Assessment of Somaclonal Variation of Calli and Regenerated Plants of Three Cucumber (*Cucumis sativus* L.) Cultivars Using Molecular Markers

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Abstract: During indirect regeneration of plants, chances of somaclonal variations may arise. These variations should be identified to produce true to type plants. Inter-simple sequence repeat (ISSR) and sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE) were used to assess the somaclonal variation among regenerated plantlets, an embryogenic calli produced from leaf explants compared to *in vivo* plants of three cucumber (*Cucumis sativus* L.) cultivars (Waffir F1, Beith Alpha and Fares). Five ISSR primers yielded 34 distinct and reproducible band patterns with an average of 6.8 bands per primer. Jaccard's similarity coefficients revealed presence of a relatively high genetic similarity (75%) among regenerated plantlets and *in vivo* plants of Beith Alpha cultivar compared to other cultivars. However, relatively high genetic variability have been recorded among calli and *in vivo* plants of Waffir F1 (62%) and Fares (45%) cultivars. Protein profile derived by SDS-PAGE analysis revealed that there are variation among *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars. The molecular weights of detected bands for all samples ranged from 23 to 107 KDa. A hundred percent (100 %) similarity between *in vivo* plants of Waffir and Beith Alpha cultivars as well as between their calli has been noted. The regenerated plantlets of Beith Alpha showed a relatively higher similarity of 78% with *in vivo* plants. Variations in protein pattern were recorded between calli and regenerated plantlets of Waffir (70%) and Beith Alpha (57%) cultivars from one side and between calli and *in vivo* plants from another side (67% in both cultivars). We think that leaf explant culturing influenced by the cucumber cultivar genotype and some genotypes are more susceptible to somaclonal variation, or that the in vitro instability is actually a consequence of a genotype versus culture medium interaction. Our results suggested that rapid propagation of cucumber Beith Alpha cultivar via leaf organogenesis is an acceptable method with a lower risk of somaclonal variation.
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Keywords: *Cucumis sativus* L.; ISSR; regenerated plants; calli; SDS-PAGE

1. Introduction

Cucumber has received much attention as a model plant for Cucurbitaceae. Production of transgenic cucumber carrying agronomically important traits and reverse-genetic studies using transgenic cucumber has been infrequently reported. The reasons are thought to be that difficulties for the transformation of cucumber remained, although transformation of cucumber has been reported by several groups. Accordingly, Nanasato *et al*. (2013) attempted to develop a more efficient and reproducible cucumber transformation via direct organogenesis. The ability to regenerate plantlets in vitro could provide opportunities for genetic transformation and development of transgenic plants expressing novel traits (Tejavathi and Shailaja, 1999). Evaluation of genetic variation in regenerated plants compared to the plants of origin is essential for them to be of use for the maintenance of selected genotypes.

Molecular markers are widely used to detect and characterize the genetic variation at the DNA level. Inter-simple Sequence Repeat (ISSR) is one of molecular markers which has an important role in studying the somaclonal variation and genetic stability through *in vitro* propagation. ISSR is a general term for a genome region between microsatellite loci (Verstrepen *et al*., 2005). ISSR is PCR-based technique which is dominant and targets simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome (Nagaoka and Ogihara, 1997). ISSR is a useful marker system which has been successfully employed to assess the genetic stability/instability in *Robina ambigua* (Guo *et al.,* 2006), *Prunus mume* (Ning *et al.,* 2007), *Swertia chirayita* (Joshi and Dhawan, 2007), *Emmenopterys henryi* (Li and Jin, 2008), *Glycyrrhiza uralensis* (Yao *et al.,* 2008), London plane tree (Huang *et al.,* 2009), *Clivia miniata* (Wang *et al*., 2011) and *Phalaenopsis gigantea* (Samarfard *et al*., 2013), *Scutellaria altissima* (Grzegorczyk-Karolak *et al*., 2013) and synthetic seeds of cucumber (Adhikari *et al*., 2014).

Among the studies on genetic stability in *in* vitro propagation in cucumber, Elmeer and Hennerty (2007) used RAPD marker to study the genetic

stability/variability in somatic embryos of cucumber hybrids. They are concluded that no significant difference was obtained in somatic embryos relative to mother plant. Moreover, Diao *et al.* (2009) studied the genetic stability in regenerated double haploid plantlets of cucumber resulted from ovary culture. They are found that regenerated plants were homozygous using SSR marker. Tabassum *et al*. (2010) found a resemblance between mother plants and synthetic seeds of cucumber using AFLP marker. Cheruvathur *et al*. (2012) found that ISSR analysis of *Rhinacanthus nasutus* mother plant and regenerants showed low degree of variation and statistically it is not significantly different. At the same direction, Ahmed *et al*. (2012) observed presence of somaclonal variation between callus and more than one regenerated plant in three cultivars of date palm by using 16 ISSR primers. A more recent study analyzed the genetic stability of *Arum palaestinum* Boiss regenerated plants compared to the mother plants through ISSR marker. They are reported presence of variation between the regenerated plants and their mother plants (Farid *et al.,* 2014).

Otherwise, ISSR analysis of shoots developed via callus organogenesis on *Scutellaria altissima* showed their genetic similarity to shoots originated from the seeds (Grzegorczyk-Karolak *et al*., 2013). Also, Adhikari *et al*. (2014) applied 16 RAPD and 10 ISSR markers to analyze the genetic stability of the plants regenerated from synthetic seeds of cucumber with the mother plant. A total of 106 loci amplified and all loci were found to be monomorphic in nature indicating the homogeneity among the regenerants.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) is still used in protein studies. It is considered a low-cost, reproducible, and rapid method for quantifying, comparing, and characterizing proteins. The relative ease of performing SDS-PAGE along with its wide applications has made it an important analytical technique in many fields of research (Hafez, 2005). SDS-PAGE marker has been used in many plants for numerous applications such as evolutionary and taxonomic studies, in breeding, estimating crossing frequencies, varietal fingerprinting, synthetic value, genetic stability studies and cultivar identification (Maalouf *et al*., 1999; El-Baz *et al*., 2003; Orabi, 2004). It has been used for the assessment of variation and stability of regenerated plants in wheat (Kiarostami and Ebrahimzadeh, 2001)), broccoli (El-Kazzaz and Taha, 2002), garlic (Bekheet, 2004), *Glycine wightii* (Silva *et al*., 2005) sugar beets (Bekheet *et al* 2007), *Citrullus colocynthis* (Mohamed *et al*,. 2011), Ginger (Taha *et al*., 2013), grasspea (Barpete *et al*., 2014), *Arum palaestinum* (Farid *et al.*, 2014) and *Silybum marianum* (Rady *et al*., 2014).

Kiarostami and Ebrahimzadeh (2001) found dissimilarities between plantlets produced through tissue culture techniques and those germinated from seeds of wheat.

Bekheet *et al*. (2007) reported that plantlets derived from shoot tips, via direct and indirect organogenesis, are identical and they are similar to those *in vivo* grown plantlets of sugar beets in protein content. Barpete *et al*. (2014) studied the differences in protein content among regenerated plantlets and seeds of grasspea. Their results recorded absence of somaclonal variation in regenerated plants. Farid *et al.* (2014) investigated the genetic stability of tissue culture-raised plants via shoot regeneration of *Arum palaestinum* through SDS-PAGE biochemical marker. Their results revealed presence of differences in protein profiles in the examined samples, suggests that a real genetic change might have occurred.

The aim of this study was to detect the probable somaclonal variation in cucumber tissue culture derived plants and their calli resulted from indirect organogenesis culture of leaf explants using an efficient molecular markers including inter-simple sequence repeat (ISSR) and sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

2. Material and Methods

2.1. Plant material

Seeds of three cultivars of cucumber (Waffir F1, Beith Alpha and Fares) were obtained from the Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. We are used basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose to obtain aseptic seedlings after 4 weeks. Leaf explant was excised from seedlings and used as source of production of callus. Four segments of explant (1cm) were cultured on basal MS medium.

The embryogenic calli of three cucumber cultivars (Waffir F1, Beith Alpha and Fares) were obtained from the calli which originated from leaf explants cultured on MS medium supplemented with 1 mg/l NAA + 1 mg/l BA after 6 weeks of cultivation (Figure 1). At least three samples of calli from each cultivar have been used in ISSR and SDS-PAGE analysis.

The rooted shootlets of the three cucumber cultivars were transferred to MS media free of plant growth regulators for elongation. The elongated plantlets the three cucumber cultivars were subjected for acclimatization after 3-4 weeks of cultivation (Figure 2).

The rooted shootlets of the three cucumber cultivars were transferred to peat moss-sand mixture

and covered with polyethylene bags which perforated after 2-3 weeks till removing after 4 weeks of transferring. The highest percentage of survived plantlets (95%) was obtained in acclimatized plantlets of Waffir F1 cultivar. The percentage of survived plantlets of Beith Alpha cultivar (80%) was a little bit lower than Waffir cultivar. Furthermore, the lowest percentage of survived plantlets (75%) was recorded in acclimatization process of Fares cultivar plantlets (Figure 3). At least three samples of regenerated plants from each cultivar have been used in ISSR and SDS-PAGE analysis.

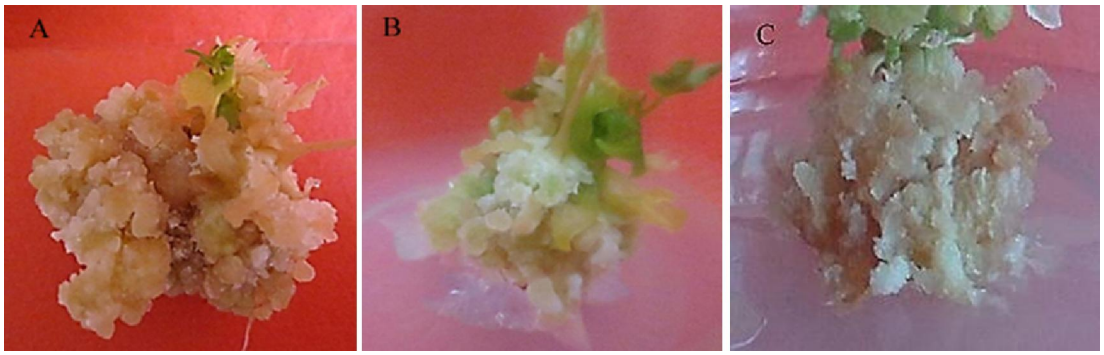

Figure 1. Develoment of an embryogenic calli for the three cucumber cultivars after 7 weeks of cultivation: (A) Waffir F1 cv., (B) Beith Alpha cv. and (C) Fares cv.

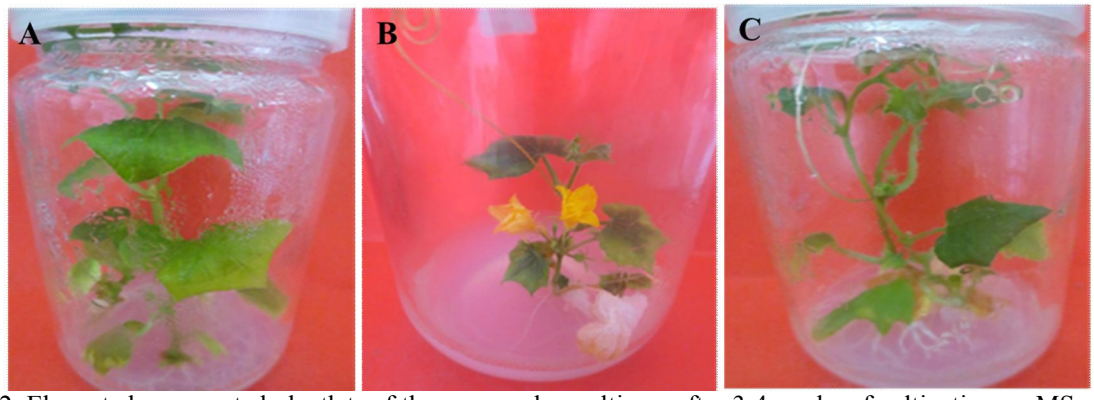

Figure 2. Elongated regenerated plantlets of three cucumber cultivars after 3-4 weeks of cultivation on MS medium free of plant growth regulators: (A) Waffir F1 cv., (B) Beith Alpha cv. and (C) Fares cv.

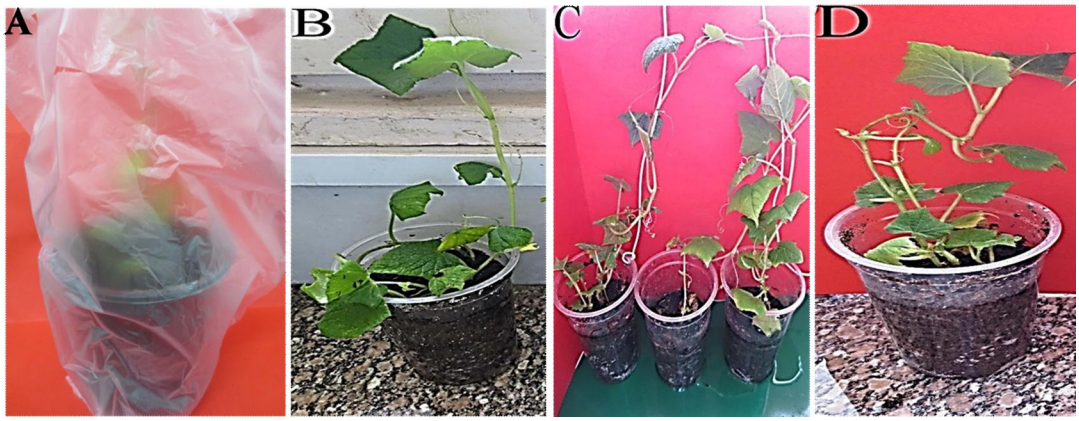

Figure 3. Acclimatization of regenrated plantlets for three cucumber cultivars after 4-5 weeks of transferring to peat moss-sand mixture: (A) plantlets covered with perforated polyethylene bag, (B) Waffir F1 cv. (C) Beith Alpha cv. and (D) Fares cv.

2.2. Inter simple sequence repeat (ISSR) analysis

Approximately 100 mg of fresh leaves were immersed in liquid nitrogen for DNA extraction using DNeasy plant Mini Kit (QIAGEN Hilden, Germany). PCR reaction was performed in 30µl volume tubes contained dNTPs (2.5 mM), $MgCl_2$ (25 mM), 3 µl Buffer, 2 µl Primer, 0.20 µl Taq DNA polymerase (5U/µl), 2 µl Template DNA and 16.80 µl distilled H_2O in an automated thermal cycle (Techno 512) programmed for one cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C. The reaction was finally stored at 72°C for 10 min. Based on the clear and accurate amplified bands profiles, different five primers were selected (Table 1). The reaction products were analyzed by electrophoresis on 1.2 % agarose gels, stained with ethidium bromide and photographed under UV transilluminator by digital camera with UV filter adaptor. The DNA ladder 100 bp (Pharmacia) was employed as molecular markers for bands molecular size. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel.

Table 1. List of ISSR primers sequences used for analysis of *in vivo* plants, an embryogenic calli and regenerated plantlets

Primer code	Primer sequence
44B	5′- CTC TCT CTC TCT CTC TTG- 3′
HB-10	5′- GAG AGA GAG AGA CC- 3′
HB-11	5′- GTG TGT GTG TGT TGT CC- 3′
HB-12	5′- CAC CAC CAC GC- 3′
HB-15	5′- GTG GTG GTG GC- 3′

2.3. Protein electrophoresis (SDS-PAGE)

Total protein was extracted from the healthy leaves of the mother plants (*in-vivo*) and regenerated plantlets (*in-vitro*) as well as the calli of the three cucumber cultivars. Then, it was ground to fine powder with pestle and mortar. Ten mg of powered flour was homogenized thoroughly with 400 µl extraction buffer using vortex. The extraction buffer was prepared by dissolving 0.6 g Tris base, 0.2 g Sodium Dodecyl Sulfate (SDS) and 30 g of urea in 50 ml of double distilled water. One ml of β-mercaptoethanol was added and then the solution was diluted to 100 ml with double distilled water. The mix was kept overnight at 4°C and then centrifuged at 13000 rpm for 10 minutes at room temperature (Sigma 3K 18 Bench Top centrifuge). For qualitative analysis of protein, 20 µl of the extracted protein was boiled in a water bath for 3-5 min and loaded on Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) containing 12.5% resolving gel and 4% stacking gel (Lammeli, 1970) using 3 µl bromophenol blue as tracking dye. The samples were then loaded in equal amounts (15 µl) and a molecular weight marker standards (5 µl) (Prism Ultra Protein Ladder) were loaded at on to each well. Electrophoresis was carried out at 150 V and 25 mA until tracking dye reached to the bottom of the gel. The gels were destained in solvent composed of 40 ml of methanol, 10 ml glacial acetic acid and 50 ml distilled water. The gel was stained overnight in 25 ml of Coomassie Brilliant Blue (R-250) staining buffer. The gels were photographed and the molecular weights of the polypeptide bands were estimated by correlating position of the molecular weight marker standards.

2.4. Data analysis

Somaclonal variation among the mother plants, calli and regenerated plants of the three cucumber cultivars was estimated following the Jaccard's similarity matrix (Jaccard, 1908) using binary data for both of ISSR and SDS-PAGE banding patterns using Community Analysis Package (CAP). Profiles were considered different when at least one polymorphic band was identified. Bands were scored as'1' if it's present or '0' if it's absent based on standard marker using Community Analysis Package (CAP) software. The similarity coefficients were used to construct a dendrogram depicting genetic relationship using the unweighted pair group mean average (UPGMA) method (Sneath and Sokal, 1973).

3. Results and Discussion

3.1. Detection of somaclonal variation using ISSR

A major problem encountered with the *in vitro* culture is the presence of somaclonal variation. In this study, ISSR profiles were used to check genetic variation among regenerated plantlets, an embryogenic calli that are generated from leaf explants and - *in vivo* – mother plants of three cucumber cultivars (Waffir F1, Beith Alpha and Fares) . 10 ISSR primers were screened using a mother plant DNA sample, out of these 10 primers, only 5 primers (44B, HB-10, HB11, HB-12 and HB-15) produced more than four clear and scorable bands, and were used in further PCR analysis.

Analysis of ISSR data showed presence of different levels of genetic variation. The total number of detected amplified fragments using five ISSR primers was 34, while the number polymorphic fragments were 28 (Table 2). ISSR HB-10 produced maximum number of amplified products (13) and ISSR 44B; ISSR HB-12 and ISSR HB-15 produced the least (5). The highest percentage of polymorphism (92.31%) was observed in HB-10

primer. The lowest percentage of polymorphism (60%) was obtained in HB-12 primer. The number of bands per each primer varied from 4 to 13, with an average of 6.8 bands per primer. The total number of amplified fragments in *in vivo* plants was 17, 19 and 16 in Waffir F1, Beith Alpha and Fares, respectively. Furthermore, the total number of amplified fragments was (23 and 22), (22 and 19) and (15 and 18) in Waffir F1, Beith Alpha and Fares (calli and regenerated plantlets), respectively (Table 3). The levels of polymorphism were different with different primers among the regenerated plantlets, embryogenic calli and mother plants.

Jaccard's similarity coefficient values of ISSR were calculated between regenerated plants, an embryogenic calli and the mother plants of the three cucumber cultivars using all primers are presented in (Table 4). The highest similarity value (0.75) was found between *in vivo* plants of Beith Alpha and Fares and between *in vivo* plants of Fares and regenerated plantlets of Beith Alpha as the closet but the lowest value (0.33) was found between *in vivo* plants of Waffir F1 and calli of Fares as most distant.

The dendrogram of genetic distances among the *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars based on band polymorphisms generated by ISSR after using all primers is shown in (Figure 5). The dendrogram separated all tested plants into two clusters. First cluster was further divided into two subclusters, first subcluser formed a separate cluster with callus of Fares cultivar and second subcluster included calli of both of Waffir F1 and Beith Alpha cultivars. Second cluster was further divided into two subclusters, first subcluster formed a separate subcluster with *in vivo* Waffir F1 cultivar and second subcluster included both of *in vivo* Beith Alpha and Fares; regenerated plantlets of the three cultivars.

Table 2. Total number, monomorphism and polymorphism of amplified fragments generated using five ISSR primers

Primers	TAF	MF	PF	Polymorphism (%)
44B	5	1	4	80
HB-10	13	1	12	92.31
HB-11	6	1	5	83.33
HB-12	5	2	3	60
HB-15	5	1	4	80
Total	34	6	28	82.35

TAF: Total Amplified Fragments; **MF**: Monomorphic Fragment**; PF**: Polymorphic Fragment

Table 3. Total number and polymorphic fragment for *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars

Plant Samples	TAF	PF	Polymorphism (%)
Waffir *in vivo* plants	17	11	64.71
Waffir calli	23	17	73.91
Waffir plantlets	22	16	72.73
Beith Alpha *in vivo* plants	19	13	68.42
Beith Alpha calli	22	16	72.73
Beith Alpha plantlets	19	13	68.42
Fares *in vivo* plants	16	10	62.5
Fares calli	15	9	60
Fares plantlets	18	12	66.67

TAF: Total Amplified Fragments; **PF**: Polymorphic Fragment

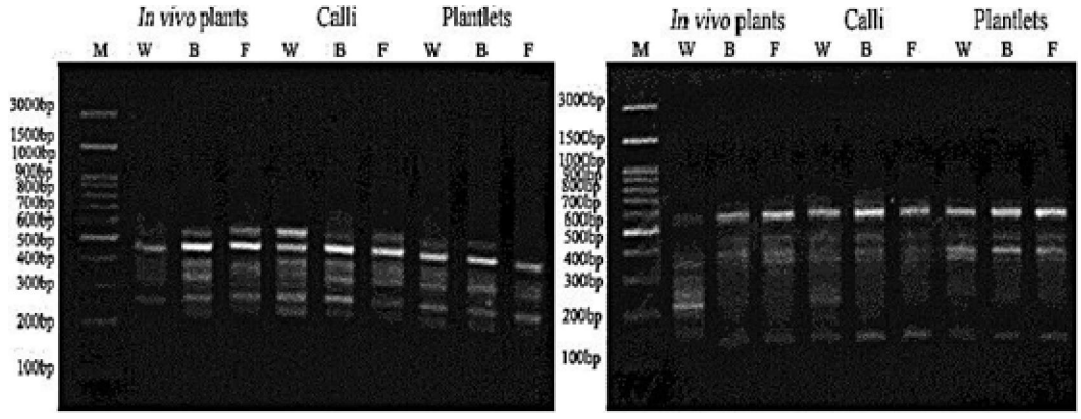

Figure 4. A band pattern produced by primer HB-12 (left) and primer HB-15 (right) for *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars. Letters indicated the name of cultivars as following; W: Waffir F1, B: Beith Alpha and F: Fares. Line M represents DNA marker

Table 4. Jaccard's similarity coefficient for *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars

Plant Samples		*In vivo* plants			Calli			Plantlets		
		W	BA	F	W	BA	F	W	BA	F
***In vivo* plants**	**W**									
	BA	0.44								
	F	0.43	0.75							
Calli	**W**	0.38	0.56	0.50						
	BA	0.39	0.64	0.52	0.67					
	F	0.33	0.55	0.55	0.52	0.54				
Plantlets	**W**	0.50	0.71	0.58	0.45	0.57	0.48			
	BA	0.57	0.73	0.75	0.50	0.58	0.48	0.71		
	F	0.46	0.68	0.62	0.41	0.60	0.43	0.54	0.6087	

W: Waffir F1 cv.**; BA:** Beith Alpha cv.**; F:** Fares cv.

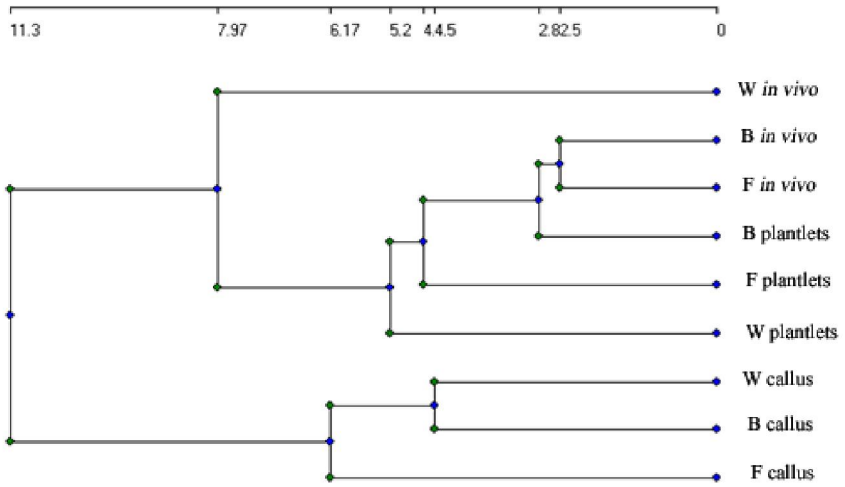

Figure 5. UPGMA dendrogram derived from Jaccard's similarity coefficients calculated among *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars (W: Waffir, B: Beith Alpha and F: Fares) based on amplification profiles generated by ISSR marker

The obtained results showed apparent genetic variations between calli and *in vivo* plants of three cucumber cultivars when subjected to ISSR analysis. These results are in agreement with those of Hu *et al.* (2008) who noticed that ISSR primers could produce a high-frequency polymorphism in detection of somaclonal variation in *Amorphophallus rivieri.* At the same orientation, Zucchi *et al.* (1996) reported that recognition of DNA polymorphism in meristem culture derived somaclones of sugarcan cultivars could be attributed to pre-existing polymorphism in source plant.

The dedifferentiation of *in vivo* plants tissues forming calli was considered to be the main reason for the genetic variability detected in calli. The cause of dedifferentiation was the use of different plant growth regulators. Genetic variation was observed in calli of *Jasminum auriculatum* induced in MS medium containing 2,4-D and kinetin alone or in combination (Joy and Raja, 2010). Furthermore, Jose *et al*. (2012) concluded that the genetic similarity between mother plants and calli of *Jatropha curcas* was largely dependent on the concentration of IBA and BA used. The morphological characters, nature and high rates of cell division of calli were predicted as results of genetic variations from *in vivo* plants.

Somatic embryogenesis has been considered as an effective pathway of plant regeneration because of its high regenerative potential and lower risk of chimeric mutations (Chen and Chang, 2000). The results obtained demonstrated that regeneration of cucumber cultivars through somatic embryogenesis, maintained relatively high genetic variation. There are many reports demonstrating such higher variations from mother plant (Eshraghi *et al.,* 2005; Qun *et al.,* 2009). The genetic variation of regenerated plants from the mother plant depends on many factors like the genotype involved, type of explant, culture media, conditions etc. (Shuangxia *et al.,* 2008).

On the other hand, the regenerated plantlets of three cucumber cultivars showed relative genetic resemblance to *in vivo* plants. These results were in line with a study on *Amorphophallus rivieri* Durieu of Hu et al. (2011). They are observed presence of low somaclonal variation in the regenerated plants propagated via corm organogenesis. Also, our results were in agreement with a study on *Viola patrinii* of Chalageri and Babu (2012). They are reported that RAPD profile of the regenerated plants showed similar banding patterns to that of the mother plant, thus they are demonstrating the homogeneity of the micropropagated plants. Furthermore, regenerated plantlets of potatoes (*Solanum tuberosum*) showed high genetic fidelity to mother plants by more than 96% using AFLP marker (Tiwari *et al*., 2013) and 100 % using ISSR marker (Al-Maarri *et al*., 2014). Similar results in the point of genetic similarity between mother plants and regenerated plantlets from calli were observed in tomato (Soniya *et al*., 2001), date palm (Eshraghi *et al*., 2005), *Anoectochilus formosanus* (Zhang et al., 2010), *Nothapodytes foetida* (Chandrika et al., 2010) and *Hydrangea macrophylla* (Liu et al., 2011) *Clivia miniata* (Wang *et al*., 2011) and *Dendrobium nobile* (Bhattacharyya *et al*., 2014).

From this analysis it is clear that a majority of the regenerated plants of three cucumber cultivars exhibited a relatively moderate genetic stability or extremely low genetic variation, and that genetic instability was restricted to tissue culture raised callus. The higher somaclonal variation we observed can be attributed to the choice of material, fast growing calli, which are likely to produce more extensive DNA variation. Together, these observations suggest either that some genotypes are more susceptible to somaclonal variation, or that the in vitro instability is actually a consequence of a genotype versus culture medium interaction.

3.2. Detection of somaclonal variation using SDS-PAGE

The protein profile system revealed the biochemical variation and evolutionary relationship among *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars was demonstrated in Figure 6. The molecular weights of detected bands for all samples ranged from 23 to 107 KDa. There were nine bands detected at molecular weight 23, 26, 37, 43, , 56, 64, 70, 80 and 93 KDa in the mother plants (*in vivo* plants) of three cultivars, in addition to presence of one band detected at molecular weight 75 KDa in Fares cv. *in vivo* plants. Only three polypeptide bands of molecular weight 23, 37 and 43 KDa have been detected in the calli of Waffir F1 and Beith Alpha and absence of other polypeptide bands. Ten polypeptide bands with molecular weight 23, 26, 30, 37, 43, 49, 64, 70, 80 and 107 KDa appeared in calli of Fares cultivar (Table 5).

On the other hand, band with molecular weight 56 KDa was absent in the regenerated plantlets of Waffir F1 cv. and detection of two new bands with molecular weight 30 and 49 KDa relative to that observed in *in vivo* plants. The regenerated plantlets of Beith Alpha differ from its *in vivo* plants in absence of two polypeptide bands with molecular weight 26 and 93 KDa. Furthermore, the regenerated plantlets of Fares showed the appearance of two new polypeptide bands with molecular weight 30 and 49 KDa and absence of four polypeptide bands with molecular weight 70, 75, 80 and 93 KDa compared to *in vivo* plants (Table 5).

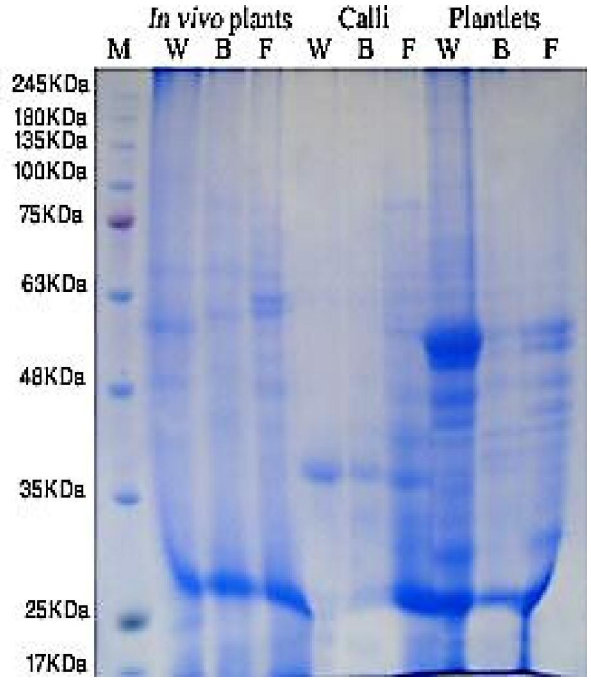

Figure 6. The protein profile of *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars (W: Waffir, B: Beith Alpha and F: Fares) using SDS-PAGE marker

As shown in Table (5), the total number of bands detected with SDS-PAGE was thirteen while the number of polymorphic bands (including two unique bands) was ten. It was noted that the percentage of polymorphism was 76.92 percent. The similarity coefficient values between the *in vivo* plants, calli and regenerated plantlets of three cultivars of cucumber based on SDS-PAGE analysis were presented in (Table 6). The highest similarity value (1.0) was observed between *in vivo* plants and calli of Waffir F1 and Beith Alpha as the closet but the lowest value (0.3) was found between Waffir F1

and Beith Alpha calli and *in vivo* plants of the three cultivars as most distant.

The dendrogram of genetic distances among the *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars based on polypeptide bands generated by SDS-PAGE is shown in (Figure 7). The dendrogram separated studied samples into two clusters. First cluster formed a separate cluster with calli of both Waffir F1 and Beith Alpha cultivars. Second cluster was further divided into two subclusters; first subcluster formed a separate subcluster with regenerated plantlets of Waffir F1, regenerated plantlets and callus of Fares cultivars. The second subcluster included *in vivo* of the three cultivars and regenerated plantlets of Beith Alpha cultivar.

Protein profile of *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars showed 100% of similarity between *in vivo* plants of Waffir and Beith Alpha cultivars and also between their calli. A bit lower similarity (90%) was observed between *in vivo* plants of Fares cv. and the two other cultivars. These results indicated that the genetic material is more stable at protein level than at DNA level under normal conditions.

On the other hand, a moderate variation in protein profile was observed between calli and *in vivo* plants of Waffir (67%), Beith Alpha (67%) and relatively lower in Fares (46%) cultivars. A quite similarity of 64% has been detected between calli cultures and regenerated plantlets of Fares cultivar while calli of Waffir and Beith Alpha showed the lowest similarity with their regenerated plantlets.

On the other side, the regenerated plantlets of Beith Alpha showed a relatively higher similarity of 78% with *in vivo* plants. A little lower similarity in Waffir 73% while that of Fares showed 50% similar to its *in vivo* plants. These results were in agreement with Barpete *et al.* (2014) in that similarity in protein profile of grasspea regenerated plantlets ranged between 37% and 85%. Also, our results coincide with Farid *et al.* (2014), where tissue culture-raised plants via shoot regeneration of *Arum palaestinum* revealed differences in protein profiles in the examined samples, suggests that a real genetic change might have occurred.

Table 5. The binary matrix showing the protein pattern of *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars based on SDS-PAGE marker

Band No.	**RF**	**MW**	***In vivo* plants**			**Calli**			**Plantlets**			**Frequency**	**Band type**
			W	**B**	**F**	**W**	**B**	**F**	**W**	**B**	**F**		
1	0.223	107.469	0	0	0	0	0	1	0	0	0	0.111	U
2	0.279	93.072	1	1	1	0	0	0	1	0	0	0.444	P
3	0.335	80.603	1	1	1	0	0	1	1	1	0	0.667	P
4	0.362	75.203	0	0	1	0	0	0	0	0	0	0.111	U
5	0.388	70.345	1	1	1	0	0	1	1	1	0	0.667	P
6	0.424	64.132	1	1	1	0	0	1	1	1	1	0.778	P
7	0.474	56.403	1	1	1	0	0	0	0	1	1	0.556	P
8	0.527	49.225	0	0	0	0	0	1	1	0	1	0.333	P
9	0.574	43.627	1	1	1	1	1	1	1	1	1	1	M
10	0.638	37.014	1	1	1	1	1	1	1	1	1	1	M
11	0.717	30.217	0	0	0	0	0	1	1	0	1	0.333	P
12	0.761	26.988	1	1	1	0	0	1	1	0	1	0.667	P
13	0.809	23.857	1	1	1	1	1	1	1	1	1	1	M
Total number of proteins			**9**	**9**	**10**	**3**	**3**	**10**	**10**	**7**	**8**		

Table 6. Jaccard's similarity coefficient based on SDS-PAGE technique for *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars

Plant Samples		***In vivo* plants**			**Calli**			**Plantlets**		
		W	**BA**	**F**	**W**	**BA**	**F**	**W**	**BA**	**F**
***In vivo* plants**	**W**									
	BA	1								
	F	0.9	0.9							
Calli	**W**	0.33	0.33	0.3						
	BA	0.33	0.33	0.3	1					
	F	0.58	0.58	0.54	0.3	0.3				
Plantlets	**W**	0.73	0.73	0.67	0.3	0.3	0.82			
	BA	0.78	0.78	0.7	0.43	0.43	0.55	0.55		
	F	0.55	0.55	0.5	0.38	0.38	0.64	0.64	0.5	

W: Waffir F1 cv.; **BA**: Beith Alpha cv.; **F**: Fares cv.

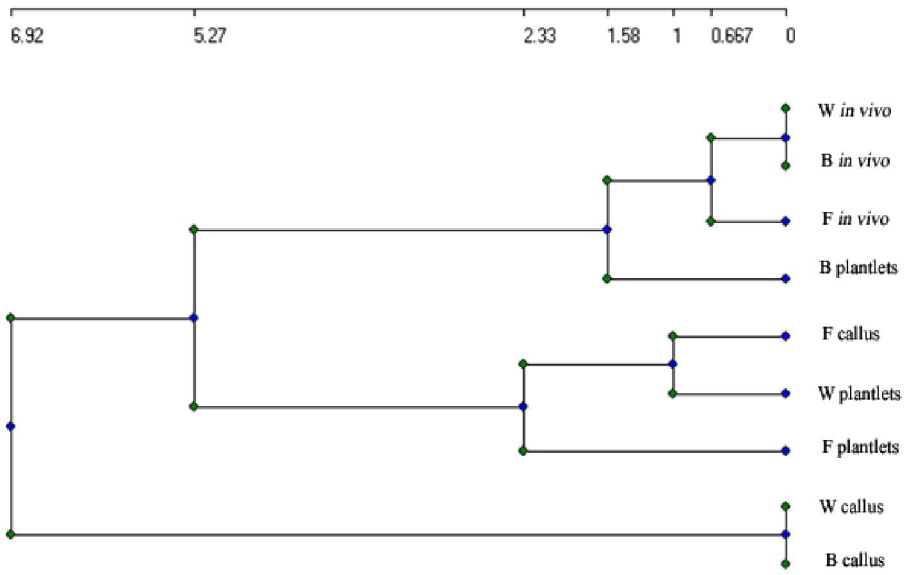

Figure 7. UPGMA dendrogram derived from Jaccard's similarity coefficients calculated among *in vivo* plants, calli and regenerated plantlets of three cucumber cultivars (W: Waffir, B: Beith Alpha and F: Fares) based on amplification profiles generated by SDS-PAGE marker

However our results were in contrary with the study of Rady *et al*. (2014) in the point of that no or little differences in protein profile were obtained between *in vivo* plants and regenerated plantlets of *Silybum marianum*. Similar results in the point of great similarity between *in vivo* plants and regenerated plantlets were obtained in Ginger (Taha *et al*., 2013). Great similarity in protein profile between *in vivo* plants and regenerated plantlets of sugar beet (Bekheet *et al*., 2007), broccoli (El-Kazzaz and Taha, 2002) and garlic (Bekheet, 2004) has been reported.

We can explain the causes of protein variation among in vivo plants, an embryogenic calli and regenerated plantlets for cucumber cultivar to one of following reasons or to all combined. This reasons included media composition; procedure of planting and conditions of incubation; callus subcultures and regenerated plants show appearance of new synthesized proteins as well as of course to the different in genotype for a cultivar. Kairong *et al*. (2002) reported that addition of hydrogen peroxide in the media induced the synthesis of new protein in the embryogenic callus of *Lycium barbarum*. Ishizaki *et al*. (2002) found a new synthesized protein in calli grown from segments of spinach (*Spinacia oleracea* L.) root in the presence of gibberellic acid (GA_3) plus auxin compared to absence of GA_3 and presence of auxin. Elavumoottil *et al*. (2003) obtained new proteins in callus cultures of *Brassica oleracea* L. var. *botrytis* grown in media supplemented with different concentrations of sodium chloride. Silva *et al*. (2005) investigated that the protein content in the callus of *Glycine wightii* depends on the incubation period. Mohamed *et al*. (2010) found that salt stress caused an induction in the synthesis of some new polypeptides in potato plantlets compared to control one. Furthermore, Mohamed *et al*. (2011) concluded that changes in the protein pattern appear to correlate with colocynth (*Citrullus colocynthis*) callus percentage and different combinations of plant growth regulators.

In conclusion, we think that leaf explant culturing increase the rate of polymorphism, which is also influenced by the cucumber cultivar genotype.

4. Conclusion

In the present study, Both of ISSR and SDS-PAGE analysis reported presence of somaclonal variation between *in vivo* plants, an embryogenic calli and regenerated plantlets generated from leaf explants of three cucumber cultivars. Our results revealed that Beith Alpha cultivar appeared lower level of somaclonal variation compared to other two cultivars. Thus, certain genotypes such as Waffir F1 and Fares cultivars might be require more appropriate conditions for culturing. In general, an efficient protocol was established for somatic embryogenesis and plant regeneration from leaf explants of cucumber Beith Alpha cultivar with relatively lower somaclonal variation.

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