Middle East Journal of Agriculture Research
Volume: 11 | Issue: 02| April – June| 2022

EISSN: 2706-7955 ISSN: 2077-4605
DOI: 10.36632/mejar/2022.11.2.21
Journal homepage: www.curresweb.com
Pages: 324-339

Assessment of phenotypic and genetic stability in strawberry plants cv. Festival derived from different *in vitro* propagation methods using SSR and ISSR markers

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Received: 03 March 2022 **Accepted:** 05 April 2022 **Published:** 15 April 2022

ABSTRACT

Different propagation methods using two explants types, subculture number and growth regulators type and concentration were studied to examine their effects on organogenic potential, phenotypic and genetic stability in strawberry cv. Festival. Our results proved that the significant highest shoot number was achieved after 7th subculture of meristem tip on MS medium containing 0.5 mg/l BA and direct regeneration of shoots from leaf disc explants on TDZ containing medium. However, the significant lowest shoot number was obtained after 12th subculture of meristem tip using 2 mg/l BA. Shoot number produced through an indirect regeneration method using 2,4-D+BA significantly decreased in comparison to the direct regeneration method using TDZ-containing medium. *Ex vitro* phenotypic test showed that leaf morphology, serration pattern and stomata number were similar among plants obtained from different propagation methods comparing with mother plants. In the same direction, SSR analysis indicated that plantlets of cv. Festival obtained from meristem-propagation method subculture up to 12th generation using high BA concentration (2.0 mg/l) were genetically stable (zero% polymorphism) as compared with control plantlets and *ex vitro* mother plants. Also, direct regenerated and callus derived plantlets were also genetically stable using SSR markers. However genetic differences were detected using ISSR technique (32% polymorphism), where plantlets from 12th subculture using high BA (2.0 mg/l) and callus derived plantlets were genetically differed from each other and from control plantlets, mother plants as well as plantlets derived from 7th subculture using 0.5 mg/l BA. Micro-propagated shoots from meristem culture up to 7th subculture and regenerated ones from leaf disc using TDZ may be produce genetically stable plantlets in the commercial production nurslings of cv. "Festival".

Keywords: *Fragaria* x *ananassa*, *in vitro* propagation methods, subculture number, BA concentration, genetic stability.

1. Introduction

The commercial important Strawberry (*Fragaria* × *ananassa* Duch.) belongs to the family *Rosaceae*. It's an economically important, highly popular crop and is in great demand for fresh markets as well as in the fruit processing industry. Also popularity can be judged from the fact that their production has increased considerably in recent years (Husaini and Zaki, 2016). In addition strawberry is valued for its low-calorie carbohydrate, high fiber contents and good sources of natural antioxidants such as carotenoids, phenols, vitamins and flavonoids (Giampieri *et al.,* 2015; Amatori *et al.,* 2016; Afrin, *et al.,* 2016)

Strawberry is one of the main plants of horticultural interest, which is multiplied by micro-propagation, compared to conventional planting methods. Among the various methods developed to micro-propagate plants, the improvement of culture of axiliary branching has become the most important method of propagation. This method is especially advantageous, because it is simple and the propagation rate is relatively high. More importantly, it is generally considered to be an *in vitro* culture

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system with low risk of genetic instability (Debnath, 2013; Palei *et al.,* 2015; Naing *et al.,* 2019), because the organized mersitems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Vasil & Vasil, 1980).

The results of Diengngan *et al.,* (2016) showed that the reproductive responses of *in vitro* propagated plants cv. Festival in the field were superior over the runner propagated plants. The economics of strawberry tissue culture industry are directly dependent on the number of propagation subcultures. As a number of subcultures increases (i.e. more than 8) then the number of plantlets obviously increases which gives more profit. It is generally recommended not to exceed four to five subcultures to avoid loss of trueness-to type of the propagated material (Faedi *et al.,* 2002). Libek and Kikas (2003) reported that the younger mericlones produce plants with higher runner production rates than older ones. During micropropagation, a high rate of proliferation is achieved in relatively shorter periods and leads to more frequent subculturing. El-Sayed *et al.,* (2017) reported that increasing subculture number up to 6 subculture had the highest multiplication rate in three strawberry cultivars. Also, Mohamed *et al.,* (2018) found that the highest multiplication rate in strawberry cvs. Camarosa, Fortuna, Kamela and Amiga up to 6 or 7 subcultures using 0.5 or 1 mg/l BA, depending on the cultivar in comparison with twelfth subcultures using 2 mg/l BA. However, Biswas *et al.,* (2009) reported that the multiplication rate did not differ significantly among meristem culture, after second and twelfth subcultures. Also, somaclonal variants appeared from the twelfth subculture as reported by Biswass *et al.,* (2009). However, no somaclonal variants were appeared up to 10 subcultures in cvs. "Festival" and "Rubigem" (Ayvaz-Sönmez and Kafkas, 2016) and up to twelfth subcultures in cv. "Aromas" (da Fonseca *et al.,* 2013).

Also from the advantages of *in vitro* culture, the direct and indirect shoot regeneration systems allow the production of variant plants, of which, some might show unique characteristics as new somaclones. Direct regeneration was observed in leaf discs using 9.8 µM TDZ (Husaini and Abdin, 2007). Also, Debnath (2005) reported an efficient *in vitro* system to regenerate shoots on excised sepals (calyx) of greenhouse grown strawberry using 2-4 µM TDZ-containing medium. The results of Mohamed *et al.,* (2007) generally proved that the shoot regeneration occurred directly in medium amended with TDZ alone and the highest shoots number was in cv. "Festival". Regarding indirect regeneration, Biswas *et al.,* (2010) found that highest shoot regeneration rate from leaf disc explants was achieved using 1.5 mg/l BA + 4.0 mg/l NAA, then 3 mg/l BA. Karim *et al.,* (2011) were able to induce callus from immature *in vitro* leaves with 3.0 mg/l 2,4-D and 0.5 mg/l BA, then shoots indirectly regenerated on a medium amended with 0.5 mg/l BA + 0.25 mg/l NAA. Also, Karim *et al.,* (2015) produced callus on MS medium supplemented with 2 mg/l NAA + 0.5 mg/l BA and shoot regenerated indirectly by transferring the callus to the MS medium containing 1.5 mg/l BA + 0.75 mg/l NAA + 0.5 mg/l Kin. In the same direction, Palei *et al.,* (2017) studied callus induction and indirect regeneration from different strawberry explants. Callus-derived on MS medium + BA, indirectly regenerated shoots on MS + 2.5 mg/l BA + 0.5 mg/l NAA. Recently, strawberry leaf explants successfully induced green compact calli with 1 mg/l 2,4-D + 0.5 mg/l BA, then the calli subcultured on medium containing 1 mg/l TDZ and 0.2 mg/l 2,4-D produced 3.8 microshoots (Chomboon and Lohasupthawee, 2022).

In Strawberry, the genetic stability of *in vitro* plantlets is often questioned because there are frequent studies on the occurrence of somaclonal variations not only in non- directive regenerated plants but also in micro-propagated plants ones (Biswas *et al.,* 2009; Nand Kishor Sharma and Kumar, 2012; Sutan *et al.,* 2009; Ayvaz-Sönmez and Kafkas 2016; Mohamed *et al.,* 2017).

In this context, molecular markers, such as randomly amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) are useful, suitable and have proven to be efficient in detecting genetic stability in regenerated and micro-propagated plantlets in strawberry plants (Biswas *et al.,* 2009; Sutan *et al.,* 2009; Hosseini *et al.,* 2013, Ayvaz-Sönmez and Kafkas 2016; Mohamed *et al.,* 2017).

In spite of, inter simple sequence repeats (ISSR) markers are used to detect genetic stability in regenerated shoots and micropropagated material in other plants such as Banana, Gooseberry and Greek sweet cherry (Ray *et al.,* 2006; Venkatachalam *et al.,* 2007; Ganopoulos *et al.,* 2011; Jung *et al.,* 2021; Wójcik *et al.,* 2021), no previous research was found to detect genetic variability among different *in vitro* propagation methods in strawberry using ISSR markers. However inter simple sequence repeats (ISSR) markers are used to detect genetic variability among strawberry genotypes (Corrêa *et al.,* 2016; Kaleybar *et al.,* 2018; Saridas *et al.,* 2021).

Among the various molecular markers (based-DNA) developed for assessment of genetic stability/variability, microsatellites or simple sequence repeats (SSRs) are a relatively new class of DNA markers based on tandem repeats of short (2 - 6 nucleotides) DNA sequences that occurs within or between gene sequences. This marker is especially helpful compared with the others, because it is highly polymorphic, even among closely related genotypes, specific markers, rapid, dependable abundant, co-dominant, evenly dispersed along the genome, highly reproducible, somatically stable and easy to assay using PCR (Sánchez-Pérez *et al.,* 2005). More importantly, Gur-Arie *et al.,* (2000) reported that SSRs existing either in the coding regions of DNA sequences (expressed sequence tag-SSR) or in non-coding regions (genomic DNA sequences). Whereas, the EST-SSR markers are designed from highly conserved genomic regions and more linked to genes of agronomic importance (Jung *et al.,* 2005). However, genomic DNA sequences (g SSR) markers are scattered in abundance in the genome and more easily transferred across taxa, in addition to highly evolutionary regions (Varshney *et al.,* 2005). In the same context, ISSR markers based on di-, tri-, tetra- or penta-repeated nucleotides and abundant throughout the plant genome in addition to these sequences are existing in coding or non-coding regions (Wang *et al.,* 1994). While, Wang *et al.,* (1998) declared that these markers are dominant following simple Mendelian inheritance. Generally, these markers are considered to be highly polymorphism, more reproducible than RAPD markers possibly due to, high annealing temperature and the primers sequences are longer (16-25 bp) and are random-type markers, moreover these sequences are highly evolutionary regions and characterized by a high level of mutagenicity (Reddy *et al.,* 2002). Korbin *et al.,* (2002) observed that ISSRs were more informative than RAPDs in fruit plants (strawberry, apple and Ribes species) for the evaluation of genetic diversity. Kojima *et al.,* (1998) reported that the microsatellites (SSR or ISSR) themselves are possibly nonfunctional sequences and selectively neutral, but they are known to be linked to coding regions, so that ISSRs are likely to mark important agronomic genes rich regions.

Therefore, our aims are: firstly, investigate the effect of increasing subculture number up to 12th generation, BA concentration up to 2.0 mg/l and the composition of direct and indirect regeneration media on plantlets number of cv. Festival. Secondly, assess the effect of different tested *in vitro* propagation methods on phenotypic and genetic stability/variability of cv. Festival using SSR and ISSR markers

2. Materials and Methods

2.1. *In vitro* propagation

2.1.1. Plant materials and conditions

This study was conducted at the Plant Tissue Culture Laboratory of the Department of Horticulture, Faculty of Agriculture, Suez Canal University, and Ismailia, Egypt. Shoot tips of strawberry (*Fragaria* x *ananassa*) cv. Festival were isolated from runners of greenhouse grown plants. The shoot tips were washed with running tap water several times, and surface sterilized by immersion in 70% (v/v) ethanol for a few seconds, and then soaked in a 20% Clorox (Commercial bleach 2.5% sodium hypochlorite) for 15 min with two drops of tween 20, which was used as a wetting agent. Thereafter, shoot tips were rinsed three times in sterile distilled water. The pH of the medium was adjusted at 5.6- 5.8. Meristem tips (5 mm long) were isolated under binuclear and cultured into glass culture jars (50 ml) containing 10 ml of the establishing MS hormone-free medium (Murashige and Skoog 1962). Derived shoots were obtained from the previous stage were excised and subcultured onto 200 ml jars containing 30 ml of multiplication medium containing 0.5 mg/l BA up to fifth generation (subculture). Each treatment consisted of three replicates, each replicate consisted of three jars, which arranged in complete randomized design. All cultures were incubated at 25±2°C under a 16 h day length with illumination of 100 µmol/m^{-2}/S^{-1} Osram cool white18 W fluorescent lamps. Growth observations were taken on shoots clump weight (g) and shoots number/clump.

2.1.2. *In vitro* multiplication up to 12 subcultures

Fifth subcultured derived shoots were transferred to MS medium containing 0.5 and 2.0 mg/l BA from sixth generation up to twelfth generation. The proliferated cultures from subculture number 7 and

12 of cv. "Festival" were transferred into the rooting media (MS+ 0.5 mg/l IBA) in 375 ml jam jars amended with 40 ml medium for 45 days.

2.1.3. Direct and indirect regeneration

The organogenic potential of leaf disc explants (5 ×5 mm) were obtained from *in vitro* plantlets of fifth generation using two media types for direct (M1) and indirect regeneration (M2). MS salts and vitamins + 2.5 mg/L TDZ for 40 days, then transferred to MS+0.75 mg/l IBA for elongation and rooting for direct regeneration. However, for indirect regeneration, the medium composition was MS salts and vitamins + 1 mg/l 2,4-D + 0.5 mg/l BA under darkness for two weeks, then callus transferred to medium containing MS + 1 mg/l BA + 1 mg/l IAA for two weeks. Regenerated shoots were transferred to hormone-free MS medium for rooting.

Two leaf disc explants were cultured with the adaxial surface touching the medium on 50 ml glass jars amended with 10 ml medium in a laminar air- flow hood. Plantlets from each treatment obtained in the first stage were transferred into 200 ml jars containing 30 ml medium.

2.1.4. *Ex vitro* characteristics of tissue culture-derived plants.

The proliferated cultures from subculture number 7 and 12 were transferred into the rooting medium (MS+ 0.5 mg/L IBA) in 375 ml jam jars amended with 40 ml medium for 45 days. Rooted cultures were isolated from the culture jars, washed thoroughly in running tap water to remove any remaining of agar from the medium. Plantlets were moved to the greenhouse for acclimatization in 5 cm plastic pots containing soilless medium (peatmoss + vermiculite 1:1 v/v), covered with plastic sheet and mist irrigated daily for a period of two weeks. Misting intervals were gradually decreased until plantlets were fully acclimatized after 8 weeks. Plants were subcultured into larger plastic pots (15 cm) containing peatmoss and sand (1/1 v/v) and allowed to grow in the greenhouse.

Leaf morphology and growth habit from the different propagation methods plus mother plants were examined by testing the following parameters after 8 -10 weeks of acclimatization:

- Leaf width (W)
- Number of teeth in central leaflet
- Leaflet weight (g)
- Stomata length (µm)
- Number of stomata
- Leaflet length (middle leaflet) (cm)
- Leaf weight (g)
- Leaf petiole length (cm)
- Stomata width (µm)

2.1.5. Statistical analysis

The results were evaluated using descriptive statistics and analysis of variance (ANOVA). Using one-way ANOVA, the effect of BA concentrations, number of subcultures and their interactions were evaluated by Fisher's F-test, followed by Duncan's multiple range test at 0.05 level of probability for comparing the means separation. All tests were performed at a significance level α of 0.05. Calculations were carried out using the software package Statistica™ for Windows version6.1 (Statsoft Inc., 2001, Tulsa, Oklahoma, USA).

2.2. Genetic Stability Assessment

DNA samples were tested for genetic stability/variability including; control plantlet on MS media free-hormones, plantlets from subculture no. 7 and no. 12 using 0.5 mg/l BA, direct regenerated plantlets, callus derived plantlets, plantlets from subculture no. 12 using 2.0 mg/l BA and mother plant (Festival cultivar) from open field.

2.2.1. DNA extraction

Total genomic DNA was extracted from the tissue of frozen juvenile leaves, according to the CTAB method (Rogers and Bendich, 1985), with slight modifications by Porebski *et al.* (1997). The resultant DNA was stored in 0.1XTE buffer. Based on spectrophotometric measurements, purity of DNA was estimated (260/280 nm factor) and their concentration was calculated.

2.2.2. SSR amplification

The polymerase chain reaction (PCR) was carried out in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) thermal cycler, using 5 SSR primers were obtained from Metabion international AG, Martinsried/Deutschland (Table1). SSR amplification was performed in a 25 µl reaction volume, composed of 2µl genomic DNA (30 ng/µl), 5 µl of 5x PCR buffer, 2 µl of $MgCl_2$ (25 mM), 2.5 µl of each of the four dNTP`s (2 mM), 2µl of 10 pmol/µl of each primer (forward and reverse), and 0.2µl Taq DNA polymerase (5 U/µl) and then the final volume was adjusted using dd.H_2O. PCR amplification was programmed with a hot start at 94ºC for 5 minute, followed by 35 cycles each cycle consisted of a denaturation step at 94ºC for 45s, an annealing step at 55°C for 50s, an elongation step at 72ºC for 90s and a final extension at 72ºC for 7 min.

2.2.3. ISSR amplification

Six arbitrary primers, 15 – 20 mer for ISSR (Sangon Biotech. Inc., Shanghai) were screened in ISSR-PCR. Two of the tested primers were selected for analysis, which produced good and reproducible bands (Table 2). ISSR-PCR amplification were performed in a T100Thermal Cycler (Bio-Rad, Hercules, CA, USA).The reaction was performed in a total volume of 25 µl containing 30 - 50 ng template DNA, 1X PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmole primer, 2 Units Taq DNA polymerase. The PCR parameters were as follows: an initial denaturation step at 94°C for 5 min, followed by 40 cycles, each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 53-55 °C for 40 second and an extension step at 72°C for 2 min, following by final extension for 7 min at 72 °C.

The PCR products of the microsatellites (SSR) and (ISSR) markers were resolved by electrophoresis in a 2% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at constant voltage (95 V) at room temperature, run for approximately 2-2.5 h. The PCR reaction with both SSR and ISSR markers was repeated at least twice via each primer to establish the reproducibility of banding pattern.

Table 1: A list of 5 microsatellite (SSRs) markers and their nucleotide sequence.

Loci code/primer name	SSR type	Sequence (5→3)	Repeat motif	Reference
FAC-012	Genomic	F: TACACGTGTCCTAGGGTTTTCA R: AGCGGAGAATGAGTGACGATAG	(CCT)6	Lewers *et al.,* 2005
UAFv7648	EST	F: AACCAGAGCCAGAGCCAG R: CGACAGTGATGTAGAGGAAGA	(CT)12	Bassil *et al.,* 2006
SF-2H12	EST	F: CCTGCATATCTTCTGCAACAAC R: AAGCAGCACCACCTTCAGTAGT	(TC)15	Njuguna *et al.,* 2011
SF-1B07	EST	F: GGAGAGACAGACCTCAAAGGTG R: GAGGGGTTCTGTTTTTGACAAG	CTs(AG)7	Njuguna *et al.,* 2011
SF-5G02	EST	F: CTTTTGCTGCTAGCTCTTTGTG R: TACGTACTCCACATCCCATTTG	(TC)11	Njuguna *et al.,* 2011

Table 2: A list of 2 Inter Simple Sequence Repeats (ISSR) primers and their nucleotide sequence

Primer Name	ISSR type	Primer sequence (5→3)	Ta	Reference
HVH(CA)7T	3'-Anchored	CACACACACACACAT	53	Fang and Roose (1997)
HVH(TCC)5	Unanchored	TCCTCCTCCTCCTCC	55	Fang and Roose (1997)

2.2.4. Detection of SSR and ISSR products

A DNA molecular weight marker (100 bp or 1 kb DNA ladder, Fermentas) was used as size markers. After the run, gels were photographed under UV light inside gel documentation system model (Gel-Doc 2000 with Diversity Database software Ver. 2.1, Bio-Rad Laboratories, Hercules, California, USA) for gel analysis.

2.2.5. Data analysis

The PCR products from SSR and ISSR analyses were scored as presence (1) or absence (0). The genetic information was assessed only for single locus SSRs using the following parameters, observed number of alleles per locus (NA). The polymorphism information contents (PIC), which also known as heterozygosity index, was calculated for each locus depends on number of alleles and the allele frequency from the formula: PIC = 1- $\sum pi^2$ where pi is the frequency of the ith allele and summation extends over n alleles (Nei 1973). The power of discrimination (PD) was calculated as above formula for each locus, but the allele frequency was replaced by the bands frequency, according to Kloosterman *et al.,* (1993), effective number of alleles (Ne) = (1/1-He) were calculated for each marker according to (Hart and Clark 1997).

3. Results

3.1. *In vitro* micro-production and regeneration

Micro-shoots were obtained through different tissue culture techniques including micro-propagation using meristem culture, direct and indirect (via callus) regeneration from leaflets explants (Table 3 and Figure 1). The indicated results showed that non-significant highest shoot number per explants and clumb weight were found after 7th subculture of meristem tips on MS supplemented with 0.5 mg/l BA, however the significant lowest value of shoot number per explants and clumb weight were found after 12th subculture in higher BA concentration (2.0 mg/l). Direct regeneration medium supplemented with TDZ resulted non- significant highest shoot number without callus from the surface of leaf segment explants. While, indirect regeneration medium amended with 2, 4-D and BAP, then BAP and IAA produced shoots from callus tissue. Shoots derived from direct regeneration were significantly higher in number compared to those derived from callus phase, while, clumb weight was on the contrary (Table 3 and Figure 1).

Table 3: Multiplication and regeneration potential using different *in vitro* culture methods and growth regulators in strawberry cv. Festival

Methods of propagation	Number of shoots/ explants	Clumb weight (g)
7th subculture with 0.5 mg/l BA	16.00 a	0.32 a
12th subculture with 0.5 mg/l BA	4.33 c	0.179 c
12th subculture with 2.0 mg/l BA	3.00 d	0.104 d
Direct regeneration	16.75 a	0.29 b
Indirect regeneration	12.00 b	0.31a

Values followed by the same letter within a column are not significantly different at the 0.05% level of probability according to Duncan's multiple range test.

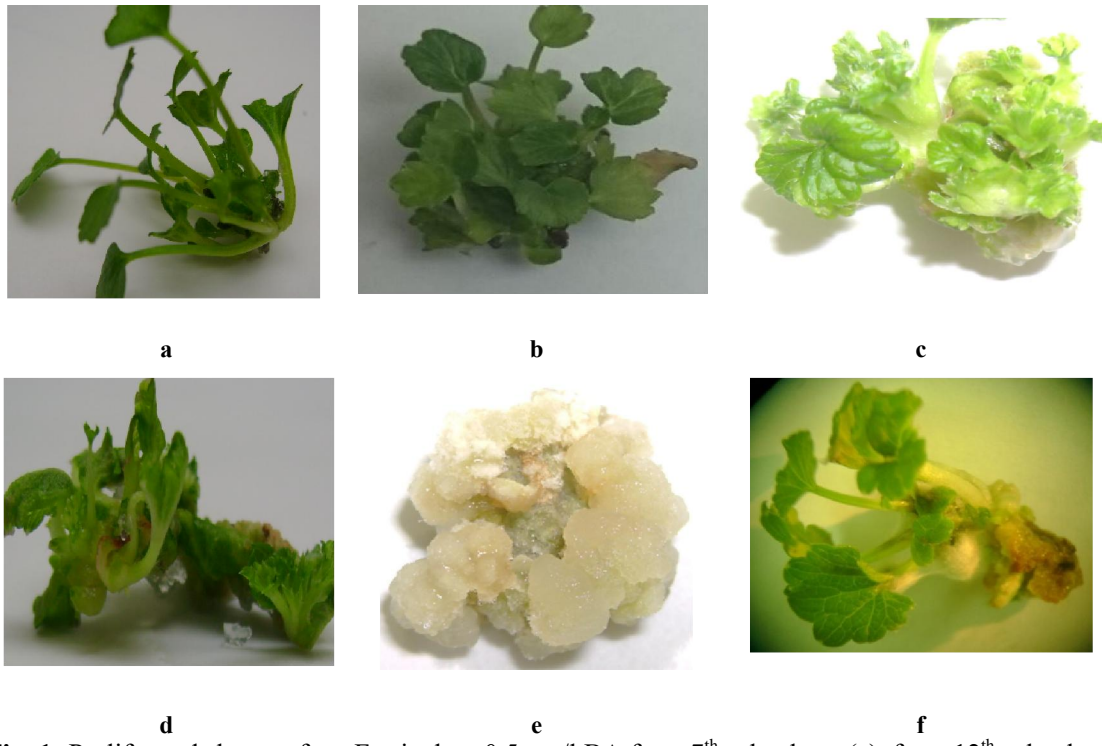

Fig. 1: Proliferated shoots of cv. Festival on 0.5 mg/l BA from 7th subculture (a), from 12th subculture (b) and on 2.0 mg/l BA from 12th subculture (c). Regeneration of shoots in cv. Festival, direct on MS plus TDZ (d), callus on MS plus 2, 4-D+BA (e) and regenerated shoots from callus on MS plus BA+IAA (f)

3.2. *Ex vitro* leaf characteristics of tissue culture-derived plants.

Plants of strawberry cv. "Festival" derived from runner-propagated mother plants (RP), meristem derived plants after two cycles, 7th and 12th subcultures were planted in small plastic pots for acclimatization in the greenhouse under humid conditions, provided by plastic tunnels over the plantlets or by misting. After 8 -10 weeks, plants were fully acclimatized and the cover plastic sheets were removed to allow plants to grow further under normal greenhouse growing temperature (25±1 0 C°) and RH (65 -70%).

Leaf growth measurements, including leaf weight, leaflet weight, leaf petiole length, leaf width, leaflet length, tooth number/middle leaflet, stomata number, length and width of stomata were measured as an indicator of variation from normal leaf morphology. Results in Table (4) show that the leaf weight, leaf petiole length, leaf width, leaflet length, tooth number/middle leaflet (Figure 2), stomata number (Figure 3) and width of stomata leaf were almost the same for the different two subculture cycles and mother plants.

Table 4: Morphological characteristics of cv. Festival leaves derived from two different *in vitro* subculture cycles in comparison with mother plants

	Methods		
Character	**RP**	**7th subculture**	**12th subculture**
Leaf weight (g)	1.127 a	1.323 a	1.401 a
Leaflet weight (g)	0.155 b	0.179 a	0.175 ab
Leaf petiole length (cm)	4.07 a	4.27 a	4.37 a
Leaf width (cm)	3.03 a	3.13 a	3.40 a
Leaflet length (cm)	3.67 a	3.50 a	3.47 a
Teeth No./middle leaflet	19.00 a	18.33 a	17.00 a
Stomata No./ mm²	58.2 a	54.4 a	54.6 a
Stomata width (µm)	10.4 a	10.0 a	10.4 a
Stomata length (µm)	21.0 a	20.2 ab	19.4 b

Values with the same letter for each character are not significantly different at the 0.05% level of probability according to Duncan's multiple range test.

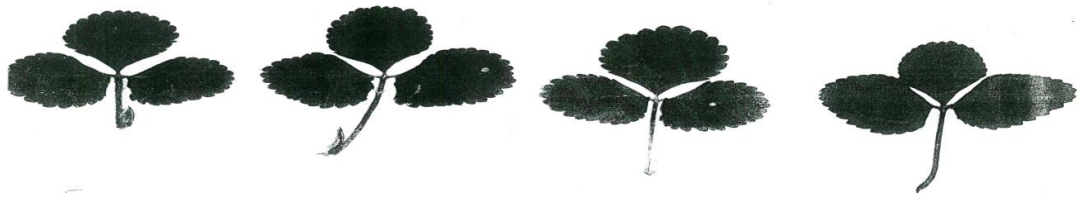

Fig. 2: Leaf morphology and serration pattern of strawberry cv. "Festival" derived from runner propagation(F0), mother plants(M), meristem after 7th(F7) and 12th subcultures(12).

However, plants from 7th subculture had the significant highest leaflet weight comparing with mother plants, while, mother plant leaves had the significant highest stomata length than plants from 12th subcultures.

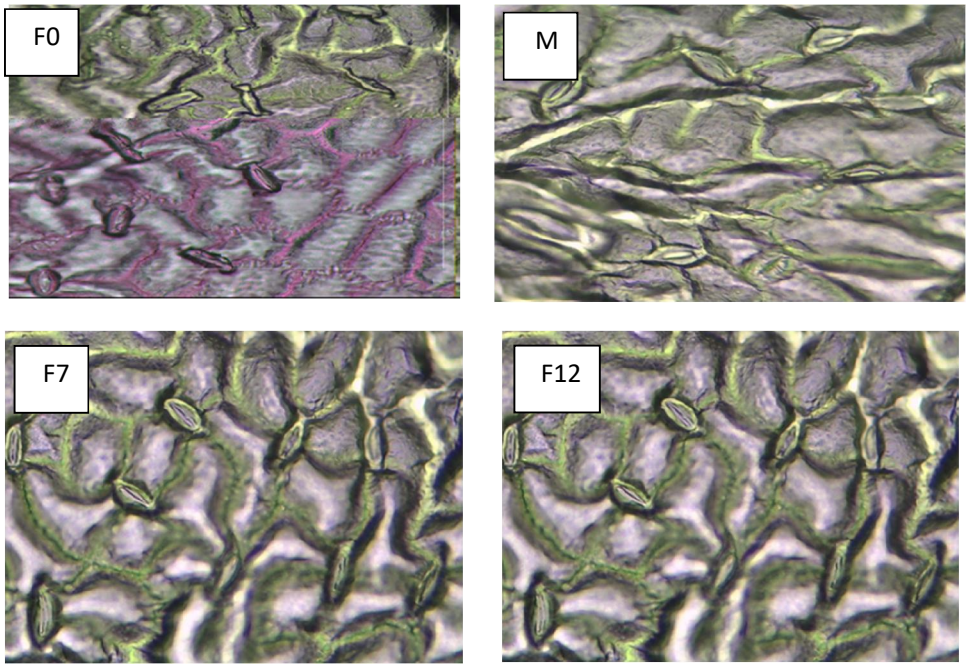

Fig. 3: Stomata morphology of strawberry cv. "Festival" derived from runner propagation (F0), mother plants (M), meristem after 7th (F7) and 12th subcultures (F12).

3.2. Analysis of genetic stability/variability

3.2.1. Using SSR markers

Analysis of the five SSR loci among the tested *in vitro* propagation methods produced 8 scorable bands only (Figure 4). Bands of the used SSR markers were monomorphic in nature and detected percentage of zero % polymorphism. The band patterns of *in vitro* plantlets from different propagation methods, control plantlets on MS free-hormones and mother plant were similar in all aspects.

The number of observed and effective alleles per locus ranged from one to two with an average of 1.6. Also, Polymorphism Information Content (PIC) or gene diversity, which express the appreciation of the discriminatory power of a marker ranged from 0.00 to 0.50 with mean 0.30, while based upon discriminating power (DP) the most informational loci were UAFv7648, SF-2H12 and SF-5G02 (Table 5).

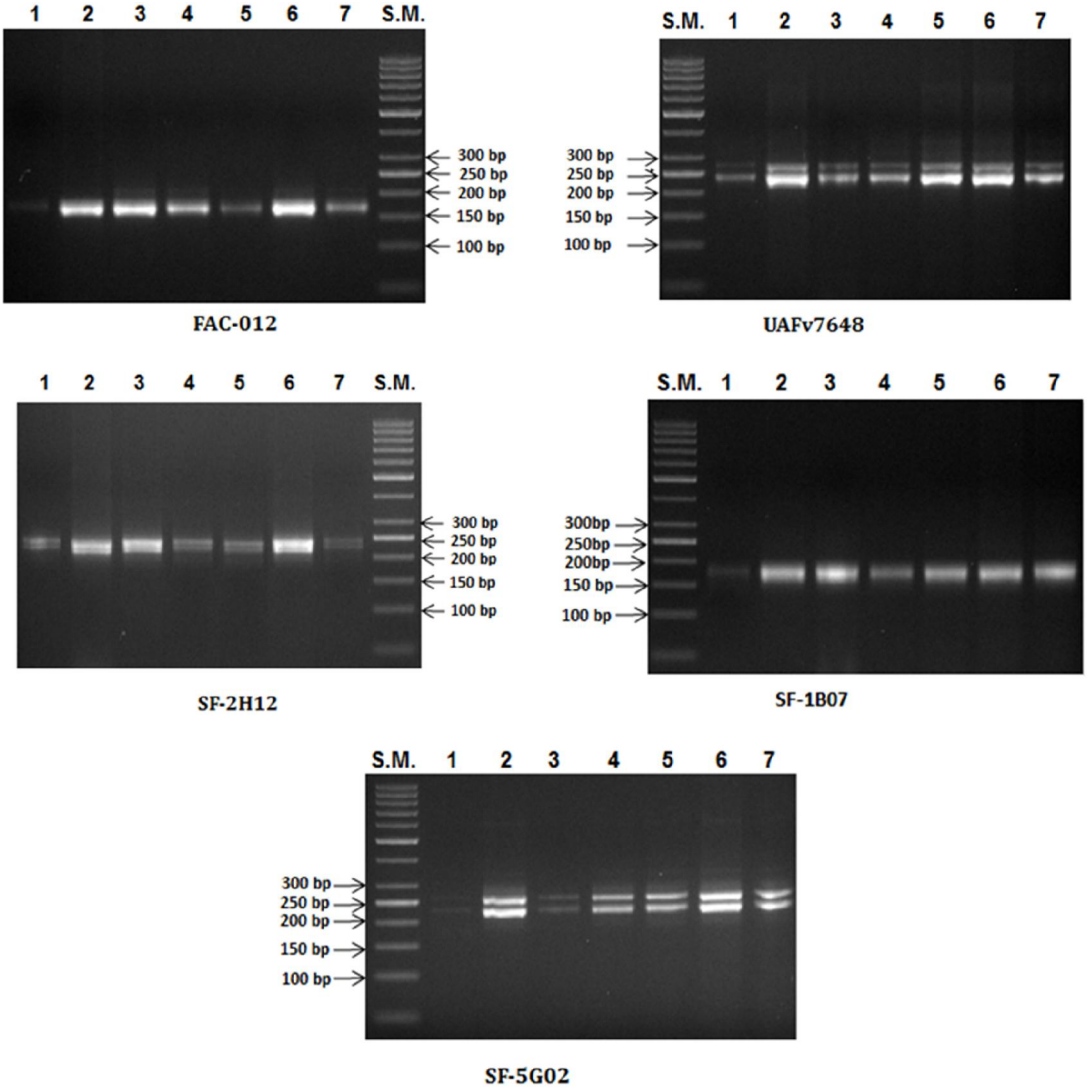

Fig. 4: SSR profiles as detected with loci FAC-012, UAFv7648, SF-2H12, SF-1B07 and SF-5G02. Whereas, S.M. refers to DNA ladder, lane 1 refers to plantlet on MS media without hormones, lane 2 refers to plantlet from subculture no. 7 with 0.5 mg/l BA., lane 3 refers to plantlet from direct regeneration with TDZ, lane 4 refers to plantlet from indirect regeneration with 2,4-D., lane 5 refers plantlet from subculture no. 12 with 0.5 mg/l BA., lane 6 refers to plantlet from subculture no. 12 with 2 mg/l BA., and lane 7 refers to original mother plant.

3.2.2. Using ISSR markers

Out of six arbitrary ISSR primers initially screened, only two primers generated 22 clear and scorable bands. Bands for primer (HVH (CA) 7T) and (HVH (TCC)5) were 10 and 12 respectively with an average of 11 bands per ISSR primer, ranging in size from 240–1280 bp. Fifteen bands were monomorphic and 7 were polymorphic with polymorphism(32%) (Table 5).

Generally, the scored bands of both ISSR primers used to clearly indicate that plantlets from testing propagation methods showed almost different genetic makeup compared with mother plant and control plantlets with MS free-hormones. Based on both ISSR primers, mother plant and control plantlets using MS free-hormones were genetically identical. Micro-propagated shoots using 0.5 and 2.0 mg/l BA after 12th subcultures genetically differed than control plantlets, mother plants and shoots obtained after 7th subcultures on 0.5 mg/l BA, whereas locus HVH (CA) 7T scored the present band at 900 bp. Also, based on locus HVH (TCC)5 micro-propagated shoots after 7th and 12th subcultures scored the present band at 1280 bp which absent in control plantlets and mother plants (Figure 5).

Table 5: Molecular characterizations of SSR and ISSR markers for 6 tested *in vitro* propagation methods of strawberry cv. "Festival"

Loci code/primer name	Na	Pb	NE	Allele/band size range (bp)	PIC	DP	Number of specific alleles
				With the SSR Loci			
FAC-012	1	0	1	200	0	0	0
UAFv7648	2	0	2	220 -270	0.5	0.5	0
SF-2H12	2	0	2	230-240	0.5	0.5	0
SF-1B07	1	0	1	160	0	0	0
SF-5G02	2	0	2	260 -290	0.5	0.5	0
Mean	**1.6**	**0**	**1.6**		**0.3**	**0.3**	**0**
Percent of polymorphism 0 %							
				With the ISSR Loci			
HVH(CA)7T	10	6	8.8	240 - 900	0.89	0.89	1
HVH(TCC)5	12	1	11.8	280 -1280	0.92	0.92	0
Mean	**11**	**0.32**	**10.3**		**0.91**	**0.91**	**0.5**
Percent of polymorphism 32 %							

Na =No. of alleles/bands, Pb = No. of polymorphic bands, Ne = effective No. of alleles, SR = size range, PIC = polymorphic information content and DP = discriminating power

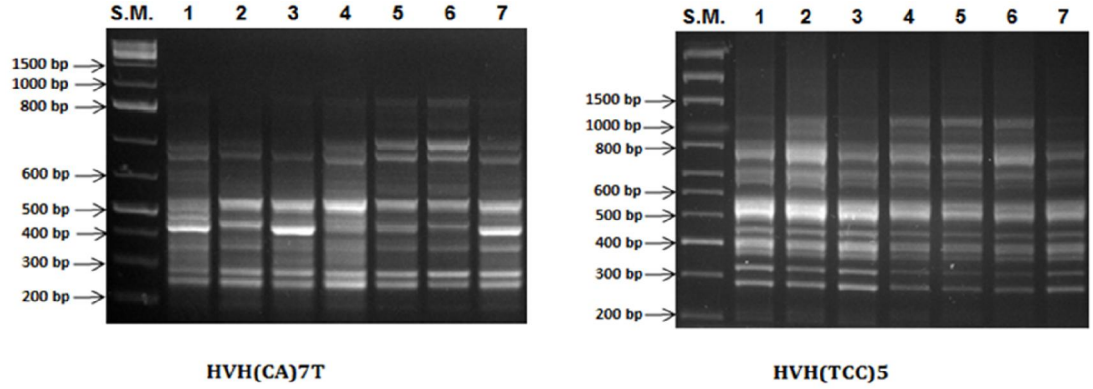

Fig. 5: ISSR profiles as detected with loci HVH (CA) 7T and HVH (TCC)5. Whereas, S.M. refers to DNA ladder, lane 1 refers to plantlet on MS media without hormones, lane 2 refers to plantlet from subculture no. 7 with 0.5 mg/l BA, lane 3 refers to plantlet from direct regeneration with TDZ, lane 4 refers to plantlet from indirect regeneration with 2,4-D, lane 5 refers to plantlet from subculture no. 12 with 0.5 mg/l BA, lane 6 refers to plantlet from subculture no. 12 with 2 mg/l BA, and lane 7 refers to original mother plant

Regarding to regenerated shoots, both direct and indirect regenerated plantlets scored absence band at 380 bp and only band at 680 bp was absent in direct regenerated shoots comparing to control plantlets and mother plants based on locus HVH (CA)7T. However, locus HVH (TCC)5 scored the present band at 1280 bp in non- directive regenerated plantlets comparing with direct regenerated ones as well as control plantlets and mother plants. Regenerated shoots (direct and indirect) scored absence bands at 380, 440 and 460 bp and only band at 680 was absent in direct regenerated shoots in comparison with micro-propagated shoots after 7^{th} and 12^{th} subcultures using low and high BA concentrations (Figure 5). Similarly, band at 1280 was absent only in direct regeneration plantlets comparing with the micro-propagated plantlets (after 7^{th} and 12^{th} subcultures) using low and high BA as well as indirect regenerated ones based on locus HVH(TCC)5 .

4. Discussion

Industrial production of strawberry nurslings completely dependent on meristem culture with restricted number of subcultures in Egypt and worldwide, because it is considered to be an *in vitro* culture system with low risk of genetic instability (Naing *et al.,* 2019). Our data proved that the highest shoot number were obtained using a low number of subcultures (7^{th} subculture) and low BA concentration (0.5 mg/l), however the significant lowest values were associated with a high number of subcultures (12^{th} subculture) and high BA concentration (2.0 mg/l). Our results regarding cv. Festival supported by the results of El-Sayed *et al.,* (2017) who found that low number of subcultures (6^{th}) and low BA concentration (0.1 mg/l) more effective for shoot multiplication. Moreover, our previous results on strawberry cultivars, namely Camarosa, Fortuna, Kamela and Amega indicated that the highest shoots number were attributed with lower number (6 or 7) of subculture and low BA (0.5 or 1 mg/l) concentrations. Also, the indicated results showed that the lowest multiplication rate was found at 12^{th} subculture and this result was supported by the findings of Libek and Kikas (2003) who reported that the younger mericlones produce plants with higher runner production rates than older ones. Organogenic potential did not differ significantly between 7^{th} subcultures and direct regeneration method using TDZ. These findings were supported by our previous investigation in case of cv. Kamela (Mohamed *et al.,* 2018). High regeneration capacity obtained in this study with TDZ as the cytokinin source in the culture medium may be explained on the basis of its extremely high cytokinin activity and stability in plant tissue (Dinani *et al.,* 2018). TDZ is able to induce diverse morphogenic responses ranging from tissue proliferation to adventitious shoot and somatic embryo formation (Wang *et al.,* 2015; Cappelletti *et al.,* 2016; Ghosh *et al.,* 2018; Chung and Quyang 2021; Tung *et al.,* 2021; Chomboon and Lohasupthawee 2022).

Regarding to *ex vitro* evaluation, our results presented in Table (4) in case subculture up to 12^{th} generation using both BA levels are supported by the results of da Fonseca *et al.,* (2013) who found that the increasing number of subcultures that enables multiplication on a large scale, without the occurrence of changes in phenotypic characteristics of clones through the process of *in vitro* micropropagation of cvs. 'Aromas', 'Camarosa' and 'Camino Real. In the same way, there were no dwarfism, chlorosis or white striping of leaves, multiapexing and stem fasciations, intensified vigour, hyperrunnering and abnormal flowering by the increasing number of subcultures to 10^{th} subculture on the phenotypic level (Ayvaz-Sönmez and Kafkas, 2016).

The use of morphological traits to confirm the identity of strawberry cultivars are clearly influenced by environmental factors, therefore this method often does not yield clear and accurate answers concerning discrimination and distinguish of plant genotypes or even estimating the differences that may be few due to multiple generations of agriculture (subculture number). For these reasons, molecular markers, essentially those based on DNA are powerful and widespread recently to analyze the genetic stability, follow phylogenetic origin in strawberry propagated *in vitro*. These techniques include the use of molecular markers which are useful in comparing the DNA from different propagation methods for the discrimination in plants due to sequence variation by identifying random polymorphisms. Microsatellites or simple sequence repeats (SSR) have proved to be specific marker, highly reproducible, co-dominant and highly polymorphic molecular markers (Powell *et al.,* 1996). The use of inter-simple sequence repeats (ISSR) to evaluate genetic stability is well documented (Leroy *et al.,* 2001; Jung *et al.,* 2021; Wójcik *et al.,* 2021) and according to their study, these markers are considered enough to detect the genetic variations among plantlets derived from *in vitro* culture. Assessment of genetic stability/variability among different *in vitro* propagation methods using two types of molecular markers that amplify different regions of the genome is a more reliable and useful method (Martin *et al.,* 2006; Venkatachalam *et al.,* 2007).

In spite of the most SSR loci used in the present study were repeated nucleotides (2 to 3 bp), however no polymorphism was detected among different propagation methods in comparison with mother plant and control plantlets (Figure 4). These results are in contradiction with the results of Gadaleta *et al.,* (2007) who found that the SSR markers in case of the few numbers of repeated nucleotides (2 bp) are highly polymorphic than in the case of a high number (3 bp) ones. Therefore, the drop in the percentage of polymorphism (Zero %) presented in our results may occur due to a high stability or low variability of the DNA sequences in the coding regions of the Festival cultivar genome under the influence of different propagation methods.

Five SSR loci used in this study were none useful for the evaluation of genetic stability/variability among propagation methods, even though, locus FAC-012 which designed for strawberry and from genomic DNA sequences with the potential for a high rate of mutations and diversity (Lewers *et al.,* 2005), however it was monomorphic. Low number of alleles per locus, probably due to low effect of the propagation methods tested or nature of amplified SSR loci, that most of them are expressed sequence tags (EST-SSR) in transcriptions regions. It's considered to be low evolutionary regions, more conserved and less polymorphic than those from intergenic regions (Genomic DNA sequences) as reported by Jung *et al.,* (2005). Our results are in contradiction with the results of Barceló *et al.,* (2019) who detected genetic variations using 10 EST-SSR markers among the lines derived from strawberry protoplasts of cv. 'Chandler.

The polymorphism information content within the loci (Table 5) were low in the most of SSR loci used, could be due to low mutational rate and then less informative. This confirms that there is no effect of the tested *in vitro* propagation methods under this study on the emergence of genetic variations at the level of these five SSR loci. Might be the previous conclusion was supported by the findings of Hosseini *et al.,* (2013) who used the same SSR markers to study the genetic diversity among17 local strawberry cultivars and found a high genetic variation. Consequently, the performance of a given SSR primer pairs to measure the genetic stability/variability, is not dependent only on diverse of germplasm or effect of the tested *in vitro* propagation methods, but also dependent on the primers itself, where it designed. That is why ISSR markers were chosen to measure the genetic stability/variability among propagation methods under this investigation.

ISSR markers were chosen to detect genetic variability among *in vitro* derived plantlets from testing propagation methods comparing with control plantlets and *ex vitro* mother plant. Based on two loci (HVH (CA) 7T and HVH (TCC) 5, control plantlets using MS free-hormones and mother plant were genetically identical and genetically differed from plantlets produced from micro-propagation, direct and indirect regeneration especially using the first locus (HVH (CA) 7T. This result was supported by the fact that the di-nucleotide repeats, anchored either at 3' or 5' end reveal high polymorphism as reported by Reddy *et al.,* (2002). Whereas, the nature of locus (HVH (CA) 7T is di-nucleotide repeats and anchored at 3' end, however, the second locus (HVH (TCC)5 is tri-nucleotide repeats and unanchored. Also, our results are in accordance with the results of Yuan *et al.,* (2009) who reported that ISSR markers were showed remarkable differences among the regenerated shoots of *Saussurea involucrata*. Contrary to our results Jung *et al.,* (2021) and Osena *et al.,* (2017) found no polymorphisms based on ISSR markers among the tested plant materials in *Lycium chinense* and Cassava, respectively. Our study has confirmed the effectiveness of propagation methods for genetic stability since the variation was higher among regenerated plants obtained from leaf explants compared to meristem. This might be explained by the initial heterogeneity of leaf tissue (epidermis, mesophyll, and parenchyma). Morozova (2002) reported a 9% abnormal leaf morphology of meristem-micropropagated plants while the percentage increased to 21% in plants regenerated from leaf explants of *Fragaria vesca*. The genetic examination did not support by the phenotypic evaluation under this study and this may be due to changes in number repetitions of the repeated units in the non-coding regions of these two ISSR loci

The reasons accounting for somaclonal variations remain unclear although factors such as; culture duration, number of subcultures (Borse *et al.,* 2011), explants type (Chuang *et al.,* 2009) and culture medium composition (Lutts *et al.,* 1998). This could explain the presence or absence some bands with the tested *in vitro* propagation methods in the present study. This action is probably due to the culture medium composition or/with the number of subcultures, direct and indirect regeneration through possible mechanisms such as; transposable element activity, changes either in the sizes of the template DNA fragments (e.g., deletion and insertion mutations). Therefore, miscarriage of the successful amplification of a target DNA fragment or variations in the amplified products. Also, from changes that could have altered the sequences of the primer binding sites (unanchored/anchored nucleotides). In addition, may be due to changes in number repetitions of the repeated units in the coding/non-coding regions of these two ISSR loci. Amplified ISSR markers highly useful tool for examining the genetic instabilities, may due to identify of some economic importance functional genes at early stages of *in vitro* culture as reviewed by Rakoczy-Trojanowska and Bolibok, (2004)

5. Conclusion

Increasing subculture number up to 7th subculture using low BA concentration (0.5 mg/l) and direct regeneration using TDZ are suitable for multiplication and regeneration, respectively of strawberry cv. Festival with quite similar number of plantlets. Both propagation methods produced plantlets genetically comparable to mother plants. The obtained results indicated and confirmed that, the ISSR markers were more useful than SSR markers for evaluation and determining genetic stability/variability of different tissue-cultured products, which might be helpful in genetic improvement and breeding programs of strawberry. However, a further investigation is needed to examine more SSR and ISSR markers as well as evaluating the phenotypic characteristics during vegetative, flowering and fruiting stages.

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