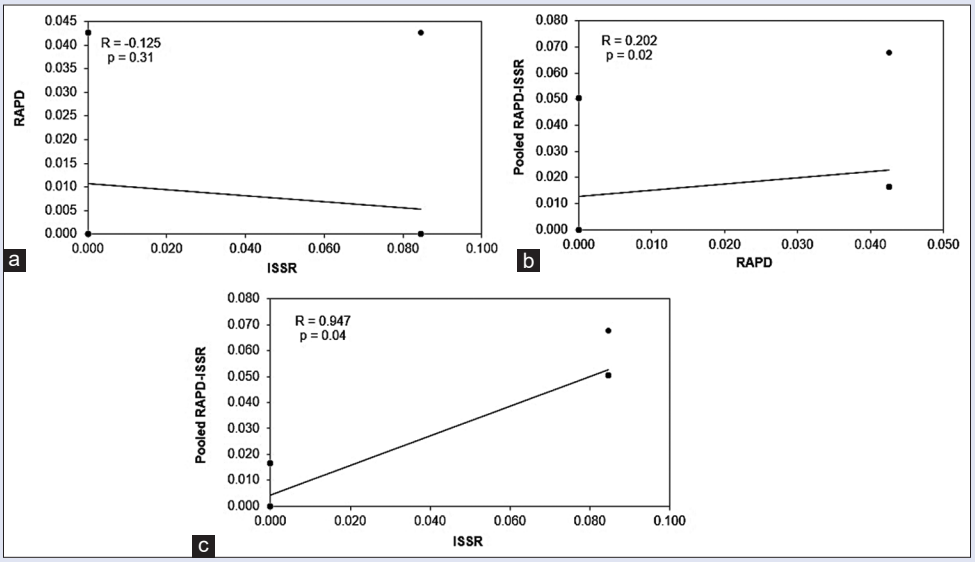

Figure 7: Mantel test displaying the correlation between the molecular markers based on genetic distance matrices (a) Random amplified polymorphic DNA (RAPD) versus inter-simple sequence repeat (ISSR), (b) RAPD versus pooled RAPD-ISSR data, and (c) ISSR versus pooled RAPD-ISSR data

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