



RESEARCH ARTICLE

In vitro propagation and somaclonal variation study of *Phlogacanthus thyriformis* Nees an ethnic medicinal plant

Tikendrajit Baro & Sandeep Das*

Department of Biotechnology, Bodoland University, Kokrajhar-783 370, India

*Email: sandeep_dna2003@yahoo.co.in



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Abstract

Bodoland Territorial Region is very rich in natural bioresources, and the Bodo tribes of the region use numerous ethnic medicinal plants to treat different kinds of diseases. *Phlogacanthus thyriformis* Nees is an important medicinal shrub species used in the region to treat different kinds of diseases. The objectives of the present study were to develop an efficient *in vitro* mass propagation technique of the species using nodal explant and study genetic stability in the genome of *in vitro* propagated plantlets by different RAPD markers. Effective explant surface sterilisation resulted at 2 min of treatment with 0.1% mercuric chloride. Explant responses were found most effective in the full strength MS + 1mg/l BAP (6- benzyl amino purine), and explant highest shoot proliferation multiplication and rooting were found in the media MS + 1 mg/l BAP + 0.250 mg/l NAA. *Ex vitro* rooting of micro propagated plants was most effective when the explants were dipped in 1 mg/l IBA for an hr. RAPD assays were conducted using eight sets of random primers (OPC02, OPC05, OPC07, OPC08, OPC09, OPX06, DK2 and OPA01). All the primers except OPC07 and OPA01 formed monomorphic DNA bands in gel electrophoresis and polymorphism was detected by OPC07 and OPA01 primer.

Keywords

explants culture, molecular marker, MS media, plant regeneration, tissue culture, RAPD

Introduction

The global population mostly relies on ethnic medicine (about 80% of the total population) for primary healthcare, most of which is taken from plant extracts (1). Traditional medicines play a crucial role in the health care and treatment of diseases in most developing countries (2). *Phlogacanthus thyriformis* Nees (Barsukha) is a shrub species of the Acanthaceae family. It is 3–7 feet in height. It is used as folk medicine in Assam and its distribution ranges from Bhutan to the North-Eastern states of India in the Himalayas and Indo-China, southern China and Sulawesi (3). The shrub is used to treat allergies, cough, cold, chronic bronchitis, rheumatoid arthritis, asthma etc. also the leaf extract is used as anti-bacterial and also effective in inhibiting the growth of HeLa cells (3). From the survey, it was found that the flowers of the plant are also consumed by local peoples considering medicinal importances like- anti gastric, anti- diabetes, anti-cough to treat cold fever.

Because of the presence of bioactive potential in medicinal plants, their demands are being higher day after day. Further, due to deforestation,

the availability of important plants is declining from the natural habitat. Plant tissue culture is an important technique for mass propagation and production of uniform, disease-free and good quality plants, genome transformation and production of plant-derived metabolites and important compounds (4, 5). Plant growth regulators (PGR) are synthetic or natural compounds that are key factors in developing plant tissues or organs in the micro-propagation process. Proper combination and ratio of the PGRs are required for rapid growth and development of the tissues or organs in tissue culture process.

The plant species produced by the tissue culture process may undergo genetic variation or phenotypic variation. The studies reported that all the *in vitro* propagated plantlets were not always clonal copies of the mother plant (6, 7). In the entire process, the explants receive several treatments, including the application of plant growth regulators, *in vitro* conditions and stress factors that induce genetic alterations resulting in genetic instability in the cultured cells, which is commonly known as somaclonal variation (8-12). The use of molecular markers can detect the genetic variation in the micro propagated plants, i.e., RAPD (Random Amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSCP (single-stranded conformational polymorphism), etc. From the therapeutic application point of view, somaclonal variation may greatly impact secondary metabolites production capabilities of the plants, somaclonal variations in the genome during tissue culture of *P. thyriformis* also may have an impact on secondary metabolite production capability of the plant parts (flowers, leaves, roots).

RAPD technique is often used to analyse somaclonal variation because it can analyse variation at many loci requiring only small quantities of DNA without prior knowledge of DNA sequence or involvement of radioactivity (13). Somaclonal variation has various applications in agriculture in crop improvement like resistance to disease, pest resistance, stress resistance etc. *In vitro* shoot proliferation and plant regeneration technique for *Phlogacanthus thyriflorus* Nees using shoot tip explant (14) had been previously developed. As per one study, hairy root cultures of *P. thyriflorus* were also done (15).

There are no reports yet been published on genetic stability (somaclonal variation) study of micro propagated *P. thyriformis*, and somaclonal variation in the genomes of the plant may have great impact on secondary metabolite production capability of the plant. Therefore, the present study aimed to develop an efficient *in vitro* mass propagation technique of *P. thyriformis* and to study genetic variation (somaclonal variation occurred during tissue culture) in the micro-propagated plantlets.

Materials and Methods

Plant material collection and explant preparation

The disease-free and healthy apical nodal explants of the *P. thyriformis* were collected from Baksa District, Assam

(26.64°N, 91.57°E) and the voucher specimen is deposited at Bodoland University Botanical Herbarium. The species was then identified at the Department of Botany, Bodoland University, Assam. The collected explants were washed in running water. The leaves and sub-branches were removed. Then, the nodal explants were trimmed to 3-4 cm length containing 3-4 nodes. Then the explants were washed in tween 20 for 15 min, followed by 70% alcohol for 30 seconds. Then the explants were introduced to 0.5% Bavistin for 60 min and shook continuously after 5 min. For surface sterilisation, 0.1% mercuric chloride is preferred over sodium hypochlorite for its better disinfecting characteristics. The explants were introduced into mercuric chloride for 1-5 min. Then the explants were washed in sterile double distilled water three times for 1 min, 3 min and 5 min.

In vitro explant culture

The explants after surface sterilisation were trimmed with the help of a scalpel to 1 cm length containing nodes inside the laminar airflow. These explants were inoculated into the phyta jars containing different strengths of MS media with different ratios of plant growth regulators using forceps. Then, the explants were incubated at 28 °C with 14-16 hrs of photoperiod and 75 ± 80% relative humidity for 21 days. The explants were checked daily for tissue responses and contaminations. The contaminated jars were removed from the room immediately. After 4-6 weeks of inoculation, the explants were transferred to fresh media. After successful development of shoots *in vitro* and multiplications, the shoots were taken out of the jars. The stacked agar was removed, the multiple shoots were separated and excess leaf and sub-branches were excised. After this, all the separated shoots were again inoculated into a fresh medium.

Rooting and acclimatisation

For acclimatization, the uncapped jars containing plantlets were taken out from the growth room and the cap of jars was loosened at the temperature of 25 °C±2 °C, 12 hrs of photoperiod and 75% ± 5% of relative humidity for 5 days. After 5 days the caps were removed and kept in the poly house for 5 days. Then, the explants were taken out from the culture jars, agars were removed from the plantlets, washed and grown in pots containing coco peat and soil mixture. Waters and fertilisers were sprayed daily and kept in the poly house for 6 days at the same parameters. After 6 days the temperature is increased to 28 °C ± 2 °C and the relative humidity of the poly house is decreased to 60%. Finally, after another 5 days, the plantlets were placed in open space for another 7 days outside the poly house and water was applied daily. These plantlets were then ready for growing in the open field condition.

For *ex vitro* rooting, the *in vitro* developed shoots were taken out, and the excess agar was removed in running water and separated into single shoots. Then, the shoots were dipped into the running water containing 1 mg/l to 5 mg/l IBA (indole-3 butyric acid) for 60-90 min and the shoots were grown in the pots containing clay and vermicompost fertiliser in the shed at 28-30 °C for 12 hrs of

photoperiod for 7-10 days. After 21 days, all the plantlets were checked for rooting *ex vitro* and recorded. All the experiments for *in vitro* proliferation and rooting were repeated three times. For each experiment, 5 explants were cultured in the jars. Hence, a total of 15 cultures were done per basal media. For *ex vitro* rooting, the experiments were repeated two times, and a total of 10 explants were inoculated for *ex vitro* rooting.

Genomic DNA isolation and RAPD assay

The genomic DNA from the wild plant and *in vitro* propagated (hardened and non-hardened) plantlets were extracted using the DNeasy Plant Mini kit (Qiagen) and confirmed in 0.8% ultrapure agarose gel electrophoresis 0.5X TBE buffer at a constant voltage of 80V for 30 min.

For the RAPD assay, eight primers (Table 1) were synthesised from Eurofins genomics. The PCR mix for RAPD contained 7.5 µl of water, 12.5 µl of PCR master mix (Genei), 3 µl of 50 ng/µl template and 2 µl random primer (10 pm/µl). The PCR program carries initial denaturation at 94 °C for 1 min, denaturation at 94 °C for 30 sec, annealing at 35 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min and hold at 4 °C.

Table 1. List of RAPD random primer used to study somaclonal variation in *P. thyriformis*

Sl. No.	Primer name	Sequence (5'-3')
1	OPC02	GTGAGGCGTC
2	OPC05	GATGACCGCC
3	OPC07	GTCCCACGA
4	OPC08	TGGACCGGTG
5	OPC09	CTCACCGTCC
6	OPA01	CAGGCCCTTC
7	OPX06	ACGCCAGAGG
8	DK02	CGACCGCAGT

The PCR products were resolved by gel electrophoresis using 1.5% agarose, 0.5X TBE buffer at a constant voltage of 80V for 45 min and 2 µl of EtBr (ethidium bromide) were added for staining the DNA and the bands were visualised at GelDoc E- Gel Imager. The samples were analysed based on the presence and absence of similar polymorphic DNA bands.

Results and Discussion

Surface sterilisation

Successful explant surface sterilisation of *P. thyriformis*

resulted in the 0.1 % mercuric chloride treatment. 100 % of explants got contaminated when no reagents were used for explant sterilisation. Treatment with 0.1% mercuric chloride for 1 min showed a minimum successful surface sterilisation rate showing a 46% of explant survival rate after 21 days of culture initiation. Treatment with 0.1 % mercuric chloride for 2 min to 3 min revealed the maximum explant survival rate (83 %). The contamination rate of the explant was higher at 2 min of mercuric chloride treatment (17 % contamination), but the contamination rate of explants was minimum with 0.1 % mercuric chloride treatment for 3 min, i.e. only 8.5 % of cultured explants got contaminated but 8.5 % explants damaged. Interestingly, 4 min treatment with 0.1% mercuric chloride showed 0 % contaminations in the explants, but the only survival rate of the explants was only 50 % (Table 2). It was previously reported that treatment of shoot tip and nodal explant of *P. thyriformis* with 0.1 % mercuric chloride for 5 min showed satisfactory results in surface sterilization (14). Endophytes may be present in the collected explants or they may be entering in the culture media due to improper or aseptic handling, laboratory condition. Microbes are very common problem in the tissue culture technique which is effecting the growth of the tissues (16). These endophytes are affecting adversely the tissue cultured explants and these microbes competes for the nutrients with the explant materials, presence of these microbes in the culture may lead to explant mortality, tissue necrosis, reduce growth and development of the tissues (17).

Explant initiation, shoot multiplication and rooting on different concentrations of growth regulators (BAP, IAA and NAA)

It was observed that the explants of *P. thyriformis* responded in the MS media with or without any plant growth regulators (PGR), though shoot proliferation and multiplication resulted best with the BAP in combination with NAA (Fig. 1. A). Maximum shoot proliferation and multiplication were observed in BM3 media consisting of 1 mg/l BAP (6-benzyl amino purine) and 0.250 mg/l NAA (1-naphthalene acetic acid) (Fig. 1.B & C). The highest rooting of explants resulted in the BM7 media consisting of 1mg/l IAA. Moderate shoot response and multiplication were observed in the media BM2, BM4 and BM5. Minimum average shoot multiplication per explant was observed in the control media where no PGRs were added (Table 3). Previously it was reported that shoot induction of *P. thyriformis* found best on 1mg/l BAP and 0.5 mg/l NAA in the experiment. Rooting was best on half-strength MS with 0.5 mg/l IBA and 0.5 mg/l NAA (14).

Table 2. Results of surface sterilization of *P. thyriformis* explants using mercuric chloride

Sl. No.	Reagent	Total No. of explants cultured	Treatment (in min)	Explant contamination rate after 3 weeks	Explant survival rate after 3 weeks
1	Control	0	12	100%	0%
2	Mercuric chloride (0.1%)	1	12	53.33%	46%
3	Mercuric chloride (0.1%)	2	12	17%	83%
4	Mercuric chloride (0.1%)	3	12	8%	83%
5	Mercuric chloride (0.1%)	4	12	8.33%	75%
6	Mercuric chloride (0.1%)	5	12	0%	50%



Fig. 1. *In vitro* propagation of *P. thyrsoformis*, A; explant initiation at BM3 media after 9 days of explant culture, B & C; shoot multiplication and root formation at BM3 media after 42 days of explant culture, D; Hardening.

Ex vitro rooting and acclimatisation

After complete development of the plantlets *in vitro*, these plantlets need to be transferred in to the wild. In this shifting process from the culture room to the different environmental condition, the plantlets may damage or die due to the adverse change in the environmental condition unless proper care or appropriate process were taken (18). In the *ex vitro* rooting experiment, all the explants of *P. thyrsoformis* were formed rooting. The explants formed rooting when grown in the soil with a vermicompost mixture at room temperature (25 °C) after treated in 1 mg/l to 5 mg/l IBA for 1-2 hrs. The explants formed the highest average of 32 roots per explants of *P. thyrsoformis*, and the lowest average roots per explants were observed in the 4 mg/l IBA concentration (Table 4). The shoots were grown in pots containing vermicompost and soil mixture. All the explants survived in environmental conditions (Fig. 1.D).

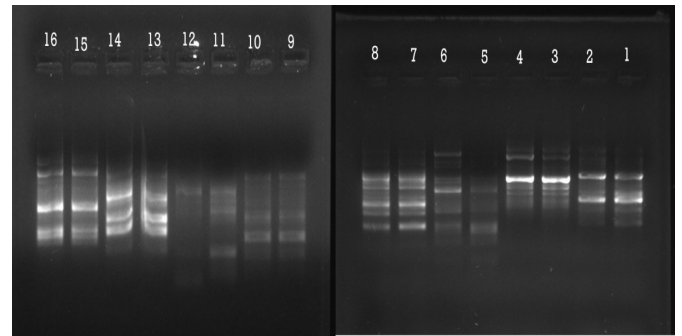


Fig. 2. Polymorphic bands formed by different random primers. Lane: 1, 3, 5, 7, 9, 11, 13, 15; wild plant. Lane 2, 4, 6, 8, 10, 12, 14, 16: micro-propagated plant. Random primers: Lane 1 & 2 (OPC 02), Lane 3 & 4, Lane(OPC 05), Lane 5 & 6 (OPC 07), Lane 7 & 8 (OPC 08), Lane 9 & 10 (OPC 09), Lane 11 & 12 (OPC 01).

RAPD Assay

For accuracy in RAPD assay result, high quality and pure genetic material, free from contaminations were isolated from the leaves of *P. thyrsoformis* using DNeasy Plant Mini kit (Qiagen). Random primers (Table 1) OPA1, OPC02, OPC05, OPC07, OPC08, OPC09, OPX06 and DK02 formed a total of 114 DNA bands in 1.5% ultrapure agarose gel in both wild and micro-propagated plants (Fig. 2). OPC07 and OPA01 formed polymorphic DNA bands (variation in DNA bands in wild and micro propagated plants) and the percentage variations were 28%. From the gel electrophoresis result, OPA01 formed a total of 6 polymorphic bands in the wild plant while only four polymorphic DNA bands in the micro propagated plant. In the case of OPC07 primer, ten polymorphic DNA bands were observed in the wild plant, but 12 polymorphic bands were observed in the micro propagated plant. The rest of the six primers formed 82 monomorphic bands, which have no variations or can be said as common in the parental genotypes. The highest polymorphic DNA bands were observed by OP07 primer in the micro propagated plant genome and the lowest polymorphic DNA bands were formed by OPA01 primer in micro propagated plant genome (Table 5). This technique has been widely used for its simplicity and rapidity also this technique does not require any genetic information of the target species. The RAPD fingerprinting results are consistent in genetic source at any age of species (19, 20). *In vitro* mutation was induced in the genomes of sugarcane, banana and potato for improving specific characteristics

Table 3. Response of *P. thyriformis* explants at different PGR ratios, Mean \pm SE of 5 replica

Sl. No.	Basal media	Growth regulators (mg/l)			No. of explant culture initiation	Rate (%) of explant producing shoots	Mean No. of shoots per explant	Mean shoot length (cm)	Mean root numbers
		BAP	NAA	IAA					
1	Control	0.0	0.0	0.0	5	100%	3 \pm 0.63	5.4 \pm 1.01	5.2 \pm 1.16
2	BM1	1.0	0.0	0.0	5	100%	6.8 \pm 1.32	9.2 \pm 0.74	4.2 \pm 0.74
3	BM2	1.0	0.2	0.0	5	100%	8.8 \pm 0.74	11.8 \pm 0.97	9.2 \pm 0.74
4	BM3	1.0	0.250	0.0	5	100%	13 \pm 0.63	14.8 \pm 1.72	15 \pm 1.41
5	BM4	1.0	0.5	0.0	5	100%	9 \pm 0.63	15 \pm 0.63	15.6 \pm 2.05
6	BM5	2.0	0.0	0.0	5	100%	8.6 \pm 1.74	11 \pm 1.78	12 \pm 1.67
7	BM7	5.0	0.0	0.0	5	100%	4.4 \pm 1.49	6 \pm 1.41	7 \pm 2.28
8	BM7	0.0	0.0	1.0	5	100%	4 \pm 0.89	8 \pm 1.09	18.2 \pm 2.78

Table 4. Effect of IBA concentrations on *in vitro* cultured shoots *ex vitro*

Sl. No.	IBA (mg/l)	Rooting	Average roots per explant
1	1.0	100%	17
2	2.0	100%	31
3	3.0	100%	22
4	4.0	100%	11
5	5.0	100%	14

Table 5. Table showing different polymorphic DNA bands formed by different random primers in micro-propagated and wild plant of *P. thyriformis*

Sl. No.	Primer	Number of Polymorphic DNA bands of wild plant produced by random primer	Number of polymorphic DNA bands of micro-propagated plants produced by random primers
1	OPC02	7	7
2	OPC05	8	8
3	OPC07	10	12
4	OPC08	9	9
5	OPC09	5	5
6	OPA01	6	4
7	OPX06	5	5
8	DK02	7	7

like disease resistance and other important characteristics to upgrade commercial values (21). Somaclonal variation may also help in crop improvement which may help the plants to obtain some properties like biotic or abiotic stress, drought, high salinity, high or low soil pH and disease tolerance (22).

Conclusion

In the micropropagation standardization protocol, checking of contamination is very important step because various contaminants like bacteria and pathogens may be present in the explants, therefore proper surface sterilisation of the collected explants need to be done in sterile condition (21). The micropropagation process surface sterilization step is found very challenging due to contaminants in the explants, therefore this step needs to be done very carefully. 0.1% mercuric chloride treatment found effective for surface sterilization process. Secondly, the explants of *P. Thyriformis* responded differently in all PGR concentrations. From the RAPD assay it was concluded that the genomes of micropropagated *P. thyriformis*

plants undergone somaclonal variation and were detected using RAPD assay. In the RAPD assay, performed using eight different random primers, epigenetic changes were observed (somaclonal variation) in the micropropagated plants by OPC07 and OPA01 primer. RAPD assay was proved to be successful to detect genetic changes in micro-propagated *P. thyriformis*. Therefore, this technique can be used for the detection of off-type plants after micro-propagation. After detection of off-type micro-propagated plants, potential comparative and evaluative studies can be designed targeting phyto-constituents and secondary metabolite production in both plants.

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Authors contributions

TB carried out the field surveys, experiments and manuscript writing. SD participated in experimental design, coordination and supervision.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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