

*Full Length Research Paper*

# Morphology, flow cytometry and molecular assessment of *ex-vitro* grown micropropagated anthurium in comparison with seed germinated plants

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**Micropropagated and seed propagated plantlets of anthurium (*Anthurium andreanum* Lind. cv. CanCan) were transferred to similar field condition and growth stage. A comparative study was conducted using morphological parameters, ploidy level assessment as well as deoxyribonucleic acid (DNA) fingerprinting using inter simple sequence repeat (ISSR) markers. The *in vitro* generated anthurium plants exhibited comparable vegetative growth and more sucker production when compared to plants propagated through seeds. No variation in ploidy level was established through flow cytometric study. Genetic assessment through ISSR showed no polymorphism in banding pattern. It was revealed that there was no significant variation between micropropagated and seed propagated plants at ploidy and molecular level assuring the trueness of the micropropagated anthurium clones and their commercial applicability.**

**Key words:** *Anthurium andreanum* Lind, *Ex vitro* performance, ISSR, morphological competence, ploidy level.

## INTRODUCTION

Amid the 108 genera and 1500 tropical herbaceous ornamental species of *Anthurium*, the most economically exploited species is *Anthurium andreanum*. Globally, it has been largely grown as cut flowers and potted plants chiefly due to its extended vase-life (Gantait et al., 2008). It is a monocotyledonous perennial herbaceous member of Araceae and is exceedingly appreciated for its striking vibrant inflorescence with straight spathe, candle-like spadix and exotic foliage (Chen et al., 2011). It can produce long lasting inflorescence all round the year under favorable environment. Although, *Anthurium* is native to Central and South America (Gantait et al., 2008), they are grown internationally for commercial production in the United States and The Netherlands (Maira et al., 2010). Thus far the demand for uniform propagative materials and new cultivars are very high. *Anthurium* is propagated by seed (Dufour and Guérin, 2003) or offshoots (suckers) and nodal cuttings (Gantait

et al., 2008), conventionally; however, the seeds cannot be stored. Seed-propagated plants are also not homogeneous (Dufour and Guérin, 2006). Clonal propagation takes years to develop clones of commercial standard (Martin et al., 2003). As an alternative approach, micropropagation of anthurium has been attained using a wide range of explants including petiole, lamina, shoot tip, leaf and spadix (Dhananjaya and Sulladmath, 2003; Martin et al., 2003; Gantait et al., 2008; Jahan et al., 2009). For commercial utilization of *in vitro* propagation, it is essential to raise an adequate amount of disease free planting material of true-to-type newly developed elite lines. The applicability and sustainable use of micropropagated plants would eventually depend on the relative field performance compared to that of the conventionally propagated plants (Zaman et al., 1997).

Therefore, field evaluation is indispensable for commercial exploitation of tissue culture which has not received due attention (Smith and Hamill, 1996). Furthermore, only few attempts have been made to study the variation in morphological characters as well as genetic integrity of micropropagated plants in general, over conventionally propagated plants, as an estimation on the

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extent of genetic fidelity and ploidy stability of micro-propagated plants can minimize the chances of somaclonal variation owing to prolonged culture period (Gantait et al., 2009; Gantait et al., 2010a, b). There are several other genetic (Singsit et al., 1990; Compton and Veilleux, 1991; Kaltsikes and Bebeli, 1993) and cytological (Puolimatka and Karp, 1993) evidences which show that both the frequency and distribution of genetic recombination events can be altered by passage, through tissue culture leading to somaclonal variation. It was not anticipated that asexual culture process would result in genetical, cytogenetical or morphological variability. The uncontrolled production of plants which are not 'true-to-type' creates problems in almost all other applications of micropropagation (Karp, 1995). Soma-clonal variation can affect the *ex vitro* performance of micropropagated plants either by limiting or improving. No single technique is ideal or sufficient, taken alone, for assessing the trueness of *in vitro* generated plantlets to ensure their identical performance *ex vitro*; a combination of several techniques should be used to evaluate the micropropagated plants (Peredo et al., 2009). Amongst a combination of different methods only certain: phenotypic analysis, estimation of nuclear DNA content, chromosome analysis and an analysis of secondary biochemical products are exploited for the detection of somaclonal variation between the regenerants (Potter and Jones, 1991; Nayak and Sen, 1991,1997). For scrutinizing the genetic stability of *in vitro* raised and conserved materials, the technique of molecular analysis is being used extensively. As DNA based markers are not influenced by environmental factors, they present the most effective way to screen the tissue culture induced variations (Peredo et al., 2009). So far as we know, there are no published reports on *ex vitro* growth and yield of anthurium plants obtained through micropropagation. A method of *in vitro* mass propagation was developed through multiple shoot proliferation from shoot tip explants (Gantait et al., 2008) of *A. andreanum* Lind. cv. CanCan. Here, we report the growth characteristics, ploidy status and genetic uniformity of the micro-propagated plants. The characteristics are compared with plants produced by conventional seed propagation method.

## MATERIALS AND METHODS

Anthurium plantlets were raised by *in vitro* culture of shoot tip explants on MS media supplemented with optimized plant growth regulators and subsequent acclimatization following the protocol, developed previously by Gantait et al. (2008). Fully acclimatized micropropagated plantlets, 45 days old with 5 to 7 cm height were transferred to earthen pots (10 cm diameter) during normal growing season in the month of August at field condition. The conventionally propagated plantlets produced from seeds, at the similar growth stage (45 days old upon germination with 5 to 7 cm height), were also transferred to earthen pots of similar sizes for the *ex vitro*

evaluation. Field trials were conducted using complete randomized block design; with 15 rows for each treatment and five plants in each row. The pots were filled with a mixture of soil, sand, charcoal and coconut fiber (1:1:1:1 v/v). After 12 months of growth period (during the month of April), important morphological attributes like plant height (cm), leaf length and width (cm), number of leaves, and number of newly regenerated suckers including the days to flower induction, etc. were recorded. All plants were kept under detailed observation at the experimental garden of Universiti Putra Malaysia, Selangor, Malaysia.

## Flow cytometry analysis

Leaf samples were obtained from the seed derived and also the micropropagated plants at 12 months field growth phase. Conventionally, propagated plantlets were used as the diploid standards. The flow cytometry analysis was attained as illustrated by Cystain ultraviolet-visible (UV) ploidy Partec protocol (Gantait et al., 2011a). The leaves were set in distilled water at 4°C and sliced into 1 cm<sup>2</sup> fragments. Suspension of nucleus was ready by chopping the leaf fragments (Galbraith et al., 1983) in 0.5 ml of Partec solution buffer containing 4', 6-diamidino-2-phenylindole (DAPI) (excitation/emission wave length: 320-385/415-520) (Shapiro, 2003), shortly after, 1.5 ml of the same solution was again added. The suspension was then filtered in nylon filter (Partec) with 30 µm mesh diameter, after 2 min. After another 15 min in the dark, the nuclear suspensions were analyzed with a Partec-PAS flow cytometer (Partec GmbH, Munster, Germany), equipped with an UV lamp emitting at 358 nm and a TK 420 filter. The equipment was cautiously calibrated and aligned by means of micro beads along with standard solutions according to the manufacturer's reference and nuclei suspension of the standard diploid plants under experimentation. For the analysis of data, FloMax software (Partec) was used. Three replications were used for ploidy level determination and more than 5000 nuclei were analyzed to carry out this process.

## ISSR assays

Apart from study on morphological competence, assessment of the genetic integrity of the micropropagated and conventionally propagated plants were also done using ISSR fingerprinting. DNA extraction followed by Polymerase Chain Reaction (PCR) amplification and subsequently ISSR analysis was done using 10 ISSR primers (Gantait et al., 2009). Primarily, genomic DNA was extracted from 80 mg tender leaves (collected from 12 months field growth stage), as per the procedure described by Chattopadhyay et al. (2008). Next, the extracted DNA samples were subjected to PCR amplification using 10 ISSR primers mentioned earlier. The 25 µl optimized PCR mixture comprised 40 ng DNA, 2.5 µl 10X Taq polymerase assay buffer, 3.5 µl 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.5 U Taq DNA polymerase (all from Chromous Biotech Pvt. Limited, India) and 200 ng of primer (Bangalore Genei Pvt. Limited., India). PCR performance consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of 45 s at 94°C, 45 s at annealing temperature and 90 s at 72°C, and final extension at 72°C for 7 min, 4°C for 5 min was done using Gene Amp PCR system 2400 (Applied Biosystems, USA). The annealing temperature was adjusted according to the T<sub>m</sub> of the primer being utilized in the reaction. The amplified PCR products, along with 50 bp DNA ladder were resolved by electrophoresis on 1.5% agarose (SRL) gel in 1X tris borate ethylene-diaminetetraacetic acid (TBE) buffer stained with ethidium bromide (10 µg l<sup>-1</sup> TBE buffer). The well-resolved and consistently reproducible amplified DNA fragments as bands were scored with



**Figure 1.** **a** Micro propagated anthurium plants established after *ex vitro* transfer; **b** Flowering initiation in micro propagated anthurium plants with vigorous growth at 12 month field growth stage.

regards to their presence or absence and photographed on Gel Logic 200 trans-illuminator system (Kodak).

#### Data collection and statistical analysis

Treatments were distributed to a Complete Randomized Block Design (CRBD), including fifteen replications with five plants per replication for *ex vitro* study of morphological competence. Each plant was considered as an experimental unit. Data on *ex vitro* evaluation studies were collected and significant difference among the treatments were tested by simple t-test using SPSS software (Statistical Package for the Social Sciences) version 10.0 (LEAD Technologies Inc., Chicago, USA). For ISSR profiles, the well-resolved and consistently reproducible amplified DNA fragments were scored in terms of their presence or absence. To detect the genetic purity, the resulting banding patterns were compared between DNA samples for each ISSR primer.

## RESULTS AND DISCUSSION

Information about field performance is necessary in order to assess the yield potential of *in vitro* generated plants. The earlier study of Gantait et al. (2008) suggests that following their established protocol, well acclimatized plantlets can be achieved from single shoot tip explants within five months in comparison to fewer number of plantlets from seeds or suckers after two to three years (Martin et al., 2003), using conventional method of propagation. Furthermore, propagation through seeds has been seldom successful in anthurium due to seed

contamination and poor germination percentage. To witness high level of germination *A. andreaenum* seeds require uninterrupted light condition which is hardly ever obtainable under field condition (Gantait et al., 2008). Hence, to get an idea of the successfulness of the established *in vitro* protocol, *ex vitro* morphogenetic efficiency of the micropropagated anthurium plants should be scanned under an array of distinguishable environments. As flower yield is the main object of an anthurium breeder, it is important to know how the various factors show their direct and indirect effects on flower production.

#### Morphological performance *ex vitro*

The *ex vitro* assessment of morphological competence proved to be the prime factor in discriminating the micropropagated and seed propagated anthurium plants. The *in vitro* generated anthurium plants were transferred to earthen pots in field condition (Figure 1a) after the successful passage of the two-step acclimatization process (Gantait et al., 2008). *Ex vitro* transferred micropropagated plants did not reveal any transplantation shock. The morphological characteristics, assessed at 12 months growth stage, are presented in Table 1. A comparison was made between the micropropagated and seed propagated plants, where both of these were not significantly different in terms of plant height, length and width of leaf, number of leaves and days to

**Table 1.** *Ex vitro* performance\* of micropropagated in comparison to seed derived anthurium.

Treatment	Plant height (cm)	Number of leaves/plant	Leaf length (cm)	Leaf width (cm)	Number of suckers /plant	Days to flower induction
Seed derived	25.63	4.43	12.87	8.77	1.33**	226.00
Micropropagated	24.03	4.94	12.63	7.83	4.13**	228.67

Data represents mean of five replicates per treatment in fifteen repeated experiments. \*Data were recorded at 12 months growth stage after field transfer.

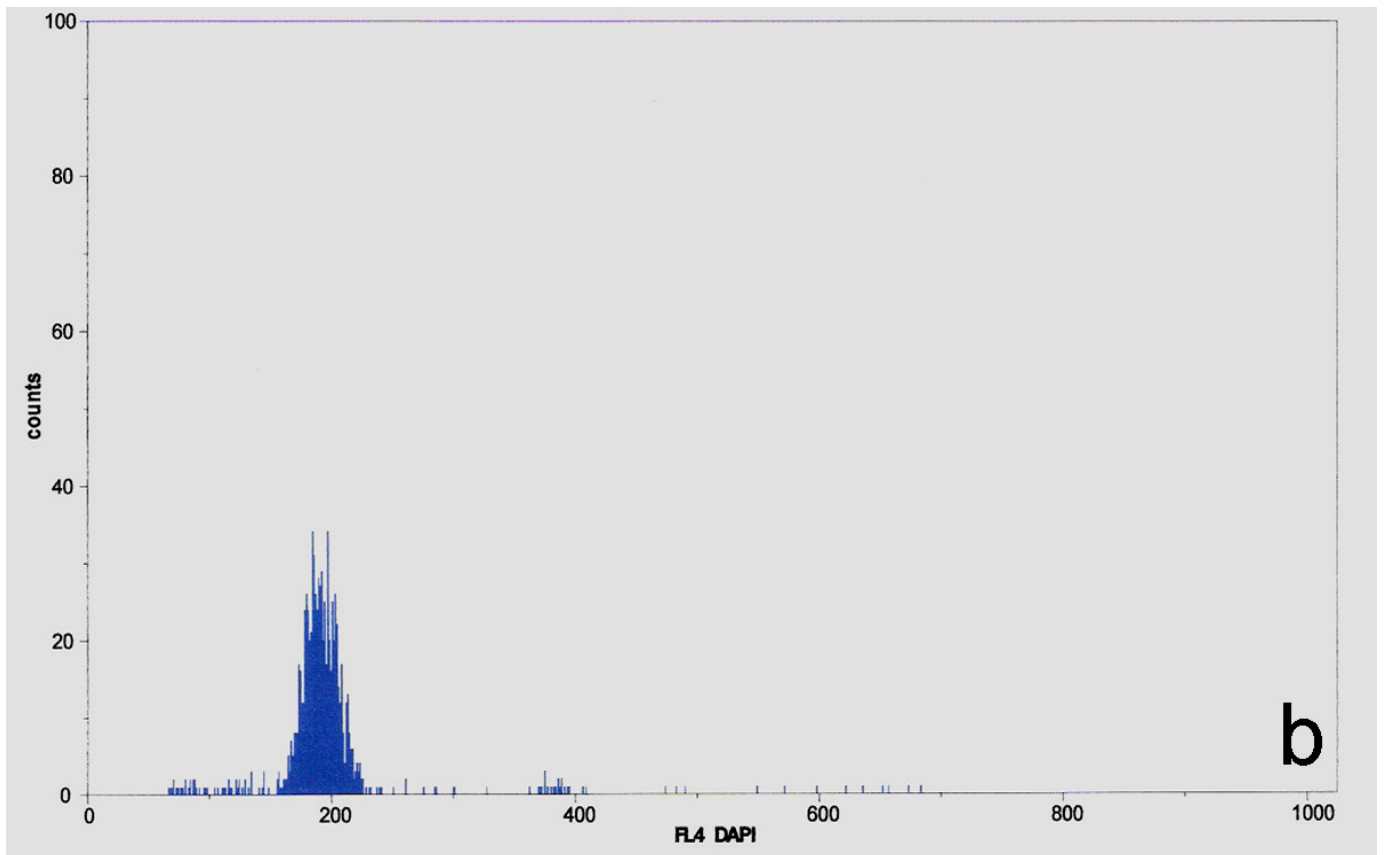
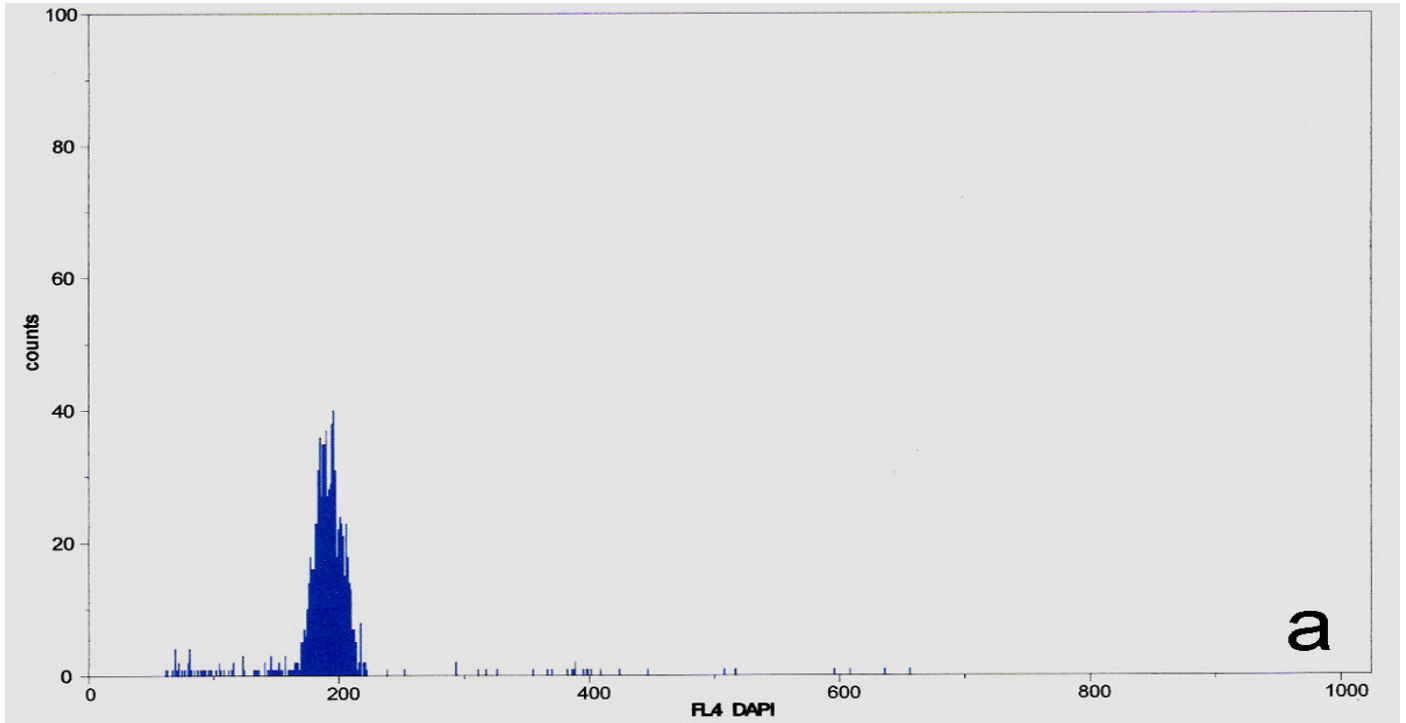
flower induction. After 12 months of growth, micropropagated plants produced around 5 leaves (4.43) per plant with average 12.63 cm length and 7.83 cm width. The average height of plants was 24.03 cm (Figure 1b). Similarly, seed-derived plants had an average plant height of 25.63 cm, leaf length of 12.63 cm and leaf width of 7.83 (Table 1). The average number of leaves was around 5 in both micropropagated plants as well as in the *ex vitro* seed derived plants. There was also a similarity in the number of days taken for flower induction, where *ex vitro* raised plants induced flower in around 226 days and in case of tissue-cultured plants flower induction (Figure 1b) was at around 229 (228.69) days.

However, the most interesting observation was that micropropagated plants produced a significantly higher number of suckers (4.13) per plant than the conventionally propagated ones (1.33) which corresponds to the earlier report of Nayak et al. (2011), in turmeric, where they found that the mean tiller number of tissue cultured plants was significantly higher than conventionally propagated plants. According to Nayak et al. (2011), the higher number of new propagules in first generation could be due to the residual effect of cytokinin present in culture media. However, this tendency of micropropagated anthurium to produce more suckers certainly attributed to the enhancement of commercial utility. With the advancement of successive field growth stage and period it would be possible to provide more number of quality propagules for further multiplication from this success achieved, expecting the significant enhancement in growth pattern of micropropagated anthurium. This is a very welcome situation for an economic plant like anthurium which exhibits very sluggish rate of conventional propagation. The enhanced sucker production observed in this study with *in vitro* propagated anthurium as compared to seed derived plants was not observed in the earlier study of Côte et al. (2000) on banana (*Musa* AAA, cv. Grande naine) where they recorded equal number of vegetative sucker from *in vitro* cultured and conventionally propagated plants. The protected *in vitro* environment during micropropagation may have favoured the plantlets to express their complete potentiality towards morphological attributes *ex vitro* too. The present study thus supports the earlier report of Gustavsson and Stanys (2000) who observed the better performance of *in*

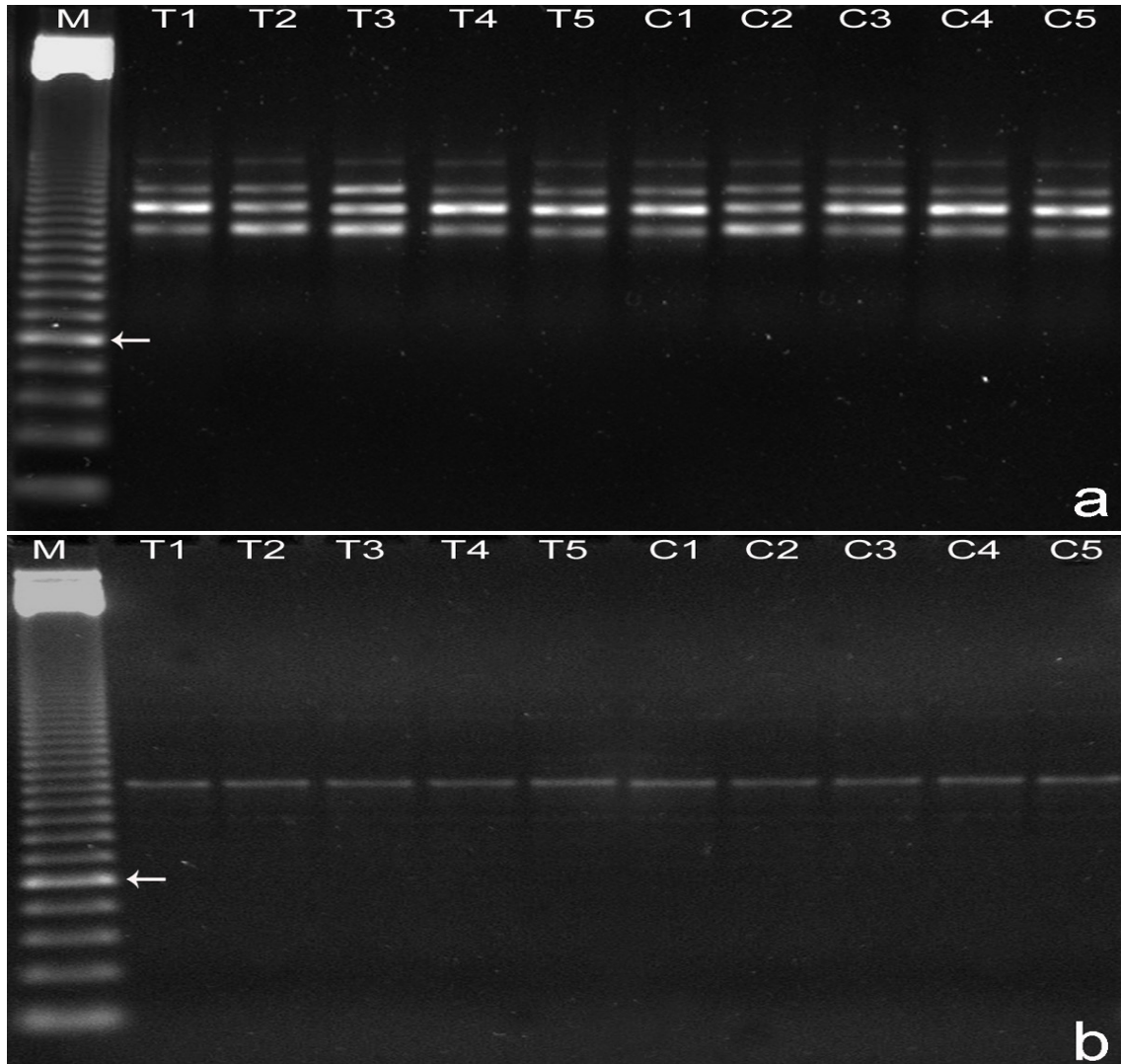
*vitro* derived lingonberry plants than the conventionally propagated plants.

### Analysis by flow cytometry

Rather than conventional methods, such as chromosome counting or stomata length measurements the use of flow cytometry to monitor the ploidy status is more accurate and faster (Nguyen et al., 2003). In the current experiment, the ploidy status of representative leaves from micropropagated, as well as seed propagated plantlets were determined with flow cytometry analysis. Figure 2 shows the results of flow cytometric analysis. Histograms from flow cytometry analysis revealed unimodal peak of the nuclear DNA content corresponding to 2x from both *in vitro* generated (Figure 2a) and seed-derived anthurium (Figure 2b), thereby confirming absence of variant nuclei. Each peak was approximately twice the fluorescence intensity (2C) in proportion to the square root of ploidy. The results of the present flow cytometric study validated that there was no alteration in diploid status as well as the DNA content between *in vitro* propagated and conventionally propagated anthurium. The assessment of ploidy status performed by the aid of flow cytometry proved to be an efficient tool to detect the ploidy level of a plant, as reported (Dickson et al., 1992; Gantait et al., 2011a). It was reported that in comparison to the other higher ploidy levels (tetraploid or hexaploid) the diploids were found to be the most stable (Winfield et al., 1993). Yet, there are several reports on the extensive alteration of ploidy level in a number of diploid species (Karp et al., 1992; Linacero and Vazquez, 1992). Unfortunately, some *in vitro* regenerated plantlets exhibit somaclonal variation, which has been demonstrated to be heritable and therefore undesirable in somatic clones. Consequently, detection of such variation is required to avoid economic disaster during commercial regeneration (Yang et al., 2011). Chromosomal alterations were successfully detected by flow cytometry in several other species also (Tremblay et al., 1999; Yang et al., 2010). Hence, in the present study, the stability of ploidy status might be attributed to the comparable performance of the micropropagated anthurium in field condition and ensured the sustainable true-to-type performance.



**Figure 2.** Flow cytometry analysis of; **a** *in vitro* propagated and, **b** seed-derived anthurium after 12 months of field growth stage showing diploid status of the plants.



**Figure 3.** Agarose gel electrophoresis of ISSR fragments of micropropagated (T1-T5) and seed-derived (C1-C5) anthurium plants at 12 month field growth stage showing monomorphic bands generated by primer. **a** IS-6, **b** IS-11. Lane M- 50 bp ladder.

### Genetic assessment using ISSR

Amid the 10 ISSR primers used, IS-6 (Figure 3a), IS-7, IS-11 (Figure 3b) and IS-61 exhibited major reproducible bands (Table 2). On the other hand, IS-8, IS-63 and IS-65 displayed a positive interaction yet failed to be reproducible. IS-9, IS-10 and IS-12 did not react with anthurium DNA. With an average of 5 (5.25) bands per primer per sample a total number of 176 (number of plants used as sample  $\times$  average number of bands per sample for all primers) reproducible monomorphic bands were scored from the micropropagated clones, including their mother. In the current study, the primers amplified distinct bands varying between 200 bp (IS-61) to 1250 bp (IS-7) molecular size ranges. Di-nucleotide SSRs motifs AG, GA, GT, TG, CT and CA were also used in the

present study. Four positive and reproducible primers (based on GA, GT and CA) amplified a distinct scorable number of bands. An analysis on genetic integrity exploiting ISSR primers was effectively attempted in various micropropagated plant species (Joshi and Dhawan, 2007; Bhatia et al., 2009; Gantait et al., 2010a, b). However, this particular study was not extensively investigated in anthurium; excepting a few reports on the application of ISSR for clonal fidelity test of tissue cultured clones only (Gantait et al., 2008). With anthurium being diploid with  $2n = 30$ , it can be assumed that the limited number of bands produced by these ISSR primers would cover the genome to only some extent.

However, none of the primers showed any variation in banding pattern. With an evaluation of the displayed monomorphic banding pattern, it can be concluded that

**Table 2.** ISSR primers used for fidelity test of *in vitro* generated anthurium clones, their sequences, anchoring, annealing temperature, mode of reaction, number and size of amplified fragments.

Oligo name	Tm (°C)	5'-3' motifs	Anchoring	Reaction to anthurium DNA	Number of scorable bands per primer	Total number of scorable bands	Size range (bp)
IS-6	52	(GA) <sub>8</sub> C	3'anchor	Positive, reproducible, monomorphic	4	40	550-1050
IS-7	50	(GT) <sub>8</sub> A	3'anchor	Positive, reproducible, monomorphic	6	60	300-1250
IS-8	52	(AG) <sub>8</sub> C	3'anchor	Positive but not reproducible	-	-	-
IS-9	46	(TG) <sub>7</sub> TA	3'anchor	Negative	-	-	-
IS-10	52	C(GA) <sub>8</sub>	5'anchor	Negative	-	-	-
IS-11	52	(CA) <sub>8</sub> G	3'anchor	Positive, reproducible, monomorphic	1	10	500-550
IS-12	52	(GT) <sub>8</sub> C	3'anchor	Negative	-	-	-
IS-61	50	(GA) <sub>8</sub> T	3'anchor	Positive, reproducible, monomorphic	11	66	200-1050
IS-63	52	(AG) <sub>8</sub> C	3'anchor	Positive but not reproducible	-	-	-
IS-65	50	(AG) <sub>8</sub> T	3'anchor	Positive but not reproducible	-	-	-

both the micropropagated and seed derived plants maintained similar genetic clonality. Micropropagation using explants, comprising an organized meristem is usually considered to have a lesser risk of genetic instability (Shenoy and Vasil, 1992). However, genetic stability is not definite amid meristem culture derived plantlets (Zucchi et al., 2002) and the same may eventually result into off-type plants with a gradual reduction in *ex vitro* performance after field transfer. Every so often, it has been suggested in some reports, based on somaclonal variation, that extended *in vitro* cell proliferation process could promote the same (Côte et al., 2000). While researching on somaclonal variation in sugarcane cultivars, Zucchi et al. (2002) reported that recognition of DNA polymorphism in meristem culture derived somaclones could be attributed to pre-existing polymorphism in source plant. In our present study, absence of polymorphism in micro-propagated anthurium may perhaps be due to nonexistence of polymorphism in source plant itself. Genetic variation in meristem culture line may well be dependent on the genotype used (Hammerschlag et al., 1987; Zucchi et al., 2002). Nevertheless, the application of different genotypes and different explants of same genotype established to have noticeable effect on the induction of somaclonal variation (Nayak and Sen, 1998). This study in anthurium is in close agreement to that of Gantait et al. (2008) in anthurium and Martins et al. (2004), Peredo et al. (2009) and Gantait et al. (2011b) showing genetic integrity among micropropagated plants of other species even

after long term field growth stage (Gantait et al. 2010c,d).

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