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FIELD PERFORMANCE AND MOLECULAR EVALUATION OF MICROPROPAGATED STRAWBERRY

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Abstract
Micropropagated and conventionally propagated plantlets of strawberry (*Fragaria* × *ananassa* Duch. cv. Chandler) were transferred to the similar field condition and growth stage. A comparative study was conducted based on morphological parameters as well as genetic assessments using ISSR markers. The *in vitro* generated strawberry plants exhibited significantly vigourous morphological growth and earlier flower induction when compared to the plants propagated through planting of runners. Genetic assessment through ISSR showed no polymorphism in banding pattern and thus it was revealed that, there was no significant variation between micropropagated and conventional propagated plants at molecular level.

Keywords: *Ex vitro* performance, ISSR, *Fragaria* × *ananassa* Duch.

Introduction
Strawberry (*Fragaria* × *ananassa* Duch. cv. Chandler), an important family member of *Rosaceae*, is one of the most popular soft fruit in the world. The strawberry fruits are of a very delicious taste and fresh aroma. The cultivated strawberry is an octaploid (2n = 8x = 56) stoloniferous perennial herb (Debnath and Teixeira da Silva 2007). It has a wide range of climatic adaptation which includes Mediterranean, temperate, subtropical and taiga zones (Hancock et al. 1991). Conventionally, strawberry is propagated by runners (Sakila et al. 2007), which is very labour intensive; time consuming and results in the transmission of viral diseases (Gautam et al. 2001). In contrast of these, mass multiplication *in vitro* through tissue culture results high yield in disease free plant material (Mohan et al. 2005) and proved to be the best alternative approach to conventional propagation method (Mahajan et al. 2001). The standardization of protocol and procedure of micropropagation of strawberry was successfully attempted by many (Kaur et al. 2005; Sakila et al. 2007; Gantait et al. 2010). But complete field performance of micropropagated plants was not studied enough where extensive field evaluation is necessary for commercial utilization of tissue culture (Smith and Hamill 1996). Furthermore, attempts have not been made to study the variation in morphological characters as well as genetic integrity of micropropagated plants over conventionally runner-derived plants. Hence, the present investigation was carried out to evaluate the performance of *in vitro* generated in comparison to *ex vitro* propagated strawberry plants in terms of both morphological and molecular aspects.

Materials and Methods
Strawberry plantlets were raised by *in vitro* culturing of runner tip explants on MS media and subsequent acclimatization following the methods developed earlier by Gantait et al. (2010). Fully acclimatized micropropagated plantlets of 35 days with 5-7 cm height were transferred from earthen pots (8 cm diameter) to the field in the month of August at field condition. The similar arrangement was provided during *ex vitro* transfer of plantlets generated through conventional method of propagation and they were of same growth stage. The field was prepared with a mixture of soil and farm yard manure (1:1 v/v). All plants were kept under detailed observation at the experimental garden of Bidhan Chandra Krishi Viswavidyalay, W.B., India and agronomic practices were applied in accordance with the plantation protocols during this period. After 5 months of growth period (during the month of January), different important morphological attributes like plant height (cm), leaf length and width (cm), number of leaves, and number of newly regenerated runners including the days to flower induction *etc.* were recorded. Apart from study of morphological competence, the genetic integrity of micropropagated and conventionally propagated plants was assessed through ISSR fingerprinting. In doing so, DNA extraction followed by PCR amplification and ISSR analysis were done using 10 ISSR primers (Gantait et al. 2010). At first genomic

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DNA was extracted from 80 mg tender leaves according to the procedure described by Chattopadhyay et al. (2008). Extracted DNA samples were subjected to PCR (Polymerase Chain Reaction) amplification using 10 ISSR primers mentioned above. The 25 µl optimized PCR mixture contained 40 ng DNA, 2.5 µl 10X Taq polymerase assay buffer, 3.5 µl 2.5 mM dNTPs, 0.5 U Taq DNA polymerase (all from Chromous Biotech Pvt. Ltd., India) and 200 ng of primer (Bangalore Genei Pvt. Ltd., 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 Tm of the primer being used 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 TBE buffer stained with ethidium bromide (10 µg l^{-1} TBE buffer). The well-resolved and consistently reproducible amplified DNA fragments as bands were scored in terms of their presence or absence and photographed on Gel Logic 200 trans-illuminator system (Kodak).

Treatments were distributed to a Complete Randomized Design (CRD) including three replicates in fifteen repetitive experiments for *ex vitro* study of morphological competence. Each plant was considered as an experimental unit. Data on *ex vitro* evaluation studies were collected and subjected analysis of variance (ANOVA) where significant difference among the treatments were tested by Duncan's multiple range test (Duncan 1955) at 5% level using WINDOWSTAT 7.5 (Indostat services, Hyderabad, India) software package from Uttar Banga Krishi Viswavidyalaya, India. 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 uniformity, the resulting banding patterns were compared between DNA samples for each ISSR primer.

Results and Discussion

Micropropagation results in uniform batches of plants, which grow, flower and fruit normally. Although it can be expected that eventually plants multiplied *in vitro* will be equivalent or superior to those propagated by traditional techniques, it cannot be assumed that their *ex vitro* growth behavior in long term will necessarily be the same (George 1996). So it is of utmost importance to assess the field performance of micropropagated plantlets to ensure their fidelity or superiority to conventional propagated plants.

Morphological performance *ex vitro*

The *ex vitro* assessment of morphological competence proved to be the potent factor in discriminating the micropropagated and traditional propagated strawberry plants. The *in vitro* generated strawberry plants were transferred to the field in the month of November after the successful passage of the two-step acclimatization process. *Ex vitro* transferred plants, both conventionally propagated and micropropagated did not reveal any transplantation shock. The morphological competences, assessed at the 5 months' growth stage, are presented in Table 1. A comparison was made between these two types of regenerated plants, where both of these were significantly indifferent performers in terms of plant height, number and length of leaf. The micropropagated strawberry plants produced on average around 8 (7.77) leaves per plant with 3.73 cm and 3.17 cm length and width respectively (Fig. 1a) (Table1). Conventionally propagated plants also produced around 8 (7.63) leaves with the length and width of 3.27 cm and 2.73 cm respectively. Significantly, the micropropagated strawberry plants have larger leaf width. Similarly, *in vitro* generated strawberry plants expressed better performance of in terms of days to flower induction and number of runner production also. Micropropagated plants produced 7.5 runners after 5 months of growth and took around 34 days (33.57) for flower induction (Fig. 1b) whereas the conventionally propagated plants produced a significantly lower number (5.8) of runners and had a longer duration (around 37 days) to induce the first flower in a similar growth stage. This is a very welcome situation for crop like strawberry which is economically important for its fruit. The better performance in vitro generated strawberry plantlets over conventional ones oppose the earlier observation of Biswas et al. (1999) in *Eucalyptus tereticornis*. It is to be noted that earlier study was based on a tree species but the present experiment was carried out on a herb. The *in vitro* generated plants develop higher number of leaves to be able to intercept incoming radiation for photosynthesis earlier than conventional propagated plants. This may explain the differences between the micropropagated and *ex vitro* runner-derived plants (Buah et al. 2000). Moreover, the better vegetative growth in micropropagated plants means the support in better establishment of plants against any biotic stress. The protected *in vitro* environment during micropropagation 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 conventional propagated plants.

Figure 1. (a) Micropropagated strawberry plants after *ex vitro* transfer, (b) Flowering in micropropagated strawberry plants with vigorous growth; Agarose gel electrophoresis of ISSR fragments of micropropagated (C1-C3) and conventional propagated (C4-C5) strawberry plants with their mother (P) showing monomorphic bands generated by primer IS-7 (c) and IS-65 (d). Lane M-50bp ladder.

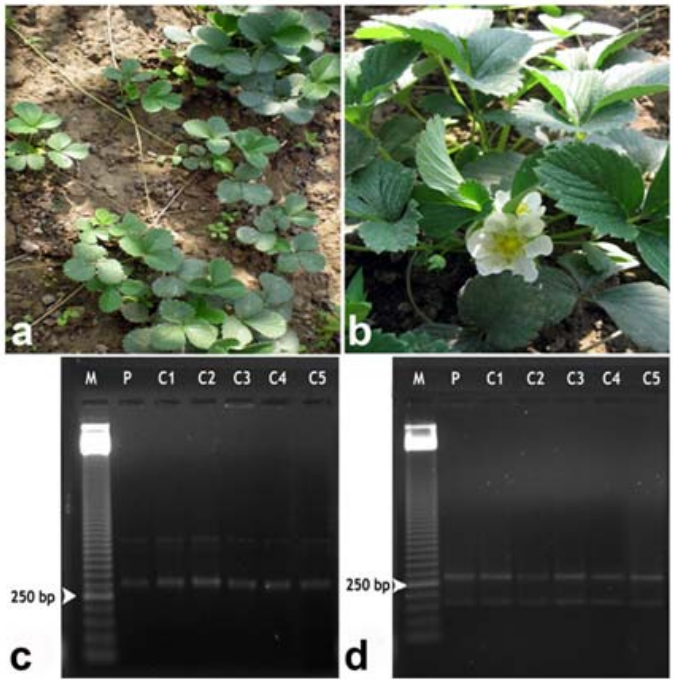

Table 1. *Ex vitro* field performance* of micropropagated in comparison to conventionally propagated strawberry

Treatments	Plant height (cm)	No. of leaves /plant	Leaf length (cm)	Leaf width (cm)	No. of runners/plant	Days to flower induction
Conventional	5.70[a]	7.63[a]	3.27[a]	2.73[b]	5.80[b]	37.03[a]
Micropropagated	5.93[a]	7.77[a]	3.73[a]	3.17[a]	7.50[a]	33.57[b]
Mean	5.82	7.70	3.50	2.95	6.65	35.30
SE (±)	0.1741	0.1548	0.1716	0.0922	0.1753	0.5531
CD at 5%	0.504	0.448	0.497	0.267	0.508	1.602

Data represent mean of 3 replicates per treatment in fifteen repeated experiments
Means within columns separated by DMRT (P=0.05)
*Data were recorded at 5 months growth stage after field transfer

Genetic assessment using ISSR

In the second phase of study *i.e.* the assessment of genetic integrity of in vitro generated as well as ex vitro runner-derived plants IS-6, IS-9, IS-10 and IS-12 did not react with strawberry DNA. The rest of the primers (*i.e.* IS-7, IS-8, IS-11, IS-61, IS-63 and IS-65) displayed a positive interaction but only IS-7 (Fig. 1c) and IS-65 (Fig. 1d) among these showed to be reproducible (Table 2). Each of these two primers generated a unique set of amplified products with the size range of 150 bp in IS-65 to 600 bp in IS-7. The number of bands from each of these successfully used primers remained same. A total number of 24 (number of plants used as sample × average number of bands per sample for all primers) reproducible monomorphic bands were scored from the clones including their mother with an average of 2 bands per primer per sample. In the present study di-nucleotide SSRs motifs AG, GA, GT, TG, CT and CA were used. Two positive and reproducible primers (one based on AG motif and one on GT) amplified a distinct scorable number of bands. Significantly, these two primers anchored at 3' end and are known to give clear banding patterns (Blair et al. 1999). The test of clonal fidelity using ISSR primers was successfully attempted in different micropropagated plant species (Joshi and Dhawan 2007; Bhatia et al. 2009). However, this particular study was not extensively investigated in strawberry though there are some reports on the use of ISSR for genetic diversity in strawberry genotypes (Reddy et al. 2004; Debnath et al. 2008). With strawberry being an octaploid, it can be assumed that limited number of bands produced by these ISSR primers would partially

cover the genome. However, none of the primers showed any difference in the banding pattern. Considering displayed monomorphic banding pattern, it can be suggested that both micropropagated and conventional propagated plants maintained similar genetic clonal integrity though micropropagated plants are better performer in *ex vitro* condition.

Table 2. ISSR primers used for fidelity test of in vitro generated strawberry 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 strawberry DNA	Number of scorable bands per primer	Total number of scorable bands	Size range (bp)
IS-6	52	$(GA)_8C$	3'anchor	Negative	-	-	-
IS-7	50	$(GT)_8A$	3'anchor	Positive , reproducible, monomorphic	2	12	300-600
IS-8	52	$(AG)_8C$	3'anchor	Positive but not reproducible	-	-	-
IS-9	46	$(TG)_7TA$	3'anchor	Negative	-	-	-
IS-10	52	$C(GA)_8$	5'anchor	Negative	-	-	-
IS-11	52	$(CA)_8G$	3'anchor	Positive but not reproducible	-	-	-
IS-12	52	$(GT)_8C$	3'anchor	Negative	-	-	-
IS-61	50	$(GA)_8T$	3'anchor	Positive but not reproducible	-	-	-
IS-63	52	$(AG)_8C$	3'anchor	Positive but not reproducible	-	-	-
IS-65	50	$(AG)_8T$	3'anchor	Positive , reproducible, monomorphic	2	12	150-300
Total					4	24	150-600

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