DOI : 10.5958/0974-0112.2019.00096.3

Indian J. Hort. 76(4), December 2019: 596-602

Studies on genetic fidelity of long term micropropagated culture derived plants of Ofra strawberry using molecular markers

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ABSTRACT

Availability of good plant material of a required cultivar 'Ofra' in a large quantity is a major limitation in expansion of strawberry cultivation. Keeping in view this problem, regeneration through *in vitro* culture has now become a viable and alternate method to conventional one. The formation of healthy shoots and higher rates of multiplication is one of the pre-requisite of an economically viable propagation. Somaclonal variations have been observed in the plants raised through tissue culture, which defeats the purpose of producing true- to- type plants. Therefore, need arises to study the genetic fidelity of tissue culture raised strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' plants. Runners of strawberry cv 'Ofra' used as explants and cultured on MS supplemented with 2mg/l GA_3, 2.0 mg/l BA, 100 mg/l of meso inositol and 30g/l of sucrose gave best results for *in vitro* shoot multiplication. DNA from fresh, young and healthy leaves of 14 *in vitro* raised plants and mother plant of Strawberry cv "Ofra" was isolated following CTAB method. Random amplified polymorphic DNA and inter simple sequence repeat analysis were carried out with 15 and 20 primers out of which 7 and 14 primers produced three and 8 monomorphic bands respectively, for genetic stability studies of the regenerated plants. Comparison of the bands with the mother plant revealed the monomorphic nature and true to type clones. The above regeneration protocol of Strawberry (*Fragaria × ananassa* Duch.) cv 'Ofra' will be useful for micropropagation and genetic transformation studies.

Keywords: *Fragaria* × *ananassa*, regeneration, microsatellites.

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most important members of family Rosaceae. The cultivated strawberry is an octaploid (2n =8x = 56) stoloniferous perennial herb. It is cultivated worldwide for its fruit. The fruits of strawberry are an aggregate of accessory fruits (not a botanical berry) which is widely acknowledged for its characteristic bright red color, juicy texture, aroma, and sweetness. Strawberry is valued for its low-calorie carbohydrate and high fibre contents. It is good source of dietary glutathionine metabolites, flavonoids, carotenoids, vitamins (vitamin B, C and it is also a good source of manganese.), phenols, and exhibit a high level of natural antioxidant capacity against free radicals that are believed to reduce carcinogens in humans and protect against tumor development. The strawberry can be consumed as a fresh food or as a prepared food such as preserves, pies, ice cream, fruit juice, milkshakes, and chocolates. The strawberry is also widely used as artificial flavorings and aromas in many products like candy, hand sanitizer and perfumes.

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Strawberry cultivation is quite labour intensive and is propagated by runners. Clonal fidelity is one of the main concerns in commercial micropropagation of cv 'Ofra'. Ofra is a new and distinct cultivar of strawberry generated by crossing between "Parker" and "111" which results in a variety that flowers several months earlier (early flowering results in early fruit production) than most other known strawberry varieties (Patent number: Plant 8746). The *in-vitro* application of propagation of strawberry is depend on the true-to-type propagules and genetic stability in the shoots. The occurrence of variations named as 'somaclonal variation' in plants regenerated from *in- vitro* cultures and have been reported for morphological and yield variation in micropropagated strawberries. There is a pressing need to recommend the use of reliable methods for studying genetic fidelity of micropropagated plants. Though variations can be studied through morphological and biochemical markers also, but DNA markers are stable and not affected by environmental and developmental stages. The superiority of molecular markers over morphological characterization in fruit species is well established and widely accepted (Sharma *and* Sharma, 13). The trueness-to-type of tissue cultured material was evaluated by using DNA markers like RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple

Sequential Repeats) (Rout and Das, 10), According to Rajdan (9), RAPD analysis can be adequately used to determine the genetic fidelity of *in vitro* grown plants and is very important as genetic variation occurs in isolated protoplasts, tissue, calli, undifferentiated cells and morphological traits of regenerated plants.

MATERIALS AND METHODS

Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' runners being maintained in the fields of Department of Fruit Science, UHF, Nauni, Solan formed the source plant material for *in vitro* propagation. Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' runners were excised from the source plants growing in the fields and sterilized with water having tween 20 for half an hour. Bavistin a fungicide and sodium hypochlorite singly or in combination were used for sterilization of explants (Table 1). After sterilization, nodal segments were cut down into appropriate sizes of 1 to 1.5 cm and blotted dry on sterile blotting paper (Goyal *et al.,* 4).

Nodal segments were cultured on sterile basal MS medium gelled with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.7 ± 0.1 with 1N HCl and 0.1N NaOH prior to the addition of agar followed by autoclaving at 121 ˚C for 20 min at 15 psi. Different concentration of growth regulators like kinetin, BA and GA_3 (Table 2) were experimented in this study. The growth regulators were added to culture medium in desired concentration after filter sterilization. Proliferation in explants and shoots were observed after two weeks. For multiplication of shoots, the obtained shoots were sub-cultured at 5-6 weeks interval on fresh medium for three years (total number of sub culture was 20) (Table 3). All the cultures were incubated at 25 ± 2°C under a 16 h photoperiod and white fluorescent lamps (3000 lux).

Table 1. Different types of treatments given for sterilization of explants.

Sr. No.	Treatment code	Sterilant	Concentration	Duration	Percentage of uncontaminated buds after 2 weeks of culturing
1	T0	Bavistin	0.3%	10 minutes	
		NaClO	-	-	0.00
2	T1	Bavistin	0.3%	5 minutes	
		NaClO	0.1%	3 minutes	66.60 (54.70)[a]
3	T2	Bavistin	0.3%	8 minutes	
		NaClO	0.1%	3 minutes	73.00 (58.71)[a]
4	T3	Bavistin	0.3%	10 minutes	
		NaClO	0.1%	3 minutes	78.00 (62.60)[b]
5	T4	Bavistin	0.3%	12 minutes	
		NaClO	0.1%	3 minutes	79.80 (63.20)[b]
			$CD_{0.05}$	2.12	

Table 2. MS medium with different concentrations and combinations of growth regulators for *in vitro* establishment of cultures.

Sr. No.	Medium (MS basal)	Kn (mg/l)	BA (mg/l)	GA_3(mg/l)	Percentage of bud proliferated
1	M-1	0.5	2	2	65.69 (54.76)[a]
2	M-2	0.5	1.5	2	66.32 (55.14)[a]
3	M-3	0.5	1	2	67.19 (55.68)[a]
4	M-4	-	0.50	2	73.93 (59.97)[b]
5	M-5	-	1	2	77.91 (62.68)[c]
6	**M-6**	**-**	**2**	**2**	**84.09 (67.66)[d]**
7	M-7	-	2.5	2	82.20(65.82)[d]
8	M-8	-	3	2	80.50 (64.23)[e]
				$CD_{0.05}$ 2.45	

Table 3. Medium composition based on MS basal medium and growth regulators for shoot multiplication.

Sr. No.	Medium (MS basal)	Kn (mg/l)	BA (mg/l)	GA_3 (mg/l)	Mesoinositol (mg/l)	Average number of shoots
1.	M-1	0.5	2	3	100	2.20[a]
2.	M-2	0.5	1.5	3	100	3.51[ab]
3.	M-3	0.5	1	3	100	2.57[a]
4.	M-4	-	0.5	3	100	4.11[abc]
5.	M-5	-	1	2	100	5.23[bcde]
6.	M-6	-	1.5	2	100	6.08[cde]
7.	**M-7**	**-**	**2**	**2**	**100**	**7.04[e]**
8.	M-8	-	2.5	2	100	6.76[cde]
9.	M-9	-	3	2	100	4.77[bcd]
			$CD_{0.05}$ 2.23			

The genomic DNA was isolated from *In vitro* Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' shoots, which were maintained from last three years in tissue culture laboratory of the Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). Cetyltrimethylammonium bromide (CTAB) method was used to isolate genomic DNA from green fresh and healthy leaves (14 *in vitro* shoots and mother plant) of micropropagated plants of Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' (Doyle and Doyle, 2). The fresh leaves were ground in liquid nitrogen and incubated for 30 min at 65°C in extraction buffer (10% CTAB; 4M NaCl; 0.5M EDTA; 1M TrisHCl pH 8.0; 2% PVP; 0.2% β-mercaptoethanol). Nucleic acids were purified by Chloroform/isoamyl alcohol (24:1) and phenol/chloroform/isoamyl alcohol (25:24:1). The DNA were precipitated with isopropanol by using a centrifuge and dissolved in the TrisHCl-EDTA buffer. The DNA concentration was determined spectrophotometrically at 260 nm. Before conducting further analyses, the DNA was diluted to 10 ng/µl.

A set of 15 RAPD and 20 ISSR markers were used to analyze genetic fidelity in 14 *in vitro* raised shoots and mother plant (i.e field grown plant). The amplification was carried out with standardized PCR protocol using RAPD and ISSR primers in 20 µl of reaction mixture which consisted of 1X Taq buffer A (with 75mM $MgCl_2$), 1mM dNTP, 10 picomoles primer, 1UTaq DNA polymerase, 50ng DNA.

To carry out PCR amplification with RAPD primer, the following protocol was used: initial denaturation at 95°C for 3 min, followed by 32 cycle's repeat of denaturation at 94°C for 30 second, annealing at 35°C for 30 second, extension at 72°C for 60 second, followed by final extension at 72°C for 10 minute (Singh *et al.,* 11). In The case of ISSR, following protocol was used: initial denaturation at 95°C for 3 minute, followed by 40 cycle's repeat of denaturation at 94°C for 30 second, annealing according to primer Tm for 30 seconds, extension at 72°C for 2 minute and final extension at 72°C for 10 minute (Samriti *et al.,* 12).

The 6X loading dye was thoroughly mixed with amplified DNA. The electrophoreses of this amplified product was then carried out on 1.2% agarose gels for RAPDs and ISSRs. The gel was run at constant voltage at the rate of 5 V/cm under submerged conditions for about 3 hours. Ethidium bromide at the rate of 0.5µg/ml was added to the gel. DNA profiles were visualized on a UV-transilluminator and photographed by using gel documentation system.

The data recorded for different parameters was subjected to completely randomized design (Gomez and Gomez, 3). The monomorphism generated by three types of DNA markers will use to determined genetic fidelity of micropropagated plants. The statistical analysis based on mean values per treatment was made using analysis of variance (ANOVA) technique for CRD.

RESULTS AND DISCUSSION

In this experiment, nodal buds were used as explants for *in-vitro* regeneration. The nodal segment is selected (because of active meristems) which gives rise to axillary buds that grows into shoot by the continuous availability of the cytokinin in the growth medium. Due to these properties, nodal culture forms the basis of vegetative propagation and proves to be suitable explants for *in vitro* regenerations (Goyal *et al.,* 4). However, by means of tissue culture techniques, the shoot multiplication rate can be increased many folds because phytohormones (auxin and cytokinins) accelerate the event.

Contamination by bacteria and fungi was the main problem that posed during the early stages of culture initiation. To get rid of this, bavistin, a fungicide and mercuric chloride singly or in combination were used for sterilization of buds. The treatment T-4 was found to be best sterilization treatment consisted of 0.3% bavistin with 0.1% sodium hypochlorite solution which give rise to 79.80% uncontaminated buds that were able to survive at the end of second week of culturing. The treatment T-0 consisting of 0.3% alone for ten minutes proved to be least effective treatment giving zero % survival of buds whereas T-2 and T-1 consisting of 0.3% bavistin for eight minutes and five minutes, respectively, followed by three minutes treatment with 0.1% mercuric chloride proved reasonably satisfactory treatments giving 73.00% and 66.60% survival of uncontaminated buds (Table 1). The treatments T-4 is statistically significant and no two treatments were found to be at par. Each treatment consisted of 5 replicates (culture flasks) and the experimental unit was 3 explants per treatment. Bondok *et al.,* (1) studied effect of sterilant on the Mariana 2624 plum rootstock explants, after sterilization of explants; next crucial step is to establish the explants on culture medium.

The percentage of bud breaking frequency was increased when basal medium is supplemented with different plant growth regulators like kinetin, BA and GA_3 (Fig. 1). Regeneration frequency of plantlets is also depended upon the type of medium used. The percent survival on different medium combinations was recorded after 2 weeks of culturing. Medium composition M-1 i.e MS basal + 2mg/l GA_3 + 2mg/l BA + 0.5mg/l Kinetin was found to be least responding for bud break whereas M-6 medium as MS basal supplemented with 2mg GA_3 + 2mg BA, was found to be best responding medium giving 84.09% bud break after 2 weeks of culturing followed by M-7 medium consisting of MS basal + 2mg GA_3 + 2.50 mg BA and giving 82.20% of bud break. Medium M-6 was observed to be statistically significant (Table 2. and Fig. 1).

The proliferated buds were transferred to MS medium supplemented with different concentrations and combinations of GA_3, BA, Kinetin and Meso inositol. MS basal medium based a total of nine medium combinations were tested, out of which M-7 media was found to be the best for shoot multiplication upon successive sub-culturing. The maximum average number of shoots obtained per explants after four week of establishment was 7.04 on M-7 medium (Table 3). The number of shoots was increases as much as 25 numbers of shoots were obtained. After sixth or seventh sub-culturing of shoots, the leaves of *in vitro* shoots turn yellowish and rate of multiplication was slow down (Fig. 2). However, the in vitro multiplication process was repeated and carried out for three years. After three years of initial culturing, in vitro multiplication was further carried out without any apparent variation, as fresh culturing are also started to enhance the overall multiplication rate.

Use of more than one marker system has been suggested for better analysis of genetic stability of plants, because they will target different regions of

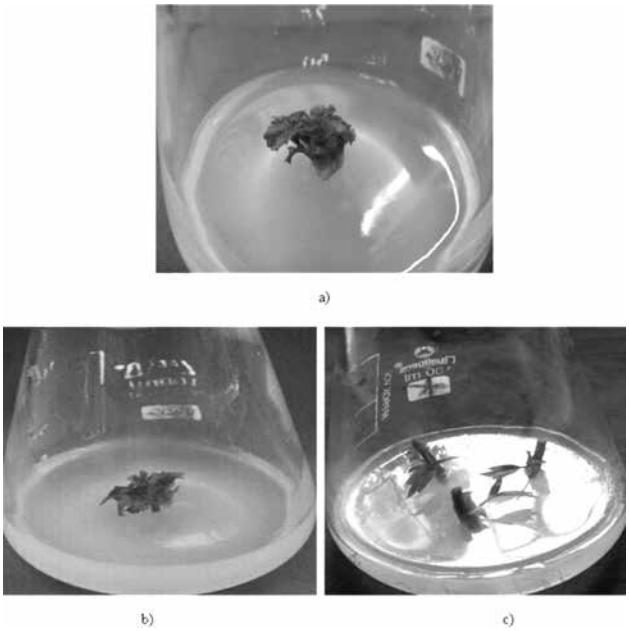

Fig. 1. a), b), c) Shoot initiation in eighteenth stage sub cultured strawberry 'Ofra' explants.

Fig. 2. *In vitro* sub culturing of strawberry cv 'Ofra'.

the genome. Therefore, two sets of primer (RAPD and ISSR) were used in present study (Table 4, 5). The fact that, RAPD (dominant, arbitrary) and ISSR (semi arbitrary, medium to highly reproducible, dominant and more stringent) markers are capable to scan the whole genome randomly and quickly.

Table 4. Data showing amplification of *in vitro* raised 14 rootstock samples and a mother plant using 15 RAPD primers.

Sr. No.	Primers	Primer sequence 5'→3'	Total number of amplified bands	Total number of amplified segments	Size range of amplified bands in base pairs
1.	5383-053	CCTCACGTCC	1	15	200bp
2.	5383-07	ACCCGGTCAC	-	-	-
3.	5383-024	ACGATGAGCC	2	30	200-300bp
4.	5383-088	CGTCCTCAGG	-	-	-
5.	5383-073	CCAGATGCAC	2	18	400-600bp
6.	5383-094	AGAGATGCCC	-	-	-
7.	5383-063	CATGACAGGC	3	20	250bp-500bp
8.	5383-064	TGGAAGAGGC	-	-	-
9.	5383-089	CACTGTTCGG	2	30	200bp-300bp
10.	5383-050	AGT TCC ACG G	-	-	-
11.	5383-085	CTCTGTTCGG	3	20	300bp-500bp
12.	168919	GGAGCCTCAG	-	-	-
13.	5383-056	GTGCTCCCTC	3	17	350bp-800bp
14.	5383-017	GGC ATG ACC T	-	-	-
15.	5383-018	TGG GCG TCA A	-	-	-
Total			16	150	

Table 5. Data showing amplification of 14 *in vitro* raised samples and mother plant using 20 ISSR primers.

Sr. No.	Primer Name	Primer sequences 5′→3′	Total number of amplified bands	Total number of amplified segments
1	UBC-840	GAGAGAGAGAGAGAGACTC	2	20
2	UBC 841	GAGAGAGAGAGAGAGACTC	1	15
3	ISSR 5	AGAGAGAGAGAGAGAGYC	1	32
4	ISSR3E	TCTCTCTCTCTCTCTCA	1	9
5	UBC 829	TGTGTGTGTGTGTGTGC	1	15
6	ISSR 808	AGAGAGAGAGAGAGAGC	1	15
7	IISRS 3M	ACACACACACACACAC	1	15
8	ISSR8	CACACACACACACACA	2	23
9	UBC894	TGGTAGCTCTTGTCAGGCAC	2	9
10	UBC 854	TCTCTCTCTCTCTCTCCAGC	1	15
11	UBC-855	ACACACACACACACACCTT	3	45
12	ISSR-4	AGAGAGAGAGAGAGAGYT	-	-
13	ISSR-2	CAGAGAGAGAGAGAGAYT	-	-
14	ISSR-844B	CTCTCTCTCTCTTGC	-	-
15	ISSR17898B	GATCGAGAGATGCT	-	-
16	UBC886	ACGAGTACGCTCTCTCTCTCTCT	-	-
17	UBC890	ACGACTACCGTGTGTGTTTGTGT	-	-
18	UBC-850	GTGTGTGTGTGTGTGTCTC	3	33
19	ISSR 7	ACACACACACACACACYC	3	11
20	UBC 848	CACACACACACACACAAGG	4	20
Total			26	277

Fifteen random decamer oligonucleotides were used for the amplification of RAPD fragments of which seven primers amplified a total of 16 scorable bands. Out of which three bands obtained were monomorphic (Fig. 3). Out of 20 ISSR used, 14 ISSR primers showed 26 scorable bands and eight were found to be monomorphic (Fig. 4). More fragments were amplified by ISSR primers compared to RAPD which is same to the result of Singh *et al*. (14), where in ISSR marker, amplified maximum number of bands compared to RAPD markers.

Various markers have already proves the suitability of ISSR for genetic fidelity studies. The scoring data of resolved bands of RAPD and ISSR markers were subjected to calculation. The amplified products exhibited monomorphism among all the *in vitro* raised plants and had been found to be similar to those of mother plant. This confirmed the true to type nature of the in vitro raised clones and authenticated that Strawberry (*Fragaria*× *ananassa* Duch.) cv 'Ofra' can remain free from somaclonal variation over a culture period of longer duration. Most of the organized cultures (pre-existing meristems) especially axillary buds and shoot tips maintain strict genotypic and phenotypic stability compared to *de novo* originating meristematic structures like adventitious buds directly derived from cultured tissues or differentiated from callus.

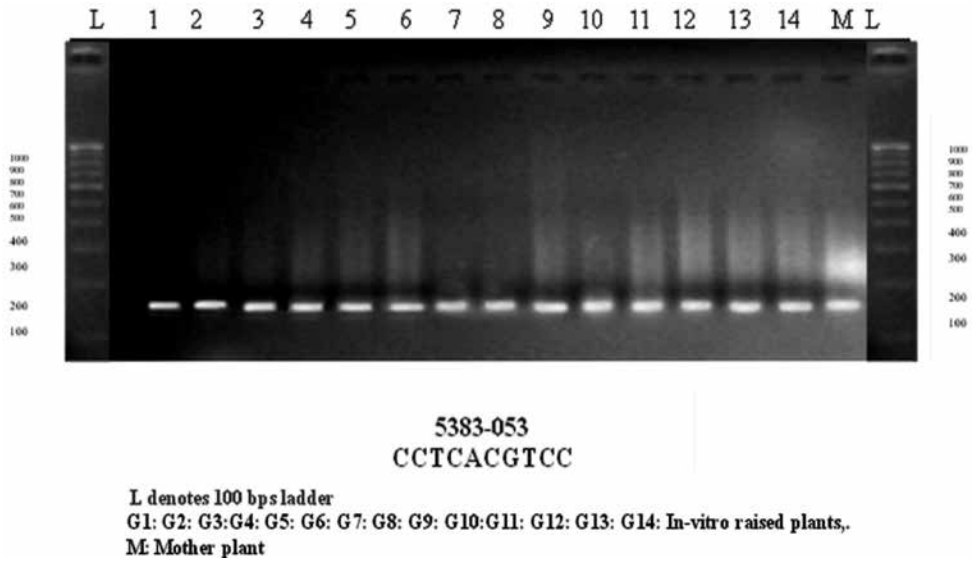

Fig. 3. Gel image of RAPD Primer 5383-053.

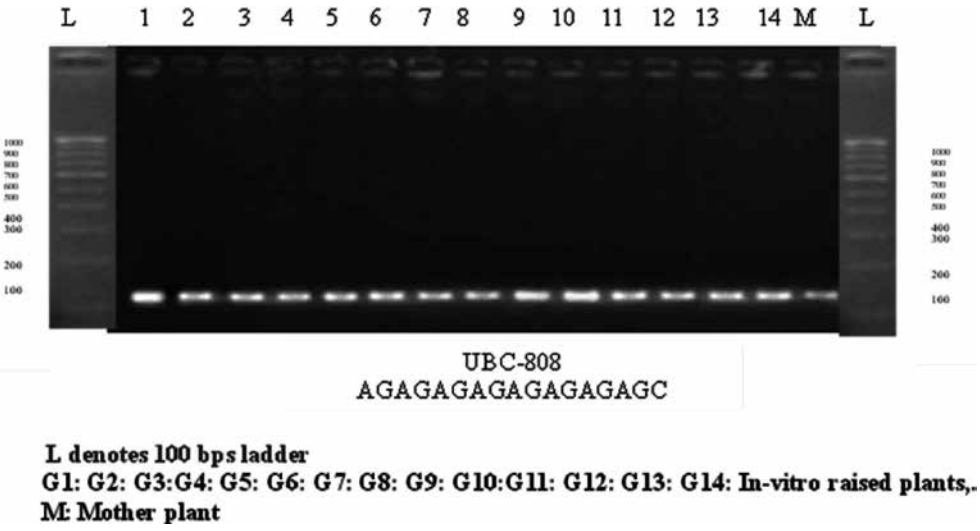

Fig. 4. Gel image of ISSR primer UBC-808.

Propagation by axillary buds evade redifferentiation and dedifferentiation of cells and tissues, outwit genomic aberrations and inevitably maintaining the clonal fidelity of *in vitro* raised plantlets studies on the length of culture been reported in almonds (Martins *et al.,* 6), *Swertia chirayita* (Joshi and Dhawan, 5), Curcuma longa L. (Panda *et al.,* 8) and bambusa balcooa Roxb. (Negi and saxena, 7) where in the *in vitro* cultures were maintained for a period of >2 years. The retention of clonal uniformity for prolonged period under *in vitro* conditions has immense commercial significance because initiation of *in vitro* cultures is difficult in Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' due to season specificity, persistent contamination, phenolic exudation etc. Therefore, due to maintenance of genetic stability in Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' rootstock for long time, it is possible to supply genetically uniform plants continuously for a prolonged duration without resorting to initiation of fresh cultures frequently, which in turn will reduce the overall cost of plant production at commercial scale.

The major concern associated with in vitro plants is the occurrence of somaclonal variations in the sub-clones of one parental line. In the present investigation an emphasis has been given to assess the genetic integrity of micropropagated Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' using DNA marker studies i.e. RAPD and ISSR. The occurrence of trueness-to-type in micropropagated plant materials with mother plant displayed satisfactory results. From this study, it is concluded that all two kinds of markers i.e RAPD and ISSR can be applied to determine genetic integrity of micropropagated plants of Strawberry (*Fragaria× ananassa* Duch.) cv 'Ofra' and micropropagated protocol can be used for long period without the risk of somaclonal variations.

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Received : August 2018; Revised : .November 2019;
Accepted : November 2019