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Assessment of Genetic Stability of Ex-vitro Lime (*Citrus aurantifolia*) Population Maintained under Nursery Condition in South Bengal

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A B S T R A C T

The South 24 Parganas of West Bengal harbours huge number of fruit nursery-cum-orchards dealing with fruit business. This nursery business leads to earning of a descent revenue and generates local employment. The nurseries are old but applies innovative species-specific techniques for sapling generation. *Ex-vitro* lime cultivation is a lucrative business producing air-layered plants within a very small timeframe. This rapid asexual regeneration of lime sapling may lead to genetic instability affecting performance. To examine the genetic performance of the clones DNA based marking was done with 10 saplings including the mother stock plant as check. 7 primers yielded 31 bands in range of 120 bp-2.1kb for both RAPD and SSR. OPA01 detected polymorphism in a single locus covering 3.4% of the total variation addressed by the marking system. The field performance and fruit quality traits were nearly uniform. The application of molecular marking technique on *ex-vitro* nursery clone examination is rare and could be included as a part of on-farm authentication programme of progressive nurseries. The periodic assessment of genetic stability in rapid clonal propagation is essential and the detected variation needs scrutiny and replacement of the off-type facilitating uniform plant material production, perfect nursery management and enhanced business growth.

***Key words*:** *Citrus aurantifolia*, RAPD, Genetic stability, Asexual propagation, Authentication, Nursery management, Quality control, Fruit business

The Amtala region of South 24 Parganas is regarded as the nursery hub of West Bengal. The region harbours large number of nurseries and majority of them propagates and sell different types of fruit. The vicinity of this region with Kolkata provides low-cost transport and timely delivery of orders. Even customized Plant-shopping malls are emerging that are only keeping diverse plant materials, cultivation equipment, manure, growing pots or small chambers, plant growth promoters, hydroponic, aquaponic business items. This region holds extreme significance for the propagation and business of horticultural plants. Majority of the nurseries are known for business of exotic fruits, predominantly imported from South East Asian countries. Though the exotic fruits fetch more price in the market but the nursery growers provide equal importance to the traditional fruits, as the choice of local customers remains unaltered for known tropical fruit materials. Though exotic fruits such as dragon fruit, Thai guava, kiwi-fruit, avocado is becoming very popular among the Bengal customers but that attraction was not able to replace the demand for local indigenous fruit. The lime, lemon, pommelo are grown and species-specific diverse asexual propagation techniques are practiced to generate huge number of saplings. These *ex-vitro* plant materials are sold to domestic or overseas market or individual customers. The progressive nurseries and orchards with central or state horticulture department accreditation nursery had a rich clientele. Renowned nurseries mainly deals with B2B business. In such a state the quality of the nursery produce is of utmost important for continuity and acceleration of business.

The nursery propagation techniques are unique for different types of Citrus. In West Bengal, farmers prefer to grow lime and lemon as the cultivation technique is simple without much input and labour. An orchard may reap profit within 4 years of formation. The lime plants are mainly developed as air-layering types but some other methods such as water-layering, pot-layering are also being practiced. In a stabilized orchard a productive plant may produce 1500-2000 fruit per plant. The demand of lime is more than lemon in local markets due to a specific fragrance. The unique

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refreshing fragrance of lime is due to presence of limonene, β-pinene and γ-terpinene class of monoterpenes, citral along with some sesquiterpenes [1]. The local consumers purchase lime over lemon especially during summer season that suits with local cuisine. The old and conventional nurseries are producing plant materials for a long period of time. The perennial well performing plants are used as mother/ stock plant and huge number of progeny plantlets are generated from them. The skilled nursery person generates copious offspring within a short tenure. Air-layering produces huge sapling within a small time-frame and also shows early bearing. In subsequent generations the clones may perform the role of mother or stock plant in other commercial nurseries or home-based cutting practices. In such a condition examination of the genetic stability of the mother and the clones requires periodic assessment [2]. Reliability and comparative low cost of PCR based DNA markers made them unanimous choice for this kind of on-farm experiments [3]. In this paper RAPD and SSR markers were used to assess the genetic stability of the plantlets generated from the mother stock plant by *ex-vitro* method under nursery condition.

MATERIALS AND METHODS

Lime (*Citrus aurantifolia*) mother plant along with 10 air-layered plants were marked inside the nursery of Amtala, South 24 Parganas. Field characteristics of the plants were recorded in the nursery. Fruits and semi-matured leaves were collected for plant DNA extraction. Simple fruit parameters were analyzed at the Genetics laboratory of the University and leaves were kept inside -20°C deep-freezer for further analysis.

Method of DNA Extraction and quantification

Genomic DNA was extracted from the collected tender leaves of the test plants with the standard CTAB method of DNA extraction with minor modifications for *Citrus aurantifolia* [4]. The quantity and amount of DNA was determined as described by Kahangi *et al*. [5].

Selection of suitable PCR primers

Arbitrary decamer RAPD primer was used after browsing through Google Scholar with a search including Citrus + DNA marker. Published papers were also consulted [6]. DNA isolated from lime leaves were used for primer screening. Ultimately 5 arbitrary decamer RAPD primer and 2 SSR primer pair TAA15 and TAA27 were selected for the trial. The screened primers of operon series yielding strong, intense, unambiguous and reproducible DNA fragment were selected for analysis of the progeny population.

PCR procedure

Amplification using all the PCR primers was achieved by the protocol outlined by Mondal *et al*. [7], with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1.5 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10X PCR buffer, and 0.5 µM primer of decamer primers (Bangalore Genei) in a total volume of 25 µL. DNA amplification using SSR primers were carried out in 25 µl reactions containing 50 ng of template DNA, 5 µl of 0.2 mM total dNTPs, 3 µl of 0.5 µM primer, 2.5 µl of 10X PCR buffer, 1.5 mM of magnesium chloride and 1 unit of Taq polymerase (Bangalore Genei, India). The amplification was performed in a thermal cycler (Gene Amp PCR System 9700, Applied BioSystems). The details of PCR protocol were given in (Table 1).

Table 1 Details of PCR protocol for different primers

RAPD Cycling protocol, 45 cycles			SSR Cycling protocol, 36 cycles	
Initial denaturation	94°C	30 seconds	94°C	4 minutes
Denaturation	92°C	30 seconds	92°C	1 minute
Annealing	38°C	30 seconds	51°C	1 minute
Extension	72°C	1 minute	72°C	2 minutes
Extended extension	72°C	5 minutes	72°C	10 minutes
Final extension	4°C	∞	4°C	∞

Electrophoresis of PCR product

Amplified fragments were separated on 1.5% (for RAPD), 2.0% (for SSR) agarose (Merck-Genei) gels containing ethidium bromide (0.5 µg per ml of Agarose gel) at 60 V for 6 hours in Tris Borate EDTA buffer. A 100bp ladder was included in each gel loading for size analysis of the amplicons (Bangalore Genei, India). The gel was visualized and photographed under UV excitation using an electronic dual wave trans-illuminator system (Ultra. Lum Inc., USA).

RAPD, SSR band scoring

Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra.Lum Inc., USA). Amplified bands from each primer were scored as present (1) or absent (0) for all the seedlings studied. The size of the fragments (molecular weight in base pairs) was estimated by using a 100 bp ladder with some manual random checking was also performed on the movement of the bands by Rf value calculation of DNA ladder.

Details of the software used for analysis

1. Excel version was used for calculating Mean value, coefficient of variation, Standard deviation.
2. TOTAL LAB software was used for calculating the fragment size of the generated amplicons.

RESULTS AND DISCUSSION

In this experiment the mother stock plant of the nursery and 10 clones (air-layered) generated from the mother plants were included in studying the genetic stability experiment. The field characters of the plants were noted in the nursery. The field traits along with fruit parameters for

all the clones are in accordance with the mother stock plant. The apparent uniformity in agronomic and morphological parameters confirms clonal stability. The details of the analyzed traits were given in (Table 2).

Table 2 Performance of the air-layered lime plants of South 24 Parganas nursery

Field characters		Biochemical Parameter	
Parameter	Mean with SD	Parameter	Mean with SD
Plant height (ft)	5.5-7.0	Fruit weight (g)	35.09 ± 1.65
Plant width (cm)	305-410	Juice content (ml)	11.51± 0.99
Plant type	Open dome	Pulp weight (g)	13.52 ± 1.00
Propagation method	Air-layering	Locule Number	10.0 ± 1.34
Number of branches	Primary -2	Seed Number	8.45 ± 0.93
	Secondary- 12-14	Seed Weight	0.45 ± 0.18
Bearing type	Thrice in a year	Fruit Diameter (mm)	10.92 ± 0.57
Fruit colour	Light Green	Vertical Length (mm)	14.07 ± 0.64
Fruit surface	slightly course	Seed/locule	0.79 ± 0.07

Plant source: Amtala nursery (South 24 Parganas)

In DNA fingerprinting experiments the primers were first tested with the DNA of the mother plant. The primers yielding unambiguous, consistent band in two consecutive experiments were selected for the trial. 5 RAPD and 2 SSR primer-pair were applied for genetic stability confirmation (Fig 1). The RAPD primers yielded 29 bands in the range of 145 bp to 2.1 kb showing uniform banding pattern (Table 3). Only with a single RAPD primer OPA01, a deviation is being noticed in one of the progeny plant of the selected population. With each profiling experiment the mother genotype was included to monitor the clonal performance. RAPD marking system provides a Polymorphic Information Value (PIV) of 3.4% with 5 primers. The apparent absence of one band in a single daughter plant with OPA01 is a very significant observation. The field performance and yield or other qualitative traits required to be closely observed to understand if the absence of that band is related to any agronomically or commercially important trait.

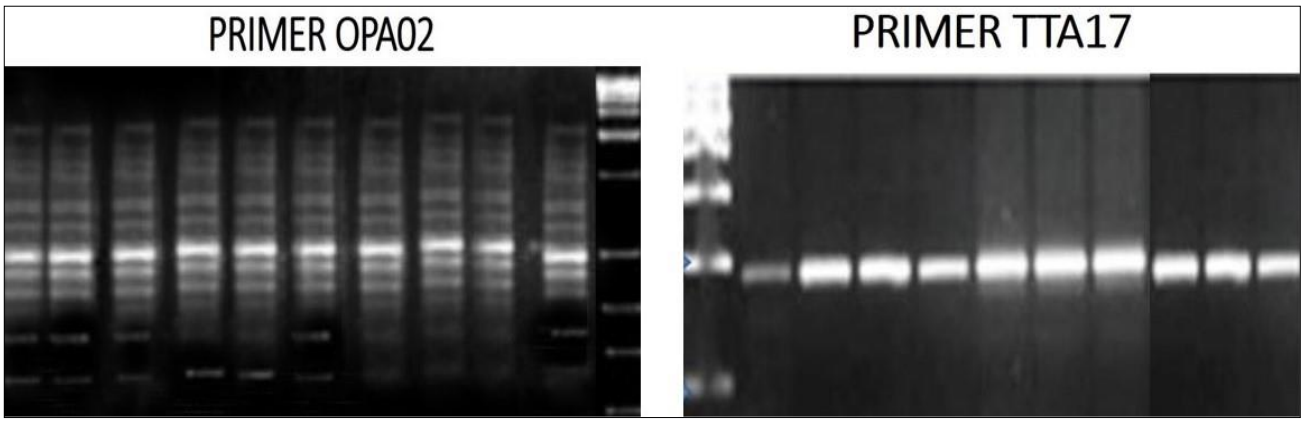

Fig 1 Molecular profile of the lime sapling along with mother stock with 100 bp ladder

Table 3 Details of the amplicon generated by RAPD and SSR primers by test plants

Primer	Sequence	Fragment size range	Total amplicon	Polymorphic bands
		RAPD		
OPA 01	CAGGCCCTTC	207bp – 930bp	4	1
OPAA 02	GAGACCAGAC	145bp – 1.3kb	5	0
OPA 04	AATCGGGCTG	157bp – 2.1kb	12	0
OPAB 17	TCGCATCCAG	219bp – 434bp	03	0
OPAG 13	GGCTTGGCGA	248bp – 987bp	05	0
	Total RAPD Marker - 5	145bp – 2.1kb	29	1
		SSR		
TTA15	GAAAGGGTTACTTGACCAGGC CTTCCCAGCTGCACAAGC	120 bp	1	0
TTA27	GGATGAAAAATGCTCAAAATG TAGTACCCACAGGGAAGAGAGC	192 bp	1	0
	Total SSR Marker - 2	120-192 bp	2	0

In a study with only 10 RAPD markers somaclonal variation was detected in micro-propagated pineapple population conforming the validity of this technique in *in-vitro* genotype assessment [8]. Similarly, in Grobogan variety of soybean, induced mutation with EMS (0.5% and 1%) was detected using 20 OPA series primers (OPA-1 to OPA-20). 11 out of 20 primers were effective to detect the variation thus confirming the potential for application of RAPD in customized breeding program [9].

SSR primer TTA15 and TTA27 generated monomorphic amplicon with size of 120 bp and 192 bp in all the clones including mother stock. These SSR markers usually amplifies single allele with high efficiency of detecting polymorphism. TTA series gave very high PIC (0.917) in examination of tomato landraces and were declared efficient in genetic dissimilarity/uniformity test [10]. In this experiment the presence of the band in all clones confirms the uniformity of the air-layered plants. As SSR markers are co-dominant and more specific for priming sites, the inclusion of more SSR primers may provide efficient result and could be utilized for on-farm species-specific authentication programme [11].

The application of molecular marker in commercial nursery management is of rare occurrence. In developed countries the utilization of molecular marker in nursery management is operative in few instances but in developing countries this trend is less [12]. In rapid *ex-vitro* asexual propagation method, the assessment of genetic stability of the daughter plants is of utmost importance. With 6 to 7 generation of clonal propagation the stability of the progeny plants may deteriorate due to occurrence of negative somaclonal variation or epigenetic as well as epigenomic changes [13]. Histone methylation, genome re-modelling, accumulation of abnormal bases, altered regulation of the cis and trans acting elements, down regulation of transcription factors leads to generation of epi-genetic variation [14]. In *in-vitro* propagation the genetic fidelity testing of the regenerated clones were practiced in tissue culture facilities but that practice is sparse in case of *ex-vitro* nursery propagation and requires urgent incorporation.

In breeding and seed certification programs the use of molecular marker is a common practice. In oil palm the genetics of shell thickness trait is known to the breeders. This Sh trait based molecular marking have been validated on 207 *dura* genotypes and 50 *pisifera* genotypes of different origins validating a general applicability of the proposed system [15].

Scientists are adopting new innovative techniques for rejuvenation of dwindling orchards and authentic nursery management. In Europe there is a huge demand for *Phytopthora cinnamon*i resistant rootstock for chestnut orchards. The nursery growers are applying innovative clonal propagation techniques for emerging genotypes for ongoing breeding programmes along with molecular study [16].

A comparable asexual propagating technique of grafting, the performance of grafted sapling could be assessed using molecular techniques. In grafting experiments the success of graft union depends on the genetic architecture of the parent plants. The phenotyping of the graft compatibility is a major problem as it requires sufficient number of individuals to precisely score a trait. The application of molecular marking with a number of available PCR based markers was able to validate graft compatibility experiments and regarded as a viable method for graft performance assessment [17].

CONCLUSION

In West Bengal huge number of government accredited and private nurseries are available. In South Bengal majority of the nursery deals with indigenous and exotic fruit business. Most of the orchards of this region are very old and producing lime fruits for long period of time. Our state earns sufficient amount revenue by export, import and innovative propagation of these fruit plants. The authentication of these asexually propagated plants are essential for quality control of produce. A random selection of some daughter plants and application of DNA based molecular profiling could be very helpful to ascertain the genetic stability of the clones. If required the molecular profile of the mother stock plants could be kept and provided to the customers during supply of plant consignments. As the nursery plants are generated by *ex-vitro* method the production the cost of cultivation is affordable. In such case *ex-vitro* propagation along with molecular certification will not significantly affect the cultivation cost. This investigation is a very unique one and it is being proposed to integrate the molecular marking technique as a part of quality control certificate issued by the nursery owners in bulk import-export of plant materials mainly related to B2B business communications.

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