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In vitro micro-propagation of *Typhonium flagelliforme* (Lodd.) Blume and its genetic fidelity using ISSR and RAPD markers

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Abstract
An araceous plant, *Typhonium flagelliforme* is in its extinct stage and require several ways to conserve its family. In the present study, an efficient *In vitro* protocol is established using apical meristem as an explant on basal Murashige and Skoog media supplemented with different concentrations of plant growth regulators. As an outcome, maximum number of multiple shoots (10.7±0.67) were generated on the media supplemented with 2.0 mg/L 6 - benzylaminopurine (BAP) and 0.5 mg/L α-napthalene acetic acid (NAA). The regenerated shoots were further sub-cultured on the fresh media containing 0.5mg/L BAP for elongation and proliferation. Media supplemented with 1.0 mg/L Indole-3-butyric acid (IBA) and 2% sucrose enhanced rooting in the proliferated shoots. In the process, an average of 90% plantlets survived, hardened and transferred to the field conditions. Further, genetic fidelity was exhibited among the tissue culture raised plants with the markers ISSR and RAPD. Thus, the modified tissue culture protocol and its standardization would be used for large-scale propagation and conservation of this medicinal plant.

Keywords: Proliferation, markers, hardening, genetic fidelity, elongation

Introduction
The family Araceae consists of 37 species and are distributed throughout the tropical and subtropical regions of the world, among which 16 species are present in India including *Typhonium flagelliforme* (Govaerts, 2002; Santapau and Henry, 1973) [4, 5]. A report by World Health Organization acclaims that the herbal plant *Typhonium flagelliforme* is used as a traditional medicine worldwide. Thus, artificial regeneration of herbal plants becomes important. Plant tissue culture technique provides a platform for large-scale production of plants.

Several media compositions with different concentrations of plant growth regulators have been reported from decades. For example, *T. divaricatum* is grown in *In vitro* in the combination of NAA, for shoot proliferation from tuber explants for mass production. Su *et al*. (2000) [18] reported Murashige and Skoog (MS) media supplemented with 0.3 mg/l BA and 0.5 mg/l Indole-3- butyric acid (IBA) as the most suitable for maximum shoot numbers, among (Murashige and Skoog, 1962), Nitsch and Nitsch (NN), Gamborg B5 (GB5) and White (W) medium. Koh and Chan (2003) reported usage of fermentor with liquid modified MS media supplemented with 2.46 µmol/l IBA, 1.33 µmol/l BA and 4% sucrose. A survey by Perry and Metzger (1980) [20] reported that the people in the country Philippines use flowers of *T. flagelliforme* to arrest bleeding and injury. A report by Su *et al*., (2000) [18] showed similar results with the usage of rhizomes extract of *T. divaricatum* to treat coughs and pulmonary illness.

It is commonly known as rodent tuber used in alternative cancer therapy including leukemia, breast, lung, rectum, liver, prostate, pancreas and cervical cancer (Mankaran *et al*., 2013; Mohan, 2008; Neoh, 1992) [16, 1, 8]. The problem associated with *T. flagelliforme* is sensitivity to natural growth conditions like moist and under shady area, which provided the biggest need to produce the plant *In vitro* in massive numbers. The usage of flowers as an anticoagulant by tribal people (Nobakht, 2009) [3] has led to poor seed germination percentage, resulting in extinct stage of the species. Hence, the present study is an attempt to propagate *T. flagelliforme* at large scale by using *In vitro* technique, it's genetic fidelity and save the plants from extinction.

Materials and Methods

Explants source and surface sterilization

The mature infructescence of *Typhonium flagelliforme* was collected from Experimental Garden of the Science Foundation for Tribal and Rural Resource Development, Bhubaneswar, Odisha.

The seeds were washed with running tap water with 2-3 drops of Tween-20 to remove the dirt adhering to the surface for 5 min and subsequently surface sterilized with 0.1% (w/v) mercuric chloride for 10 min for disinfection. Further, it washed with 4-5 times in sterile distilled water aseptically. Seeds were induced to germinate on basal MS medium containing (3% w/v) sucrose, 6 g/L of agar, the pH of the culture medium was adjusted to 5.8, autoclaved at 121 ^{0}C, 15 lb pressure for 15 min. The cultures were maintained at 25±2 ^{0}C with light intensity of 3000 lux at 16 h photoperiod. The apical meristem was used as explant source for induction of multiple shoots.

Induction of multiple shoot

The elongated shoots were transferred to MS medium supplemented with different concentrations and combinations of auxins (NAA, IBA, IAA) (0-2 mg/l) and cytokinins (BAP, Kinetin) with 0-2 mg/l for multiple shoot induction. The proliferated shoots were sub-cultured during second week interval that enhanced the rate of shoot multiplication. The proliferating shoots were excised and transferred to MS medium containing different concentrations of cytokinins (0-2.0 mg/L BAP) alone or in combination with (0-1.0 mg/l NAA) for shooting.

Induction of rooting and field acclimatization

The regenerated plantlets of 2 - 3 cm, were transferred to MS medium containing different concentrations of auxins (1.0 – 1.5 mg/l IAA and IBA) for induction of roots. The plantlets with roots were removed from culture tubes, washed thoroughly with distilled water. The plants were pre-hardened in distilled water at 25±2 ^{0}C for one week. The pre-hardened plantlets were transferred to shade net house in sterile soil media containing vermiculite and cocopit mixture in the ratio of 1:1 to get acclimatized and field establishment.

Assessment of genetic fidelity

Young, tender and fresh leaves of *Typhonium flagelliforme* were collected from the mother plant and used for extraction of DNA by modified Doyle and Doyle, (1987) method. DNA was checked on 0.8% agarose gel to estimate the quantity and purity against uncut lambda DNA known molecular weight. The tissue culture raised plantlets were compared with the mother plant for genetic fidelity using 10 RAPD primers and 7 ISSR primers (Table 1). A reaction mixture for PCR was prepared to a total volume of 25 µl comprising of 20 ng of template DNA, 2.5 µl of 10X Taq buffer, 0.25 units of Taq DNA polymerase (M/S Bangalore Genie, Bangalore, India), 0.25 µl of each dNTPs, 2.0 mM $MgCl_2$ and 20 pmol of primer. The amplification was carried out using Thermal Cycler (Bio-Rad, USA). Initial denaturation was maintained at 94 ^{0}C for 5 min, denaturation step for 1 min each at 94 ^{0}C, primer annealing for 2 min at 37 ^{0}C for RAPD primers and 41 ^{0}C for ISSR primers (45 cycles), extension of 2 min and final extension for 10 min at 72 ^{0}C. After the reaction, the PCR product was loaded on to 1.5% agarose using 1X TBE buffer containing 5µl/100ml of ethidium bromide solution and visualized under gel documentation system (UVITECH, Cambridge, UK).

Data analysis

The mean of all data recorded was calculated using Microsoft Excel 2010. The experiments were carried in completely randomized block design. The treatments of plant growth hormone were statistically analyzed for which the significance level was $P<0.05$ using Duncan's multiple range test.

Result and Discussions

Among all the growth regulator used, maximum shoot multiplication (10.7±0.67 shoots) per culture was achieved on MS medium containing 2.0 mg/L BAP and 0.5 mg/L NAA (Fig 1a). The multiple shoots were sub-cultured in 15 days interval for 4 weeks. Kinetin and/or in combination with NAA promoted multiplication of shoots but the frequency was very low. The developing buds were maintained in 0.5 mg/l BAP rich medium for elongation of shoots. The elongated shoots were transferred to on MS media with IAA or IBA and 2% (w/v) sucrose and observed dense rooting (Table 2). A high positive response was observed in 6th week of culture, while significant differences were observed among different combinations of growth regulators in terms of the number of shoots formed. On the contrary, few reports show significant response of plants after 10th week (Nabakht *et al*., 2009) of sub-culturing.

The percentage of rooting (Fig 1b and 1c) was optimum on the medium supplemented with 1.0 mg/l IBA without any callus formation at basal end. The rooted plantlets were transferred to sterile water for 15 days (Fig 1d) and acclimatized under greenhouse conditions (Fig 1e). The plantlets survived (90%) when they were maintained in vermiculite and cocopit mixture for three. The acclimatized plantlets were transferred to soil under natural field conditions. Healthy growth was observed throughout without any morphological variations among the plantlets.

The genetic fidelity analysis was conducted to check the genetic variations among the tissue culture raised plantlets in comparison with mother plant by using RAPD and ISSR markers. The leaf samples were collected randomly from 12 number of tissue culture raised plantlets grown in the greenhouse. The test was carried out using ten RAPD and seven ISSR primers (Table 1) to study the genetic fidelity (Rout *et al*., 2012) [14]. Out of tested primers 7 RAPD and 5 ISSR primers showed positive response and detect distinct and clearly resolved amplified products. Other primers showed undesirable amplified products and very confusing pattern of amplification that was difficult to analyze. Both RAPD and ISSR primers amplified a total number of 149 bands. In the present study, all primers amplified with the size between 120 bp to 1.3 Kbp. Amplification profile of tissue culture raised plantlets along with their donor plant has been shown by RAPD primers OPD-11 and OPM-20 (Figure 2a and 2b) and ISSR primers USB-810 and USB-841 (Figure 2c and 2d). The banding pattern of micro-propagated plantlets along with their donor plant was found to be monomorphic. In this direction, somaclonal variations was evaluated and reported by few scientists (Cassells and Curry, 2001; Palombi and Damiano, 2002; Bindiya and Kanwar, 2003; Gaj, 2004; Naing *et al*., 2013) [12, 21, 22, 11, 13]. The polymorphic pattern of tissue culture raised plants with mentioned primers has been recorded and represented in Table 3. No polymorphism was detected. Absence of polymorphism in RAPD and ISSR profile of micro-propagated plantlets suggests a high level of genetic fidelity and also indicates that this clonal propagation is efficient enough to maintain genetic stability of *T.*

flagelliforme. The clonal fidelity study concluded that all the regenerated plants were identical. The present study revealed that no variability present in the micro-propagated plants of *T. flagelliforme*. All the primers revealed a monomorphic banding pattern, which demonstrates genetic uniformity in the tested samples in concordance with their morphological appearance.

Thus, in the present study, the absence of somaclonal variations and confirmation of genetic uniformity among micro-propagated plants of *T. flagelliforme* has been recorded by the usage of two different of markers *viz.*, RAPD and ISSR. Hence, such markers are used to explore the genetic integrity of tissue culture raised and hardened plants for field cultivation to further enhance yield and produce disease-free plants.

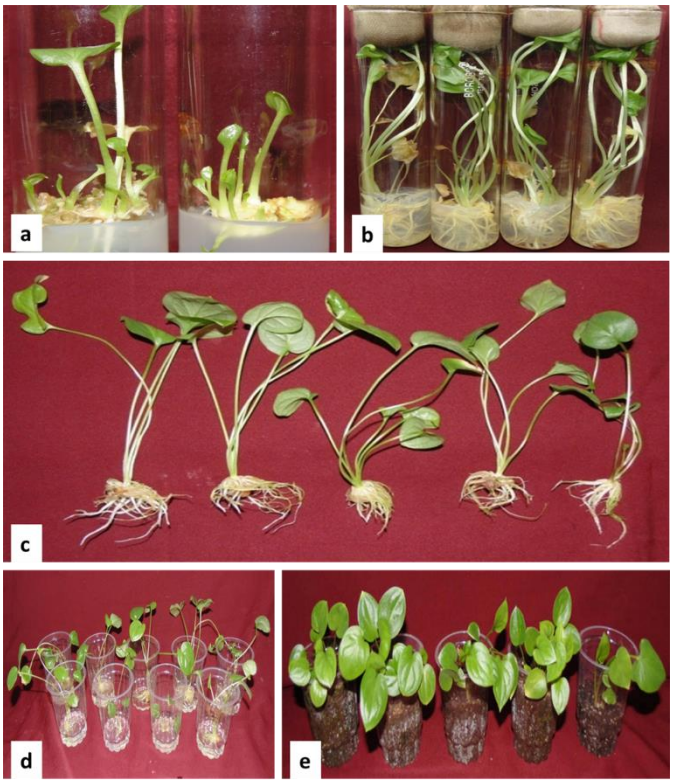

Fig 1: (a) Multiple shoot induction in *T flagelliforme* cultured on MS medium supplemented with 2.0 mg/1 BAP and 0.5 mg/L NAA. (b) Elongation of multiple shoots cultured on MS medium sub-cultured with 0.5 mg/L for rooting. (c) Root induction from micro-shoots of *T. flagelliforme* cultured on MS medium supplemented with 1.0 mg/L IBA. (d) Pre-hardening of tissue cultured *T flagelliforme* in distilled water. (e) Acclimatization of micro-propagated plants in vermiculite and cocopit mixture.

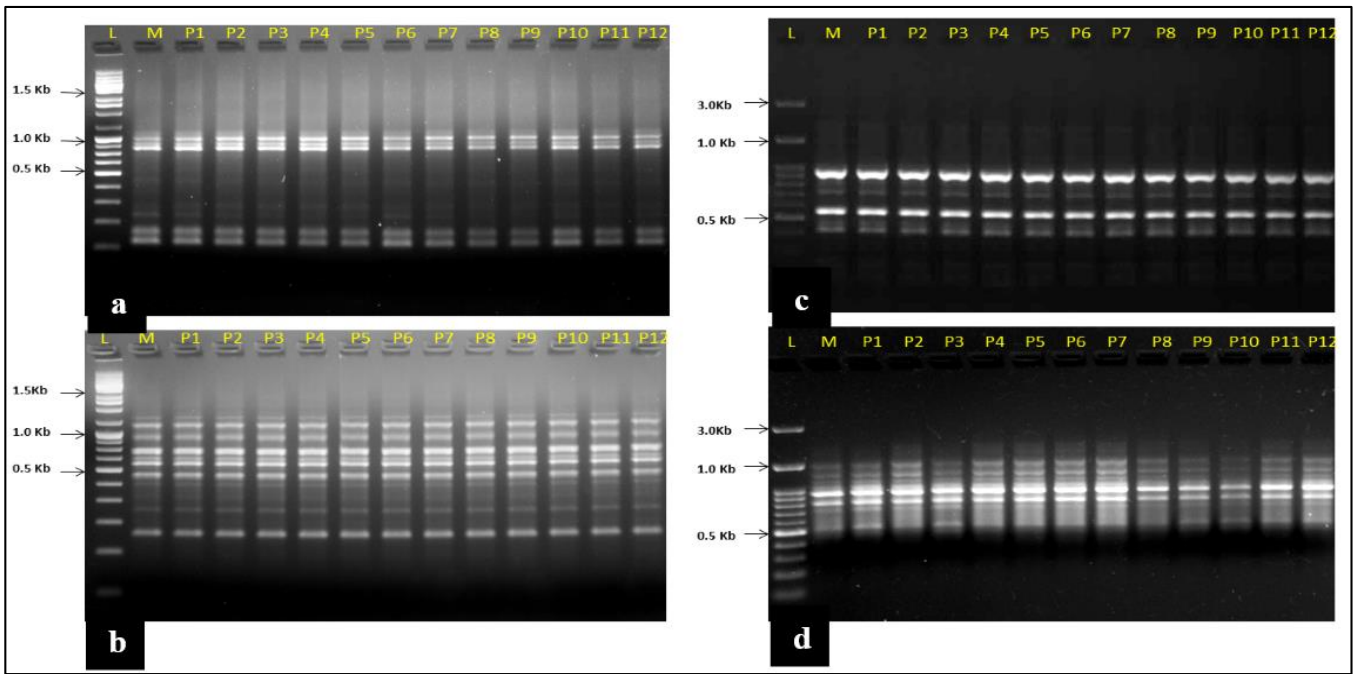

Fig 2: DNA profiling of *T. flagelliforme* by using RAPD primers (a) OPD-11, (b) OPM-20 and ISSR primers (c) USB-810, (d) USB-841 (M:Mother Plant, P1-P12: Tissue culture plantlets). The banding pattern of micro-propagated plantlets along with their donor plant was found to be monomorphic. Absence of polymorphism in RAPD and ISSR profile of micro-propagated plantlets suggests a high level of genetic fidelity and also indicates that this clonal propagation is efficient enough to maintain genetic stability of *T. flagelliforme.*

Table 1: ISSR and RAPD primers used for genetic uniformity of micropropagated and the mother plants of *Typhonium flagelliforme*

ISSR Primers	Sequence 5'-3'	Tm (^{0}C)	Total no. of bands amplified	Band range (bp)
USB 807	AGAGAGAGAGAGAGAGT	42.5	5	300-1200
USB 810	GAGAGAGAGAGAGAGAT	42.9	4	400-1000
USB 835	AGAGAGAGAGAGAGAGYC	42.7	3	450-1300
USB 838	AGAGAGAGAGAGAGAGC	42.9	5	200-1250
USB 841	GAGAGAGAGAGAGAGAYC	42.7	6	500-1500
RAPD Primers	Sequence 5'-3'	Tm (^{0}C)	Total no. of bands amplified	Band range (bp)
OPA 4	AATCGGGCTG	37.0	5	200-1000
OPC 20	ACTTCGCCAC	37.0	8	180-950
OPD 11	AGCGCCATTG	37.0	7	120-1200
OPM 20	AGGTCTTGGG	37.0	11	150-1250
OPK 18	CCTAGTCGAG	37.0	7	250-1300
OPN 7	CAGCCCAGAG	37.0	5	200-1200
OPN 11	TCGCCGCAAA	37.0	6	180-1250

Table 2: Effect of different concentrations of BAP and NAA on multiple shoot induction in MS medium after 4 weeks of culture

Basal Medium	Plant Growth Regulator (mg/l)		Number of shoots per culture (Mean±SE)*	Plant Growth Regulator (mg/l)		Number of shoots per culture (Mean±SE)*
	BAP	NAA		Kn	NAA	
MS+3% of sucrose	0.5	0.0	2.3±0.33	0.5	0.0	2.0±0.00
	0.5	0.5	3.3±0.33	0.5	0.5	2.3±0.33
	0.5	1.0	2.7±0.33	0.5	1.0	2.7±0.33
	1.0	0.0	4.0±0.58	1.0	0.0	2.7±033
	1.0	0.5	5.7±0.67	1.0	0.5	4.0±0.57
	1.0	1.0	5.0±0.58	1.0	1.0	3.7±0.88
	1.5	0.0	3.7±0.33	1.5	0.0	3.3±0.88
	1.5	0.5	6.7±0.67	1.5	0.5	4.3±0.33
	1.5	1.0	4.3±0.33	1.5	1.0	2.7±0.67
	2.0	0.0	6.7±0.67	2.0	0.0	4.0±0.58
	2.0	0.5	10.7±0.67	2.0	0.5	5.7±0.33
	2.0	1.0	10.0±0.58	2.0	1.0	6.7±0.67
	2.5	0.0	3.3±0.33	2.5	0.0	3.0±0.58
	2.5	0.5	7.7±0.33	2.5	0.5	2.3±0.33
	2.5	1.0	4.3±0.33	2.5	1.0	2.7±0.33
	3.0	0.0	6.3±0.67	3.0	0.0	3.7±0.33
	3.0	0.5	3.3±0.88	3.0	0.5	4.3±0.33
	3.0	1.0	3.0±0.58	3.0	1.0	4.0±0.57

*10 replicates for each treatment, repeated thrice

Table 3: Effect of different concentration of IAA and IBA on root induction of micro-shoots cultured on MS medium with 2% sucrose.

Medium	Plant Growth Regulator (mg/L)		Number of roots per culture (Mean±SE)*
	IAA	IBA	
MS + 2% of sucrose	0.5	0.0	13.0±1.73
	1.0	0.0	16.7±1.53
	1.5	0.0	15.0±2.64
	0.0	0.5	16.3±1.52
	0.0	1.0	21.7±1.52
	0.0	1.5	17.7±0.88

*10 replicates for each treatment, repeated thrice

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