Research Paper / 65-72

Detection of somaclonal variation in plants regenerated from different tissues of strawberry (*Fragaria × ananassa*) using ISSR marker

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Abstract

Production of genetically and phenotypically stable plantlets is the main purpose in commercial strawberry tissue culture. In this study, different tissues of *Fragaria ananassa* cv. Camarosa including stipule, apical meristem, leaf and petiole were cultured in Murashige and Skoog (MS) medium supplemented with different concentrations of N^6-benzyladenine (BA) (0.5, 1, 2, 3 mg/L) and indole-3-butyric acid (IBA) (0.1 and 0.5 mg/L). For apical meristem explants, the best regeneration rate (7.6 shoots per each explant) was obtained in the medium containing 1 mg/L BA and 0.1 mg/L IBA. Whereas stipule explants showed the highest regeneration rate in the medium containing 2 mg/L BA and 0.1 mg/L IBA. For leaf and petiole explants, the medium containing 2 mg/L BA and 0.5 mg/L IBA had the best hormonal combination. To determine the genetic variation, micropropagated plants from different tissue (after 8 subcultures) were analyzed by the inter-simple sequence repeats (ISSR) molecular marker. Among the 18 pre-selected primers, 10 displayed clear, reproducible and informative bands. A total of 88 distinct bands with a polymorphic rate of 46% were produced in the molecular profile of different explants. The highest and the lowest similarity values to maternal plants belonged to stipule and petiole explants with a similarity index of 0.738 and 0.645, respectively. Vitroplants derived from stipule and apical meristem showed the highest genetic stability with respect to maternal plants. With respect to genetic stability and regeneration rate, apical meristem can be recommended as suitable explants with the highest genetic fidelity. The findings of this study could be applied for commercial scale multiplication of strawberry, and also demonstrate that ISSR markers are eligible for detection of somaclonal variations.

Key words: *Fragaria*, Genetic stability, ISSR, Micropropagation, Molecular markers, Somaclonal variation.

INTRODUCTION

Strawberry is one of the most popular fruits for its pleasant taste and high nutritional value in the world. The commercial cultivars of *Fragaria ananassa* are octoploid in chromosomal level (Debnath and Teixeira da Silva, 2007). The common method of the large scale vegetative propagation in strawberry is separating daughter plants from mother plants in the field (Gantait *et al.*, 2010). This method leads to clone and true-to-type plants completely similar to the mother plant (Viswavidyalaya, 2011). However, there are some disadvantages of this method such as the requirement of high cultivation area, being time-consuming, being prone to fungal and viral infection

(Gantait *et al.*, 2010). Tissue culture technique can be a good alternative procedure for the propagation of different plant species (Tahmasi *et al.*, 2017; Salari *et al.*, 2018).

The production of strawberry plants through *in vitro* propagation of plants (micropropagation) offer an important alternative to conventional field-grown strawberry. Micropropagated plantlets are grown in controlled conditions (greenhouse), in less time than the field produced transplants, and are not exposed to soil-borne pathogens (Durner *et al.*, 2002). However, to achieve the desired result, the following points including propagation rate, somaclonal variation and mutation observation in the field should be considered.

The main concern of strawberry micropropagation for commercial or genetic engineering use is maintaining genetic stability with regard to the mother plant (Sahijram *et al.*, 2003). Stability of micropropagated plants is an interesting research subject from both genetic and epigenetic points of view. Both genetic and epigenetic changes, called somaclonal variations, are associated with *in vitro* propagation (Gimenez *et al.*, 2016; Olhoft and Phillips, 2018). In this regard, somaclonal variation has been reported at various levels (phenotypic, cytological, biochemical and genetic/epigenetic) in micropropagated plants (Noormohammadi *et al.*, 2014; Solano *et al.*, 2019).

Somaclonal variation which is generally induced by different genetic or environmental factors such as hormonal combination, regeneration method, and mutagenic elements can influence the uniformity of micropropagated plants (Litz, 2005). Thus, assessment of somaclonal variation or determination of genetic stability is essential for mass production of vitroplants to ensure the uniformity of individual plants within a clone population.

A wide variety of tools based on morphological traits, cytogenetical patterns, biochemical and molecular markers could be applied for assessment of genetic stability (Naing *et al.*, 2019). The use of molecular profiling to ensure the uniformity of vitroplants is more suitable than conventional methods because of their simplicity, accuracy, high reproducibility and cost-effectiveness (Agarwal *et al.*, 2008; Babaei *et al.*, 2011; Haghpanah *et al.*, 2016).

Molecular markers based on microsatellite are the best marker for studying eukaryotes genome due largely to their random distribution in the whole genome(Jahani *et al.*, 2014; Mahjoob *et al.*, 2016). Applications of ISSR markers have been increasingly developed due to their widespread distribution and high copy number in the plant genome (Shingote *et al.*, 2019; Solano *et al.*, 2019). ISSR markers were widely used for assessment of molecular stability in cabbage (Leroy *et al.*, 2000) banana (Lakshmanan *et al.*, 2007; Venkatachalam *et al.*, 2007), *Polianthes tuberosa* (Joshi and Dhawan, 2007) and bamboo (Negi and Saxena, 2010).

Although regeneration from different part of strawberry has been reported from leaf (Debnath and Teixeira da Silva, 2007), apical meristem (Ghasemi *et al.*, 2015), petiole, stipule and root (Passey *et al.*, 2003), few studies have been designed to evaluate the genetic stability in vitroplans from different explant sources. The aim of this study was to assess 1) interaction effect of plant growth regulators (BA and IBA) and explants type on the proliferation rate of *Fragaria ananassa* 'Camarosa'; 2) genetic fidelity of sub-cultured plants which originated from different tissues; 3) sensitivity of the ISSR marker to detect a genetic change in vitroplants of strawberry.

MATERIALS AND METHODS

Plant material prepration and medium composition

A commercial cultivar of strawberry, Camarosa, was selected for explants preparation. The experiments were conducted in Genetic and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University (SANRU). Different tissues of strawberry including apical meristems, leaves, petioles and stipules were separated and surface sterilized with 70% (*v/v*) ethanol (EtOH 96%, Merck) for 40 seconds. After incubation in 0.5% (*v/v*) sodium hypochlorite (NaOCl with 5 percent active chlorine) for 7 minutes, all explants were soaked in 0.1% (*v/v*) mercuric chloride ($HgCl_2$, Merck) for 5 min (Ko *et al.*, 2009). Finally, the explants were washed three times with sterile distilled water (3 min each time) and cultured onto fresh basal MS medium (Murashige and Skoog, 1962). The medium pH was adjusted to 5.8 before autoclaving. After one week, explants were transferred into glass jars containing solidified MS medium with 0.7% agar supplemented with different concentrations of plant growth regulators. Direct regeneration of different explants (apical meristems, leaves, petioles, stipules) were examined in different combinations and concentrations of N6-benzyladenine (BA) (0.5, 1, 2, 3 mg/L) and indole-3-butyric acid (IBA) (0.1 and 0.5 mg/L). All explants were kept in a growth chamber (25±1 ºC) for four weeks under a 16-hour photoperiod and photosynthetic photon flux of

200 µmol m^2 s^{-1}. For analysis of the regeneration rate, a factorial experiment with three factors including BA concentration (at four levels), IBA concentration (at two levels) and explant type (at four levels) was conducted in a completely randomized design with four replications. Four explants per replication were used in all experiments. Data for each experiment were subjected to analysis of variance (ANOVA) by the general linear model procedure using Statistical Analysis Software (SAS). Mean values were compared using Duncan's multiple range test at 5% significant level.

***Ex vitro* rooting and acclimatization**

After choosing the best medium for each tissue based on Duncan test, eight subsequent subcultures (four weeks' time of each subculture) were done in the selected medium. Thus, direct regeneration of each tissue was continued under the same conditions in the selected medium. Finally, regenerated shoots were rooted on ½ MS+supplemented with 0.5 mg/L IBA for four weeks and then were transferred to *in vivo* conditions. Acclimatization of micropropagated plants was carried out in a greenhouse under photosynthetic photon flux 300 µmol m^2 $s^{-1}$and 75% relative humidity. The fresh leaves of three-month plants were collected for ISSR analysis.

Genetic stability analysis by ISSR markers

To determine the genetic variation, the DNA of strawberry mother plants (g1) was extracted along with three-month acclimatized plants which were originated from eight subsequent subcultures of the best hormonal treatment for each tissue; apical meristem (g2), stipule (g3), leaves (g4) and petioles (g5) tissue. Twelve explants per tissue were bulked and DNA was extracted by DNeasy Plant Mini Kit (Qiagen, Germany). The quality and quantity of extracted DNA were determined using 0.7% agarose gel electrophoresis and NanoDrop spectrophotometer (BiochromWPA Biowave II, UK). ISSR profiling was carried out in 12.5 µl reaction mixture containing 15 ng DNA, 3 mM $MgCl_2$, 2 mM of each dNTP, 10 µM primer, 1 U *Taq* DNA polymerase (Thermo Scientific, USA) and 1.25 µl 10×PCR buffer (200 mM Tris HCl (pH 8.4), 500 mM KCl). The thermal cycler (MJMini, BioRad, USA) program was set as follow: 4 min at 94 ºC, 35 cycles of 94 ºC for 1 min, 55-57 ºC (depending on each primer annealing temperature) for 1 min and 72 ºC for 1 min (Mahjoob *et al.*, 2016). Finally, the last step was set at 72 ºC for 10 min. The amplified products were resolved in 1.5% (w/v) agarose (Merck, Germany) gel (0.5×TBE), stained with ethidium bromide (10 µg/ml,) and visualized under UV light scanning system (Biometra, UK).

Statistical analysis

For ISSR data analysis, the amplified bands were scored as presence (1) and absence (0) of bands across all genotypes. The similarity matrix and principal coordinate analysis were generated by NTSYS-PC version 2.02 software. Polymorphic information content (PIC) was calculated with the equation of PIC= 2fi (1 - fi), where fi is the frequency of the polymorphic bands and (1 – fi) is the frequency of monomorphic bands. Marker index (MI) was calculated according to the following formula (equation 1):

(1) $MI = Hav \times MR$

RESULTS

The results of analysis of variance showed that there were significant differences between plant growth regulators (BA and IBA), explants type (apical meristem, stipule, leaves and petioles), and their interactions (Table 1). Effect of medium composition on shoot regeneration (direct axillary shoot) was observed in all types of explant, while the callus formation was not detected in any explants. There were significant differences among interactions at 1% level and 5% for explants×plant growth regulators and BA×IBA, respectively (Table 1). From regeneration rate point of view, apical meristem cultured on MS medium containing BA (1 mg/L) and IBA (0.1 mg/L) was the best medium with the average of 7.6 shoots per explant (Table 2). Since the purpose of this investigation was to compare genetic stability of micropropagated plants derived from different explants, the best regeneration treatment was determined for each explant (bolded in Table 2), separately. The highest regeneration efficiency in stipule explants was obtained

Table 1. Analysis of variance for regeneration rate in *Fragaria ananassa*'Camarosa'.

Source of variation	df	Mean of square
Explants	3	32.87**
BA	3	18.79**
IBA	1	7.59**
Explants×BA	9	4.21**
Explants×IBA	3	9.09**
BA×IBA	3	0.79*
Explants×BA×IBA	9	0.55*
Error	64	0.21
Coefficient of variation (%)	20.5	-

* and ** : represent significant difference (P<0.05) and very significant difference (P<0.01), respectively.

Table 2. The interaction effect of plant growth regulators (BA and IBA) and explants type on the regenerated shoots per explant of *Fragaria ananassa* 'Camarosa'. Selected medium based on higher regeneration rate were bolded.

Explants type	Plant growth regulators		Shoot number/explant	Code of selected medium in ISSR analysis
	IBA (mg/L)	BA (mg/L)		
Apical meristem	0.1	0.5	4.3^{b}	
		1	**7.6^{a}**	g2
		2	4.6^{b}	
		3	2.6^{cd}	
	0.5	0.5	2.6 ^{cd}	
		1	4.3^{b}	
		2	3^{c}	
		3	1.3^{fgh}	
Leaf	0.1	0.5	1^{gh}	
		1	1.6^{efg}	
		2	2.3^{cde}	
		3	1^{gh}	
	0.5	0.5	1^{gh}	
		1	1.6^{efg}	
		2	**3^{c}**	g4
		3	1.6^{efg}	
Petiole	0.1	0.5	0^{i}	
		1	1^{gh}	
		2	1.6^{efg}	
		3	0.66 ^{hi}	
	0.5	0.5	0^{i}	
		1	1.6^{efg}	
		2	**2.6^{cd}**	g5
		3	1.3^{fgh}	
Stipule	0.1	0.5	2.3^{cde}	
		1	3^{c}	
		2	**4.6^{b}**	g3
		3	1.3^{fgh}	
	0.5	0.5	1^{gh}	
		1	2^{def}	
		2	2.6^{cd}	
		3	1^{gh}	

in the MS medium containing 2 mg/L BA and 0.1 mg/L IBA. Furthermore, the medium containing 2 mg/L BA with 0.5 mg/L IBA was selected as the best treatment for leaf and petiole regeneration. In apical meristem, the existence of shoot primordial in microscopic levels accelerated shoot regeneration by a low concentration of external hormones, while in other explants for the transition of explants from microscopic to macroscopic form the induction of shoot primordial should be triggered initially.

Among the 18 primers tested initially, 10 ISSR primers amplified clear and unambiguous bands. Out of 88 produced bands by these primers, 41 bands were polymorphic (46%). The maximum and minimum number of the bands per primer were 13 and 5, respectively.

The gel profile of ISSR-11 primer for all given explants is shown in Figure 1. The maximum number of polymorphic bands was eight, while the minimum number of polymorphic bands was 2 and average polymorphic bands per primer was 4.1. The maximum, minimum and average values for polymorphism

information content (PIC) were 0.48, 0.32 and 0.363, respectively. The highest, the lowest and the average rates of marker indices (MI) were 32, 7.4 and 18, respectively (Table 3). The ISSR-7 and ISSR-2 primers generated more informative band in this study.

Dendrogram of cluster analysis was depicted according to the result of the mantel test that showed a high level of the cophenetic correlation values (0.94) between simple matching similarity matrix and UPGMA algorithm. The highest similarity value among regenerated plants based on the SM similarity coefficient was 0.73 and 0.72, belonging to stipule and apical meristem -derived plants, respectively. The lowest similarity value was obtained from the petiole–derived plants with the value of 0.64. Based on SM coefficient a high level of similarity between regeneration plants and mother plants were observed in stipule (0.73) and (0.72) apical meristem, while the lowest level of similarity was observed between petioles and their mother plants (0.64) (Figure 2). According to the results, use of the stipule and apical meristem has the highest efficiency to strawberry regeneration under *in vitro* culture. Results of the cluster analysis showed that all examined samples were classified into

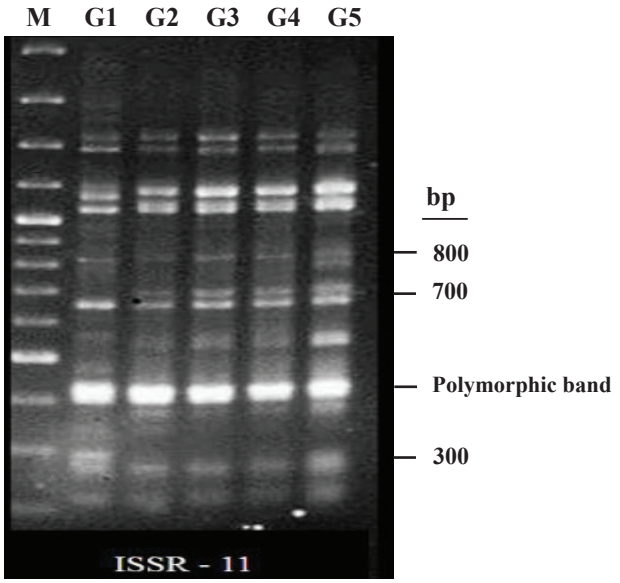
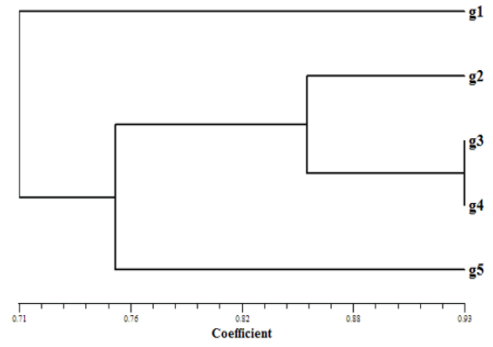


Figure 1. The profile of agarose gel electrophoresis of strawberry micropropagated plants based on ISSR-11 primer. The lanes of g1, g2, g3, g4, and g5 referred to strawberry mother plant, apical meristem, stipule, leaf, and petiole explants, respectively. Lane M devote to Gene ruler 100 bp Plus DNA Ladder.



	g1	g2	g3	g4	g5
g1	1				
g2	0.727	1			
g3	0.738	0.852	1		
g4	0.715	0.852	0.931	1	
g5	0.645	0.784	0.721	0.759	1

Figure 2. Similarity matrix and cluster analysis of 5 micropropagated strawberry explants generated from ISSR profiling data.

Table 3. Data generated from genetic stability assessment of micropropagated strawberry by ISSR marker.

Primer	Sequence (5'-3')	Number of observed band	Number of polymorphic band	Polymorphism percent (%)	PIC	MI
ISSR-1	(GA)9-A	6	2	33.3	0.32	10.7
ISSR-2	(GA)9-C	9	3	33.3	0.32	10.7
ISSR-3	(GA)9-T	12	8	66.7	0.4	26.7
ISSR-7	(GA)8C	11	4	36.4	0.37	13.5
ISSR-9	(AG)8C	11	5	45.5	0.38	17.3
ISSR-11	(GA)8C	13	3	23.1	0.32	7.4
ISSR-12	(GA)8A	6	2	33.3	0.32	10.7
ISSR-15	(AC)8G	6	6	100.0	0.32	32.0
ISSR-16	(TG)8A	5	3	60.0	0.48	28.8
ISSR-18	(ATC)6T	9	5	55.6	0.4	22.2

two main clusters, and the distance between these two groups was 0.75. Four different tissue culture-derived explants were categorized in one cluster (g1, g2, g3 and g4) while mother plants were solely located in the other clusters (Figure 2).

DISCUSSION

Molecular markers are reliable tools for the study of somaclonal variation in regenerated plants (Shingote *et al.*, 2019; Solano *et al.*, 2019). Advantages of some molecular markers such as AFLP, RAPD, MSAP and ISSR are their universality, independence of pre-sequenced data for primer construction and relatively uniform distribution in the genome (Hashemi-Petroudi *et al.*, 2010; Hashemi *et al.*, 2012; Hashemi-Petroudi *et al.*, 2014). In this study, the ISSR markers were utilized for the detection of genetic changes in micropropagated plants raised from different strawberry explants. The distinct patterns in ISSR profiles observed between the mother plants and tissue culture derived explants could be a line if evidence for the existence of somaclonal variations among given samples. The result of the similarity matrix showed that genetic variation induced in various explants was partly dependent on the types of explants. It should be noted that the observed genetic variation may be related to a genetic mutation in explants genomes. Based on results obtained from ISSR profiling, g2 (0.273) and g3 (0.262) explants had the lowest genetic distance to their maternal plants, and accordingly, it could be stated that these explants possess more genetic stability compared to other explants under *in-vitro* conditions. The explants tissue type and regeneration method (shoot organogenesis versus embryogenesis) may influence the rate of somaclonal variation (Renau-Morata *et al.*, 2005; Sahraroo *et al.*, 2019). Somatic embryogenesis is considered as the most reliable method for generating a large number of genetically stable clones (Leroy *et al.*, 2000; Sarkar and Jha, 2017). Rout *et al.* (1998) reported that micropropagated plants derived from the shoot buds of ginger did not show any somaclonal variation. Similar results have been reported in micropropagated gerbera plants (Bhatia *et al.*, 2009) and *Chlorophytum borivilianum* (Samantaray and Maiti, 2010). Genetic stability of micropropagated plants from apical meristem may be backed by the strong resistance to genetic variation during cell division and cell differentiation due to the existence of shoot preformed bud or shoot primordia (Shenoy and Vasil, 1992; Thorat *et al.*, 2018). As mentioned above, shoot primordia located in apical meristem exist as microscopic form. Once the external hormone is added, primordia can be activated. While in other explants such as petiole, leaf and stipule, the undifferentiated cells are necessary for the induction of primordia (Bacchetta *et al.*, 2008). Somaclonal variation observed in one plant can be an taken as evidence for genetic or epigenetic changes occurring in the genome during continuous subculture. This phenomenon is one of the disadvantages of tissue culture technique, but sometimes the changes can be useful in plant breeding program (Krishna *et al.*, 2016). Biswas *et al.* (2009) proved that the high concentration of cytokinin such as BA increased the somaclonal variation in strawberry so that some somaclone varieties have been identified in this approach.

The culture of the strawberry apical meristem in MS medium containing 1 mg/L BA and 0.1 mg/L NAA caused the highest regeneration value with minimum morphological and molecular changes (Gantait *et al.*, 2010). Application of BA in 5 mg/L and kinetin in 4 mg/L was the best concentration in banana tissue culture as a regenerated shoot number and the lowest genetic change was detected after three subcultures by using of RAPD and ISSR markers (Viswavidyalaya, 2011).

In this study, stipule and apical meristem as the explant had the highest genetic stability after eight subcultures, and these explants can be used for direct shoot regeneration for commercial production. However, comparing the regeneration rate between stipule (2.5 shoot/explants) and apical meristem (7 shoot /explants) showed that the apical meristem had approximately two times higher regeneration rate than stipule.

CONCLUSION

In this study, the interaction effect of explants type (stipule, apical meristem, leaf and petiole) and plant growth regulators (BA and IBA) were assessed. For leaf and petiole explants, the medium containing 2 mg/L BA and 0.5 mg/L IBA was the best hormonal combination. For apical meristem explants, the best regeneration rate was obtained in the medium containing 1 mg/L BA and 0.1 mg/L IBA. Whereas, stipule explants showed the highest regeneration rate in the medium containing 2 mg/L BA and 0.1 mg/L IBA. To determine the genetic fidelity, three-month *in vitro* acclimatized plants which were originated from eight subsequent subcultures were analyzed by the ISSR molecular marker. Based on ISSR analysis, the highest similarity coefficient belonged to stipule and apical meristem-derived plants with the value of 0.73 and 0.72, respectively.

According to the ISSR results, use of the stipule and apical meristem explants had the highest genetic fidelity in strawberry micropropagation. Overall, with respect to genetic stability and regeneration rate, apical meristem can be recommended as suitable explants for commercial scale multiplication of strawberry.

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