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Evaluation of genetic fidelity of *in vitro* regenerated Sweet flag (*Acorus calamus* L.) using molecular markers

Paraskar Shyam Purushottam, Kishor B. & Ng. Tombisana Meetei*

School of Crop Improvement, College of Post Graduate Studies in Agricultural Sciences, Central Agricultural University (Imphal), Umiam, Meghalaya, India

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Sweet flag (*Acorus calamus* L.) is an endangered, perennial wild herb known for its high medicinal properties. An efficient *in vitro* regeneration protocol for sweet flag was developed and the genetic fidelity was assessed across *in vitro* regenerants. Murashige & Skoog media fortified with different plant growth regulators (PGRs) in various concentrations and combinations were used to culture rhizome buds. For surface sterilization of explants, treatment of fungicide (0.2% Bavistin) for duration of 30 min followed by treatment with 70% ethanol for 30 s and mercuric chloride (0.1%) for 15 min was found most effective. MS solid media (0.8% agar) was found more promising than MS semisolid media (0.6% agar) in relation to explants establishment. The maximum number of shoots per explant was observed in solid MS medium supplemented with 15 µM thidiazuron (TDZ) with an average of 7.67 shoots per explants and average explant response of 60%. Shoot length response was maximum in MS containing 10 µM 6-benzylaminopurine (BAP) in combination with 5 µM 1-naphthalene acetic acid (NAA) with an average length of 6.88 cm and percentage explant response of 80%. For root induction, MS medium containing 10 µM indole-3-butyric acid (IBA) gave the maximum number of roots (6.50) and root length (3.50 cm). Plants acclimatized well with 57.14% survivability rate. Forty random amplified polymorphic DNA (RAPD) primers were used to evaluate genetic fidelity among *in vitro* regenerated plants, one from each treatment and compared with mother plant. Out of them, 20 RAPD primers produced a total of 70 fragments ranging from 350-2500 bp. The amplified bands of all *in vitro* plant samples and mother plant were observed to be monomorphic. The results showed that the *in vitro* regenerated plants were genetically stable.

Keywords: Monomorphic amplified bands, Random amplified polymorphic DNA (RAPD)

Sweet flag (*Acorus calamus* L. Fam: Acoraceae) is a tall, perennial wild herb, indigenous to India but has also been observed in Europe, Northern USA and major parts of Asia[1]. It has long, erect, sharp-pointed leaves which are sword-shaped in appearance and rhizomes which horizontally grow below the surface of soil[2]. It has small creeping roots along the rhizome which helps to absorb nutrients from the soil. This crop is propagated mainly through rhizomes. The rhizome bears small buds equidistant along the rhizome length which further grows into long leaf structure up to 2 meters height. This plant has high demand since many centuries due to its medicinal properties. Having various phytoconstituents with useful bioactivities viz. antispasmodic, antidiarrheic, carminative, anti-helminthic, antidepressant, metabolic and neurological disorders, it has been in use since the time of Ayurveda in India[3-5]. Moreover, extracts of *A. calamus* rhizomes have also been evaluated against parasitic diseases[6].

*Correspondence:
E-Mail: ntmeetei@gmail.com

Many industries have over-exploited this species for its therapeutic potential and diverse uses. The unsustainable collection of these plants from their wild habitat brought it on the verge of extinction. According to Red Data Book list of threatened species, this species has been classified as the vulnerable species[2].

Tissue culture has potential for biodiversity conservation as well as fulfilling the requirement of industries[2]. Therefore, development of efficient *in vitro* regeneration protocol is essential in order to conserve this species as well as to be able to meet the increasing demand of pharmaceutical/cosmetics industries. Genetic fidelity assessment among *in vitro* regenerants however, is important in relation to conservation studies and also in production of true to type plants in large scale. Somaclonal variation hinders genetic homogeneity of tissue culture raised plants and arises due to modification in DNA methylation, gene amplification, chromosomal abnormality and point mutation[7]. There are several factors responsible for this variation including explant

types, nutritional conditions and number of subculture cycle[8]. As a result, in order to produce clonally uniform progeny, it is necessary to check the genetic integrity of the *in vitro* regenerated plants on a frequent basis. The molecular techniques are more precise and accurate to study genetic stability of *in vitro* regenerated plants than conventional methods as it is not influenced by environmental factors. Various PCR based markers are available to check genetic fidelity such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and start codon target (SCoT)[9]. Out of all the markers, RAPD has proven to be simplest, quickest and cheapest method used in genetic fidelity study of tissue cultured plants[10]. Hence, the present study aims to develop an efficient *in vitro* regeneration protocol for *Acorus calamus* and to evaluate the genetic stability of the *in vitro* regenerated plant using RAPD markers.

Materials and Methods

In vitro regeneration of *A. calamus*

Acorus calamus plants were collected from farmers and grown in pots at the College of Post Graduate Studies in Agricultural Science, (CAU, Imphal), Umiam, Meghalaya. Young rhizome buds collected from mother plants were washed thoroughly under running tap water for 10 min to remove soil and subsequently washed with detergent solution for 5 min. After thorough washing with tap water, Bavistin (0.2%) treatment was given for varying duration ranging from 30-60 min and then further rinsed with double distilled water. Rhizome buds were then taken under laminar air hood for further surface sterilization. Initially, rhizome buds were treated with 70% ethanol for 30 s and then surface sterilized with 0.1% mercuric chloride or 2% sodium hypochlorite solution containing 5 drops of Tween-20 for varied duration to standardize an effective sterilization protocol. Explants were then washed repeatedly at least three times serially with sterile distilled water to rid of any traces of chemicals used. Excess water was removed using sterile filter paper. The surface sterilized rhizome buds were inoculated on MS medium supplemented with different concentrations (5, 10 and 15 µM) of thidiazuron (TDZ) and 6-benzyl-aminopurine (BAP) alone and in combination with 5 µM 1-naphthalene acetic acid (NAA). Readymade Himedia Murishage & Skoog powder was used for media preparation (3% sucrose, 0.8% w/v agar). The pH of the medium was adjusted to 5.8 before autoclaving by using either 0.1N NaOH or 0.1N HCl solution. The media were autoclaved at 121°C and 15 psi for 20 min and then poured in sterilized culture vessels under aseptic condition for further inoculation. The culture condition was maintained properly with temperature of 25±2°C and photoperiod as 16 h light and 8 h dark with light intensity of 2000 lux. Relative Humidity was maintained between 60-70%. Sub-culturing of regenerated shoots was done by transferring the shootlets to the same type fresh medium for shoot proliferation. Proliferated shoots were transferred to root induction medium (MS medium fortified with different concentrations (5 µM, 10 µM) of Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA)) using the same culture conditions. All the *in vitro* regenerated plants with well-developed roots were removed from culture bottles and washed thoroughly to remove media adhered to roots and subsequently transferred to artificial soil (peat:perlite:vermiculite) mixture in plastic cups and covered with polythene bags kept inside growth chamber for two weeks and later transferred to greenhouse condition. Survival percentage was calculated.

Genetic fidelity assessment

For genetic fidelity assessment, one plant from each treatment that gave explant response was selected. The young and tender leaves of the *in vitro* regenerated plants and also samples from the mother plant were collected for DNA extraction to assess the genetic fidelity by using RAPD markers. Total genomic DNA was extracted using modified CTAB method[11] and quality was checked using 0.8% w/v agarose gel electrophoresis. The samples were allowed to run in gel at 80 volts for 45 min and bands were observed using gel documentation unit. A total of 40 RAPD markers were screened for genetic fidelity assessment of Sweet flag. The PCR amplification was performed on a final volume of 10 µL containing 1 µL genomic DNA, 8.5 µL master mix and 0.5 µL RAPD primer (0.5 µM). Master mix was prepared containing 1X PCR buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, Taq polymerase (0.5 U) and volume made up with nuclease free water. The PCR condition was programmed at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 30-40°C for 1 min and extension at 72°C for 2 min and final extension at 72°C for 5 min. PCR products were electrophoresed using 1.5% agarose gel at 80 to

100 volts in 0.5X TBE and ethidium bromide staining for 2 h. DNA ladder (Fermentas) of 1 kb/100 bp was loaded @3 µL on either side of the PCR products. The gels were visualized in gel documentation system. The well resolved and consistently amplified DNA fragments as bands were scored with regard to their presence (1) or absence (0).

Experimental design and statistical analysis:

The *in vitro* regeneration experiment was performed using completely randomized design (CRD). Five explants per treatment were used and the experiment was repeated two times. Data was recorded for shoot length, number of shoots, root length and number of roots and statistically analyzed for significance using one way analysis of variance (ANOVA) at $P \leq 0.01$ level of significance using SPSS software.

Results

Figure 1 shows the *in vitro* regeneration of *Acorus calamus*.

Standardization of surface sterilization protocol

The current study had a series of sterilization stages including the use of several surface sterilizing agents for varying periods of time in order to develop an effective protocol for establishing contamination-free cultures as given in Table 1. A total of 8 different treatments were tried for sterilization of explants. The sterilized explants were inoculated on solid MS basal media and incubated up to four weeks. Out of the 8 treatments, the one with 0.2% bavistin for 30 min followed by 70% ethanol treatment for 30 s and 0.1% mercuric chloride treatment for 15 min showed the lowest percentage of contamination (20%) with maximum explant response. Another similar sterilization method with a difference in the treatment of 0.2% bavistin for 1 h also recorded less percentage of contamination but explants response was very poor.

Response to shoot elongation and number of shoots

A total of 12 different media were employed fortified with various concentrations of TDZ and BAP alone and also in combination with NAA. A control MS media devoid of any PGRs was also used. As given in Table 2, among all these media treatments, 6

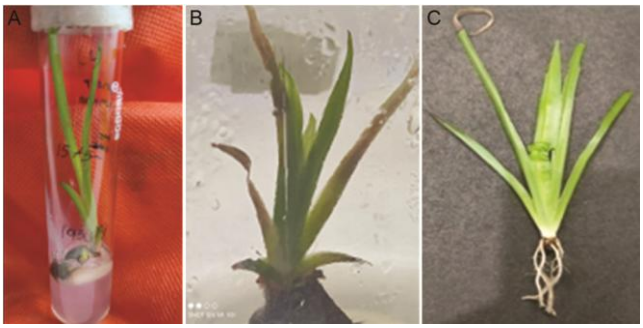

Fig. 1 — *In vitro* regeneration of *Acorus calamus* from rhizome buds. (A) Shooting; (B) Shoot multiplication; and (C) Rooting

Table 2 — Effect of various concentrations of plant growth regulators (PGRs) alone and in combination with NAA on multiple shoot induction of *Acorus calamus* using MS medium (Data recorded 45 days after inoculation)

PGRs (µM) BAP	TDZ	NAA	% of explants response (%)	No. of shoots per explants (Mean ± SE_M)	Shoot length (Mean ± SE_M)
-	5	-	80	3.25±0.25	2.68±0.24
-	10	-	60	5.00±0.58	3.60±0.21
-	15	-	60	7.67±0.33	3.67±0.33
-	5	5	-	-	-
-	10	5	-	-	-
-	15	5	-	-	-
5	-	-	-	-	-
10	-	-	-	-	-
15	-	-	-	-	-
5	-	5	80	2.75±0.48	4.75±0.26
10	-	5	80	3.50±0.29	6.88±0.43
15	-	5	60	3.00±0.58	5.60±0.32
Cont. (MS medium devoid of any PGRs)			60	0.60±0.24	0.66±0.27

[(-) No response. Values are Mean ± SE. Data is statistically significant analyzed at P <0. 01 using SPSS software]

Table 1 — Protocol for standardization of surface sterilization

SI No.	Bavistin treatment		Wash with tap water, rinse with distilled water	70% ethanol treatment (30 s)	Sterilizing agents containing Tween 20		Serial washing in sterile distilled water (3 times)	The surface sterilized explants were inoculated in control medium and kept under observation to check for contamination (if any) for up to 4 wk	% contamination and no. of days
	Conc.	Duration (min)			Sodium hypochlorite Conc.	Duration			
1					2%	10 min			100% after 3 days
2	0.2%	30			2%	15 min			100% after 5 days
3					2%	10 min			100% after 10 days
4	0.2%	60			2%	15 min			80% after 10 days
					Mercuric chloride				
5					0.1%	10 min			60% after 14 days
6*	0.2%	30			0.1%	15 min			20% after 28 days
7					0.1%	10 min			40% after 20 days
8	0.2%	60			0.1%	15 min			20% after 28 days

treatments viz., T1-T3 (MS + 5,10 and 15 µM TDZ, respectively), T10-T12 (MS + 5, 10 and 15 µM BAP + 5 µM NAA, respectively) showed positive explants response. The treatments T4-T6 (MS+5, 10 and 15 µM TDZ + 5 µM NAA, respectively), T7-T9 (MS + 5, 10 and 15 µM BAP), respectively did not show any kind of explants response. Maximum explants response was observed in treatments T1, T10 and T11 of 80% while the remaining treatments showed the explants response of 60%. Significant difference was observed in variable treatments for shoot elongation. The MS medium supplemented with BAP (10 µM) in combination with NAA (5 µM) recorded the longest shoot length with an average mean of 6.88 ± 0.43 cm. The MS medium supplemented with BAP (15 µM) in combination with NAA (5 µM) recorded the 2nd longest shoot length with average mean of 5.60 ± 0.32 cm. However, the shortest shoot length of 0.66±0.27 cm was recorded on control MS medium devoid of PGRs. Number of shoots per explant was recorded for these treatments 45 days after inoculation. The medium augmented with TDZ alone had shown better response as compared to the medium fortified with BAP in combination with 5 µM NAA. With increasing TDZ concentrations, the development of multiple shoots per explant was also increased. Treatment T3, which included 15 µM TDZ alone, produced the most shoots (7.67 ± 0.43), followed by treatment T2 (5 ± 0.58), which contained 10 µM TDZ. The control MS medium devoid of PGRs developed the least number of shoots (0.60 ± 0.24).

Response to root initiation and acclimatization

All *in vitro* raised shoots were randomly transferred to root induction medium containing IAA and IBA with concentration of 5 µM and 10 µM. Among all these treatments, MS media supplemented with 5 µM IAA along with control MS medium did not show any response to root initiation. The MS media supplemented with 10 µM IBA showed maximum response in respect of number of roots (6.50 ± 0.50) and root length (3.50 ± 0.50) as given in Table 3.

For acclimatization (Fig. 2), all rooted plants were further transferred to artificial soil mixture (peat: perlite: vermiculite) in plastic cups covered with polythene bag and was maintained at 25±2°C for two weeks. Hoagland's solution was provided in the soil mixture at every alternate day to promote proper growth and development. Of all the plants transferred to artificial soil, 57.14% plants were seen to survive and grow well. The remaining plants were eliminated due to sudden humidity shock as well as due to the effect of soil born pathogen attack after they were finally transferred to soil.

Genetic fidelity assessment

A total of 40 RAPD primers were screened for checking genetic fidelity among *in vitro* regenerated plants by comparing with mother plant of *A. calamus*. Out of total 40 RAPD primers, only 20 primers gave clear and reproducible bands (Table 4). The number of scorable bands (Fig. 3) for each RAPD primer varied from 1 (A-05) to 12 (OPL-3). The 20 primers produced 70 distinct and scorable bands with an average of 3.5 bands per primer. Each primer generated a unique set of amplification products

Table 3 — Effect of various concentrations of IAA and IBA on root induction (Data recorded 14 days after inoculation)

Media Composition	Conc. of PGRs (µM)	No. of roots (Mean±SE_M)	Root length (Mean±SE_M)
Control (MS medium devoid of PGRs)	-	-	-
MS+IAA	5	-	-
	10	2±0.58	1.33±0.33
MS+IBA	5	2.50±0.50	1.15±0.15
	10	6.50±0.50	3.50±0.50

[IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; and (-) No response]

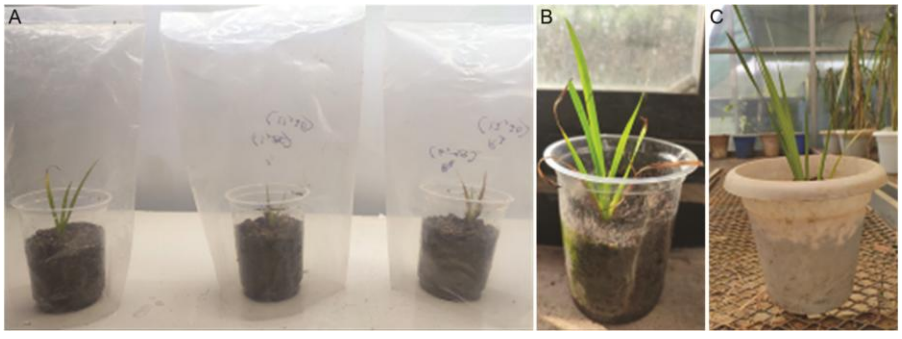

Fig. 2 — Acclimatization of i*n vitro* regenerated *Acorus calamus*. (A) Primary hardening; (B) Secondary hardening; and (C) Transfer to soil in pots

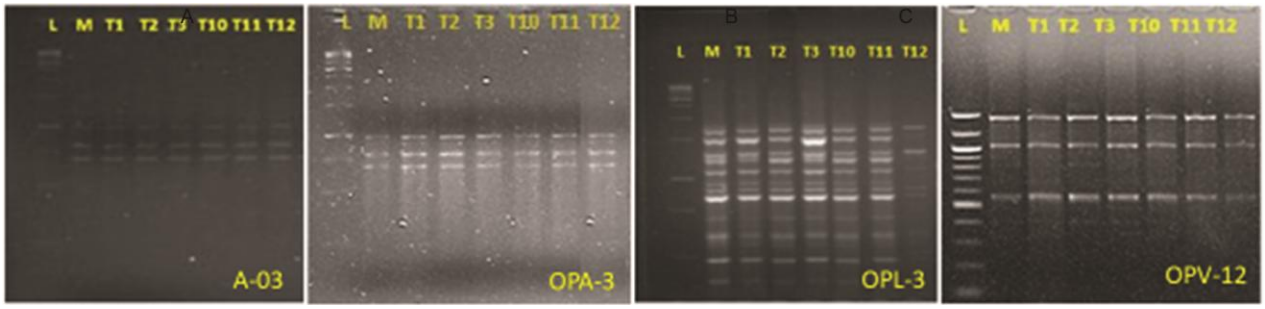

Fig. 3 — Genetic fidelity assessment of *in vitro* raised plants of *Acorus calamus* using RAPD markers. Gel picture of banding patterns of RAPD markers, namely A-03, OPA-3, OPL-3 and OPV-12. [L= 100 bp/1 kb ladder marker; M= mother plant; and T1 to T12= *In vitro* raised plants]

Table 4 —RAPD markers selected after scoring for amplification, total number of bands generated and size range

Marker	Sequence (5′→3′)	Melting temp. (°C)	Size range (bp)	Total no. of bands
OPV-06	ACGCCCAGGT	42.4	390-1700	6
OPV-12	ACCCCCCACT	40.1	550-2500	3
OPA18	AGGTGACCGT	36.2	600-2000	5
A-05	AGGGGTCTTG	32.6	800	1
OPA-3	AGTCAGCCAC	34.3	600-1000	3
OPO-10	TCAGAGCGCC	34	800-1200	2
OPA17	GACCGCTTGT	35.7	800	1
OPP-11	AACGCGTCGG	34	900	1
OPA-13	CAGCACCCAC	37.7	350-1700	6
OPA-19	CAAACGTCGG	34.2	650	1
A-11	CAATCGCCGT	36.7	650-1200	4
A-09	GGGTAACGCC	37.4	1000	1
A-03	AGTCAGCCAC	34.3	700-1000	3
A-10	GTGATCGCAG	33.1	700	1
OPN-12	CACAGACACC	34.2	800-900	2
A-02	TGCCGAGCTG	40.7	600-1200	4
OPL-3	CCAGCAGCTT	35.5	350-1700	12
OPD-08	GTGTGCCCCA	40.6	600-1100	5
OPE-20	AACGGTGACC	34.9	400-1300	8
OPN-06	CCACGGGAAG	31.6	1200	1

ranging in size from 350 bp (OPL-3) to 2500 bp (OPV-12). The allele size obtained from tissue culture raised sample was compared with the allele size obtained in mother plant which is found to be monomorphic to each other. These results have demonstrated that the genetic fidelity was maintained among all the *in vitro* regenerated plantlets.

Discussion

Acorus calamus is one of the endangered medicinal plant found predominantly in the Western Himalaya, Central India, North East India and Eastern Ghats regions of India. There are several initiatives being taken in order to conserve such endangered species. Tissue culture technology has potency to produce true to type plant and also support scaling up the production at large scale to meet the increasing demand from pharmaceutical/cosmetic industries[2]. In this study, we have made an attempt to develop an efficient *in vitro* regeneration protocol for *A. calamus* which produces true to type plants.

In vitro regeneration

For *in vitro* regeneration (Fig. 1), surface sterilization is an important key step in plant tissue culture for the successful establishment of contamination free cultures. Previous studies on *Acorus calamus* though used 0.1% mercuric chloride for sterilization of explants[12-14], they did not elaborate upon the problems associated with contaminating microbes during *in vitro* regeneration. Surface sterilization protocol was optimized in *Zingiber zerumbet* (L.)[15]. Similar finding was established in our study. For shoot induction and proliferation, TDZ and BAP, individually and also in combination with NAA was used to check their effect on explant establishment and shooting. Shoot induction and proliferation are usually done by supplementing the media with a cytokinin alone or in combination with an auxin. If a combination of a cytokinin and auxin is used, the auxin is usually added at a lower concentration as compared to the cytokinin. Successful *in vitro* clonal propagation of *A. calamus* in Murashige & Skoog medium supplemented with α-naphthalene acetic acid (0.5 mg/L) and 6-benzyl-aminopurine (2.0 mg/L) is reported. Also, BAP alone is reported to produce best shooting response[17]. Similarly, combination of Kin (2.0 mg/L) and NAA (0.5 mg/L) has been reported to be the best for *A. calamus* shoot proliferation[13].

In the present study, both BAP and TDZ provided in the medium had an impact on length of the shoot. However, the medium supplemented with BAP and NAA was found to be more effective than the medium containing TDZ. It has been observed that the right combination of BAP and NAA is required for optimal shoot elongation. The study on *A. calamus*[18] used

BAP and TDZ to check their effect on shoot formation. The results suggested TDZ used in low concentration showed highest number of shoot proliferation as compared to BAP. Contrary to the study, a high concentration of TDZ was used in the present study apart from BAP to check their effect on multiple shoot formation. Maximum number of shoots were observed in all medium containing TDZ (5, 10 and 15 µM). Finding of the present study is quite similar to the study on *Vitex trifolia*[19], where multiple shooting was observed on MS medium supplemented with TDZ in various concentrations (0.5, 1.0, 2.5, 5.0, 7.5 or 10.0 µM).

IAA and IBA are generally used as a rooting hormone in root induction medium. In previous studies, MS medium supplemented with 2.0 mg/L IBA and 1.0 mg/L IBA gave maximum root formation in *Acorus calamus*as reported by Sandhyarani *et al.*[13]. In our study, PGRs IAA and IBA were used in various concentrations (5 and 10 µM) for root induction. The maximum number of roots and root length was recorded on MS medium augmented with 10 µM IBA. IBA as a best PGR for root initiation was observed in studies on *A. calamu*[20-22].

Genetic fidelity assessment

One of the most essential goal in mass cultivation of endangered plants is to produce true-to-type plants. Genetic fidelity testing for *in vitro* regenerated plants becomes necessary as there is a strong tendency to develop somaclonal variation due to various factors including the type of PGRs used, the nature of explant, environmental parameters used and also the various chemicals used for treatments etc. For shoot proliferation, TDZ is usually used in low concentrations. A high TDZ concentration may have a negative impact on shoot growth. The current study used TDZ at concentrations of 5, 10, and 15 µM, which resulted in a higher number of shoots. As a result, assessing genetic stability is critical for maintaining the quality of tissue grown plants used in pharmacology. DNA based markers are effective in determining tissue culture-induced variations since these markers are not influenced by environmental factors[23]. RAPD and ISSR markers are widely used in the genetic homogeneity of many micropropagated plants among other molecular markers[24-28]. Genetic fidelity assessment of *in vitro* regenerated plants including *A. calamus* has been reported by different workers in several crops using RAPD[10,29,30] as well as ISSR markers[10,31,32]

In the present study, 40 RAPD primers were used to assess the genetic profile of tissue cultured plantlets developed under a variety of media treatments. All the *in vitro* samples evaluated have demonstrated genetic consistency with one another and with the mother plant. This suggests that the experimental methods used in the study can be used for the production of true-to-type plants of *A. calamus*. Similar findings have also been reported in *Zingiber officinale*[33,34]

Conclusion

The above results have shown an efficient *in vitro* regeneration protocol for the endangered medicinal plant, *Acorus calamus* developed using rhizome buds culture. The protocol developed in this study produced true-to-type plants and maintained genetic fidelity when compared with its mother plant. Thidiazuron (TDZ) was found as an alternative source to 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA) for multiple shooting. The protocol can be scaled up and recommended for future micropropagation programmes and further study can also be carried out to check their effect on the level of biochemical content.

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