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Determination of genetic fidelity of Micropropagated plants of *Zingiber officinale* cv-*Majhauley* of Sikkim Himalaya using RAPD markers

Sushen Pradhan[1*], Bharat C. Basistha[2] and Pratap J. Handique[1]
[1]Department of Biotechnology, Gauhati University, Guwahati – 781014, Assam, India
[2]Sikkim State Council of Science and Technology, Gangtok, Sikkim, India

Abstract

The present investigation was carried out for the determination of genetic conformity of micropropagated plants of *Zingiber officinale cv Majhauley* using Random amplified polymorphic DNA (RAPD) technique.Total fifteen different arbitrary decamers were used as primers to amplify DNA from *in vitro* plant material to assess the genetic fidelity of 18 months old *in vitro* cultured plantlets. Total 38 amplified reproducible bands were produced from 10 primers with an average of 3.8 per RAPD primer, amplified bands were ranging from 150-950 base pair, the highest seven number of bands found in primer CLT53 (50-875 bp) and lowest one band in CLT 112 (575 bp) and CLT 192 (650 bp) primers. All the primers were found to be monomorphic and no genetic variation was detected within the micropropagated plants. Present protocol may be used for testing of ginger propagules to ensure mass production of disease free planting materials.

Keywords: *Zingiber officinale, Majhauley,* fidelity, RAPD, Sikkim

1. Introduction

Zingiber officinale Roscoe, family *Zingibereaceae,* is rhizomatous monocot plant with 22 numbers (2n=22) of chromosome[1]. This plant is a highly important horticultural crop and plays a very important role in the pharmaceuticals, food and beverages industries from ancient times.

Ginger is a regular natural dietary ingredient of our daily food. It contains pungent phenolics, 6-gingerol one of the bio-active constituents, has been known to be have high antioxidant, anti carcinogenic properties[2] rich in secondary metabolite such as Oleoresin[3] .Gingerol also acts as anti bacterial, anti inflammatory and anti tumor promoting activities[4] found its antiangiogenic activity.

Plant breeders are totally handicapped by poor flowering and absence of seed set. Usually crop improvement propragmme of ginger was depending on selection of healthy planting material from clonal variations from natural fields. Ginger production is declining rapidly in all over the world due to highly infected with soil borne pathogenic microorganism and caused number of diseases, bacteria wilt (*Pseudomonas solanacearum*), soft rot (*Fusarium oxysporium, Pythium* sp) and nematodes (*Meloidogyne incognita*) which impact heavy loss of crops[5-7].

To overcome this problem plant tissue culture is recognized as one of the key areas of biotechnology because of its potential use to regenerate elites, conserve valuable plant genetic resources and as an alternative means of secondary metabolite production, as well as the ability to produce new and improved cultivars when combined with other tools of modern biotechnology. Conservation of germplasm of vegetatively propagated crops and considered as an alternative to conventional field gene bank to safeguard against pests and environmental vagaries[8]. Three fold increases in the production is possible with effective disease control[5] Bacteria, Fungi, viruses and nematodes are successfully eliminated from infected plants through in vitro culture of shoot tips[6,9].

Somaclonal variation mostly occurs from plantlets derived from in vitro culture is manifested in the form of DNA methylations, chromosome rearrangements, and point mutations[10,11] such varations are heritable too[12] and is therefore not desirable in clonally propagation. Several studies were conducted to screen somaclonal variations produced in tissue cultured plants such as in turmeric, *Lillium* species, neem, tea and soyabean[13-17] in case of oil palm, where aberrant flowering patterns were observed among the regenerated plants[18]. Thus it would be very important to monitor these variations quite early in the life of plant to prevent from adverse effect which may prove to be economically disastrous.

Researchers tried to assess tissue culture-induced variations can be determined at the morphological, cytological, biochemical, and molecular levels with several techniques, but most of the techniques have their own limitations. Cytological analysis cannot study in specific rearrangements of genes in chromosome level[19]. Using Polymerase chain reaction (PCR), DNA based markers are the best markers which are not influenced by environmental factors and generate reliable, reproducible results. Though Restriction Fragment Length Polymorphism (RFLP) can be used for screening genetic stability of tissue cultured plants, the method involve use of expensive enzymes, radioactive labeling and extensive care, therefore appears unsuitable. Among various methods used for such determination of genetic fidelity in that (RAPD) Random Amplified Polymorphic DNA is the simplest, cheapest, quick,

Corresponding author
Full Address :
Sikkim State Council of Science and Technology, Gangtok, Sikkim, India
Phone no. +91-9475714859
E-mail: *sushenpradhan@gmail.com*

requires only small amounts of DNA, detects rare single base mutations and deletions at the level of primer target and within the amplified fragment and appears to be a useful tool for the analysis of genetic fidelity of *in vitro* propagated plants[20-22].

In Sikkim gingers are cultivated from ancient times by folk healer of Lepcha, Limboo and Rai this showed attachment of ginger with the people of Sikkim from ancient times it is widely cultivated in all the region of Sikkim up to the altitude of 5000 ft. and has readymade market in the village level.

In this present experiment were carried out for rapid micropropagation of an elite cultivar (cv-*Majhauley*) of *Zingiber officinale* using active rhizome shoot tip and determination of genetic fidelity of *in vitro* conserved plantlets through RAPD analysis.

2 Materials and Methods

Explants for tissue culture of the cultivars of *Zingiber officinale- Majhauley* were collected from the nursery of Sikkim State Council of Science and Technology, Gangtok. Young meristemetic shoot tips of 2-3cm long were excised and collected in a Petridish. The explant was soaked in water adding two drops of tween 20 for 15 minutes. The explant was rinsed with single distilled water for 7-8 times under the laminar airflow. Outer scales of the explant was excised by sharp blade and surface sterilized with 0.1 % mercuric chloride for 10-15 minutes. The explant was washed with double distilled water for 7-8 times and its outer scales were removed, followed by sterilization with 70 % alcohol for two to three minutes and it was washed with double distilled water for 2-3 times. The sterilized explant was kept for drying in the sterilized blotting paper for inoculation on Murishage and Skoog (MS)[23] media supplemented with 2.5mg/l BAP and 0.5mg/l NAA, 3% sucrose and 2mg/l activated charcoal. The ginger propagates were subcultured after 40 days on same fresh medium because both good shoots and roots were observed in same medium. Ginger plantlets having 4-5cm roots were acclimatized in substrates having perlite, soil and farm yard manure with ratio of 1:1:1 for 20 days in hardening shed for primary and secondary hardening followed by transfer to the field conditions.

2.1 DNA Extraction

The total genomic DNA was isolated from 100mg of new fresh leaves sample ground in liquid nitrogen in a pre chilled mortar and pestle and was mixed with pre warmed 250µl DNA extraction buffer 1 M Tris-Cl (pH8), 0.5M EDTA, 5M NaCl, 2% (w/v) CTAB (cetyltrimethyl ammonium bromide, Sigma), 0.2 % β-Mecaptoethanol, 1 % PVP and vortex for 15 minutes, homogenate was incubated at 60°C for 50 minutes with proper inversion of tube in certain interval of time followed by equal volume of solvent treatment (C:l,24:1) chloroform *Iso*-amyl alcohol and supernatant was collected after centrifuged at 10,000 rpm for 15min (Eppendrof, 5804 R, Germany) and 50µl of 5M sodium acetate was added nicely mixed and equal volume of chilled *iso*-propanol was added for precipitation and incubated for 3-4 hrs at -20°C, pellet was separated by centrifuged at 12,000 rpm for 5 minutes and DNA pellet was washed with 70 % ethanol, air dried and dissolved in TE buffer (10mM Tris HCL (pH8), 0.5mM EDTA). DNA solution was purified with solvent Phenol: Chloroform: *iso*-amyl alcohol (P: C: I, 25:24:1) and centrifuged at 12 000 rpm for 10 min as discussed in Doyle and Doyle[24]. DNA was quantified in 0.8% (7 V/cm) agarose (Software Kodak Molecular Imaging version standard edition 45.1) according to a standard DNA marker in gel electrophoresis (Gel Logic 200 imaging system). The isolated DNA solution was the diluted in 100µl TE buffer and stored in -20 ºC for further use in polymerase chain reaction.

2.2 PCR Amplification

RAPD PCR reactions were generated using 15 different decamers (Clonitech technologies) for screening of primer using five clones of micropropageted ginger which were randomly picked, to determine the suitability of each primer for the study. The best conditions used for 45 cycles for single primer were as follows: 94ºC for 4 min, 35º C for 1 min and 72ºC for 2 minutes with an initial denaturation temperature at 94ºC for 1 min and an final extension at 72ºC for 5 minutes. All reactions were performed in a thermal cycler (Applied Biosystem 2070) as discussed by Williams *et.al.*[20].The total reaction mixture contained 12.5µl master mix (Promega), 10µM primer, (DNA sample was diluted at 1:10 ratio), 1.0 units of Taq DNA Polymerase (Promega), 20mM $MgCl_2$ and adjusted to final volume of 25µl with nuclease free water (Thermo scientific, D8611). All the amplified DNA were analyzed by gel electrophoresis taking 5µl of PCR product in a 1.5% agarose gel at 8V/cm for 90 min with 1X TBE buffer, visualized after ethidium bromide staining and photographed using gel documentation system.

2.3 Amplified DNA Marker Scoring

Amplified products in gels images were scored in the basis of presence (1) and absence (0) of bands images visualized under UV light (Kodak Molecular Imaging V. 45.1) individually. The amplified bands were named according to the number of primer and size (base pair) of the band was measured approximately with standard DNA marker. The bands showing low visual strength/ intensity regarded as negative control and they are not included for analysis.

3 Results and Discussion

Five samples were picked randomly, for the standardization of amplification (PCR) protocol, the concentration and volume of PCR ingredients were altered one by one to standardize the best protocol and simultaneously range of annealing temperature was also standardized before establishing the best possible reaction condition.

RAPD PCR reactions were generated using 15 different decamers (Clonitech Technologies) for screening of primer using five clones of mipropogated ginger plantlets which were randomly picked, to determine the suitability of each primer for the study. As of that 10 primers were selected by visualisized in electrophoresis bands for further analysis based on their ability to detect distinct, clear nice quality of band and showing monomorphic bands (Table 1). Rest other primers did not give good amplified outcome was rejected. Total 50 different plantlets were used for this study using 10 selected primers to examine the genetic fidelity of *in vitro* culture of *Zingiber officinale* over a period of more than 1.5 years.

RAPD analysis in micropropagated plants of *Zingiber officinale cv Majuley* was performed with total of 38 amplified reproducible bands produced from 10 random decamer primers ranging from 150-950 base pair. The number of bands per primer ranged from 1 in CLT 112 (575 bp) and CLT 192 (650 bp) to 7 bands in CLT 53 (150-875 bp). The size of amplified products varied from 150-950 base pair in size with an average of 3.8 bands per RAPD primer. All the primers were found to be monomorphic and amplified bands exhibited monomorphism within all the *in vitro* propagules and similar those to control plants (Figure 1).

Numerous analyses of somaclonal variations have been done using PCR- based techniques. No genetic variations or polymorphic amplified DNA bands were obtained after amplification by PCR-RAPD within the micropropagated plants of *Z. officinale cv Majhauley* by our culture protocol. Genetic fidelity analysis of micropropagated plants have been reported by various authors on different importants plants species using PCR-RAPD technique *Zingiber officinale*[25],

Table 1: RAPD banding pattern of micropropagated plants of *Zingiber officinale* cv *Majuley*

Primers	Sequences	Band size (bp)	Total bands
CLT 182	5′-GTTCTCGTGT-3′	250-900	4
CLT 76	5′-GAGCACCAGT-3′	450-950	6
CLT 78	5′-GAGCACTAGC-3′	150-750	5
CLT 53	5′-CTCCCTGAGC-3′	150-875	7
CLT 112	5′-GCTTGTGAAC-3′	575	1
CLT 192	5′-GCAAGTCACT-3′	650	1
CLT 25	5′-ACAGGGCTCA-3′	200-600	3
CLT 54	5′-GTCCCAGAGC-3′	300-900	5
CLT 105	5′-CTCGGGTGGG-3′	150-300	2
CLT 30	5′-CCGGCCTTAG-3′	175-500	4
		Total	**38**

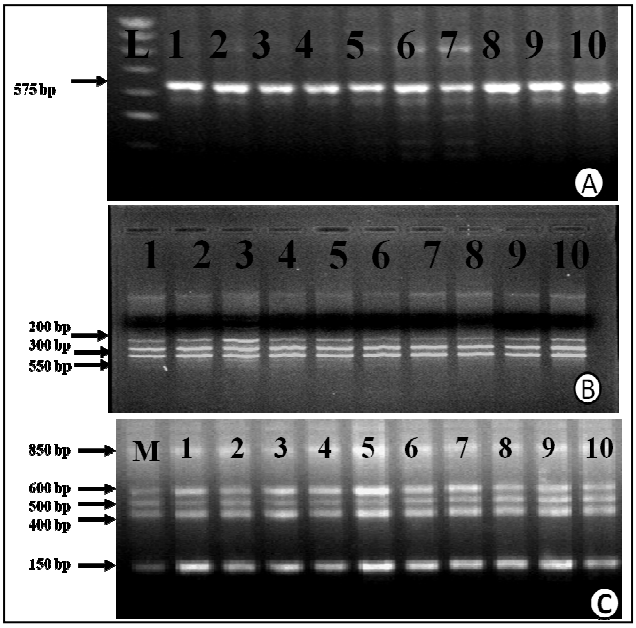

Figure. 1 (A-C): RAPD banding pattern showing monomorphism A) primer CLT 112, L represent ladder (marker) and 1-10 represent micropropagated ginger plants. B) Primer CLT 25, 1-10 represent micropropagated plants. C) Primer CLT78, M represent mother plants and 1-10 micropropagated plants. Arrow represents the size of the bands with markers.

Pinus thunbergii[26], *Plumbagao zeylanica*[21], Almond[22], *Chestnut hybrids*[27] and *Cucurma longa*[28]. We have used new merismetic axillary rhizome buds as explants for rapid multiplication of *Zingiber officinale* because it lowers the risk of genetic stability and after sub cultured for 18 months, no genetic variations were obtained after amplification by RAPD analysis. Sheony and Vasil[29] also reported that the micro propagation plants through axillary explants meristem showed with low risk of genetic stability because they are generally more resistant to genetic changes that might occur during differentation under *in vitro* condition. Similar results were coincided with same species cv-V_3S_8. by Rout *et al.*[24] reported monomorphic stability in micropropagated plantlets. Panda et al[28] *Cucurma longa* who subcultured for two years and did not find genetic variations. Same results were concurred with Martins *et al.*[22], Angel *et al*[29] in almond and cassava plantlets with no genetic variations. Few authors reported that the time in *in-vitro* give support to somaclonal variations and suggested that this variability was due to accumulating mutations during long term clonal growth[30,31]. Gould[32] reported that culture period does not give the impression to be the only parameter affecting genetic stability. From this result concluded that variations in cultured propagules possibly will be affected more by genotype than by the time in culture. Our results suggest that RAPD technique can be successfully used to assess genetic variations in micro-propagated plants of *Z.officinale* cv *Majuley* of 18 months of sub cultured plantlets.

It can be concluded that, PCR- RAPD examination showed no variations in the micropropagated plants. New axillary merismetic buds of the rhizomes may be used for rapid multiplication and conserve important germplasm resource with a minimum risk of generating somalconal variations particularly for elite clone of *Zingiber officinale* cv *Majhauley*. These cultures can be used as a source of disease free planting material and propagate improved cultivar on a commercial stage.

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