



# Indirect regeneration of *Ficus carica* by the TCL technique and genetic fidelity evaluation of the regenerated plants using flow cytometry and ISSR

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## Abstract

The fig (*Ficus carica* L.), is known as a precious fruit tree for its nutrition and medicinal values, economic importance and for sustainable production in the semi-arid and arid areas. Expanding the cultivation of fig in new vulnerable areas and the breeding programs in fig need a reliable high-efficient system for in vitro morphogenesis to meet future demands. This study was carried to develop an efficient protocol for indirect regeneration of *F. carica* L. cultivars ‘Sabz’ and ‘Torsh’ using thin cell layer (TCL) technique. The genetic fidelity of the regenerated plants was also evaluated using flow cytometry technique and ISSR markers. Stem segments of 10 mm in diameter were taken from mature plants, then explants were transversally cut into layers of 0.5–0.8 mm thickness. Callus induction was successful using Murashige and Tucker (MT) medium supplemented with 9.08  $\mu\text{M}$  TDZ plus 9.8  $\mu\text{M}$  IBA (IM3 medium) which resulted in  $50 \pm 6.11\%$  calli in ‘Sabz’ cultivar. Morphogenic calli were cut into small pieces and cultured on Murashige and Skoog (MS) medium for shoot development. Maximum shoot regeneration (45%) was observed in 17.68  $\mu\text{M}$  BAP in combination with 4.54  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA (RM2 medium), with an average of 6.9 shoots per explant. Flow cytometry and ISSR molecular marker analyses confirmed the stability of ploidy level and genetic identity of indirectly regenerated plants in both cultivars. The results of this study demonstrate that indirect regeneration of *F. carica* L. by the use of TCL system is a reliable and promising approach for future mass propagation programs as well as possible in vitro breeding objectives.

## Key message

A rapid and high-efficient in vitro method for mass propagation via callus culture in two *F. carica* cultivars was established by using TCL technique for the first time. Flow cytometry and ISSR molecular markers confirmed the clonal identity of regenerants in both cultivars.

**Keywords** Fig (*Ficus carica* L.) · Flow cytometry · Molecular markers · Morphogenic callus · Plant growth regulators

## Abbreviations

TCL	Thin cell layer	KIN	Kinetin
PGR	Plant growth regulator	2iP	6-( $\gamma,\gamma$ -Dimethylallylamino) purine
TDZ	Thidiazuron	IBA	Indole-3-butyric acid
BAP	6-Benzyl amino purine	NAA	1-Naphthalene acetic acid
		2,4-D	2,4-Dinitrophenylhydrazine
		GA <sub>3</sub>	Gibberellic acid
		ISSR	Inter simple sequence repeats
		CTAB	Cetyl trimethyl ammonium bromide

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## Introduction

The common fig (*Ficus carica* L.) belongs to the Moraceae family (Flaishman et al. 2008) and it is proposed as one of the first domesticated horticultural plants through human civilization (Kislev et al. 2006). The well adaptation to various climates and soils, drought tolerance, high nutritional and medicinal values (Badgujar et al. 2014) and high economic importance (Crisosto et al. 2011), makes the fig as a valuable and favorable fruit tree for sustainable production in semi-arid and arid areas (Doaa et al. 2015; Ibrahim et al. 2017).

Mass propagation and crop improvement through biotechnological methods have been the most important applications of in vitro techniques for the last two decades (Al-Khayri et al. 2018). Most of the woody plant species, including fruit trees, are recalcitrant and hard to propagate under in vitro conditions which cause barriers for their breeding. The establishment of an efficient micropropagation technique for fig tree could be important for its commercial production (Loyola-Vargas and Ochoa-Alejo 2016) and breeding programs as well.

The TCL technique encompasses cutting a thin cell layer from any plant tissue longitudinally (lTCL) or transversally (tTCL) (Ramírez-Mosqueda and Iglesias-Andreu 2016), which was originally developed by Tran Thanh Van in tobacco (Chattopadhyaya et al. 2010). Over the past few decades, methods based on TCLs were developed for numerous explants and successfully applied to various plant species for in vitro mass propagation, genetic transformation, production of artificial seeds, cryopreservation, and in vitro selection (Teixeira Da Silva and Dobránszki 2019). A considerable number of in vitro recalcitrant plant species, particularly fruit trees that could not be easily propagated, can now be successfully mass multiplied using this technique (Nhut et al. 2003; Boliani et al. 2019).

Steinmacher et al. (2007) reported that the TCL system dramatically increased the amount of calli and somatic embryos of peach palm (*Bactris gasipaes*) compared to other conventional regeneration techniques. They reported a maximum embryogenic callus production of 43% when the TCL explants from shoot meristem were cultured on MS medium supplemented with activated charcoal and 300 µM Picloram. The average number of somatic embryos per embryogenic callus was  $34 \pm 4$  in the maturation stage and finally, 80% of plantlets were acclimated. AFLP analyses revealed that 92% of the regenerated plantlets were true to type. Sabooni and Shekafandeh (2017) successfully set a protocol for the regeneration of two blackberry genotypes using tTCL technique. They reported that embryogenesis typically occurs on calli from dermal and central parts of the explants. The highest level of embryogenic

callus initiation in both genotypes occurred in half-strength MS medium containing  $60 \text{ g l}^{-1}$  sucrose,  $9.76 \text{ µM}$  KIN plus with  $7.99 \text{ µM}$  BA. Dobránszki and Teixeira da Silva (2011) investigated the organogenesis response of a conventionally easy-to regenerate ('Royal Gala') and a difficult-to-regenerate ('Freedom') apple cultivars using leaf tTCL. Maximum bud regeneration (100%) with an average of 8 shoots/explant was observed when tTCL of the second leaves of 'Royal Gala' cultivar was used as explant. However, the regeneration percentage in 'Freedom' cultivar was much less (39%) with an average of 2.1 shoots/explant from tTCL explants of the first leaves.

Somaclonal variation is a great challenge for clonal propagation, especially in plant regeneration via callus mediated protocols. It can be a result of epigenetic or permanent genetic changes in cells or tissues that might lead to losing the genetic fidelity of regenerants (Ali et al. 2019). Furthermore, flow cytometry can confirm whether in vitro regenerated plants and parental plants have the same nuclear DNA content (Zafar et al. 2019). Molecular markers have also been chosen during the last decades as the most desirable tool for ensuring genetic identity of the in vitro propagated plants. Among the DNA molecular markers, inter-simple sequence repeat (ISSR) is a simple, fast, affordable and reliable tool for the determination of genetic identity of the regenerants (Raji et al. 2018).

To the best of our knowledge, this is the first report of using the TCL technique in *F. carica* L. to investigate its potential for callus induction and shoot regeneration and possibly to develop a reliable method for mass propagation of this important fruit tree. The goals of this study were to (i) development of a high-performance protocol for indirect regeneration of two fig cultivars (i.e. 'Sabz' and 'Torsh') based on the TCL technique (ii) selection of the best culture medium and optimizing the PGR combinations for callus induction and plant regeneration, and (iii) assessment of their fidelity using flow cytometry technique and ISSR marker analyses.

## Materials and methods

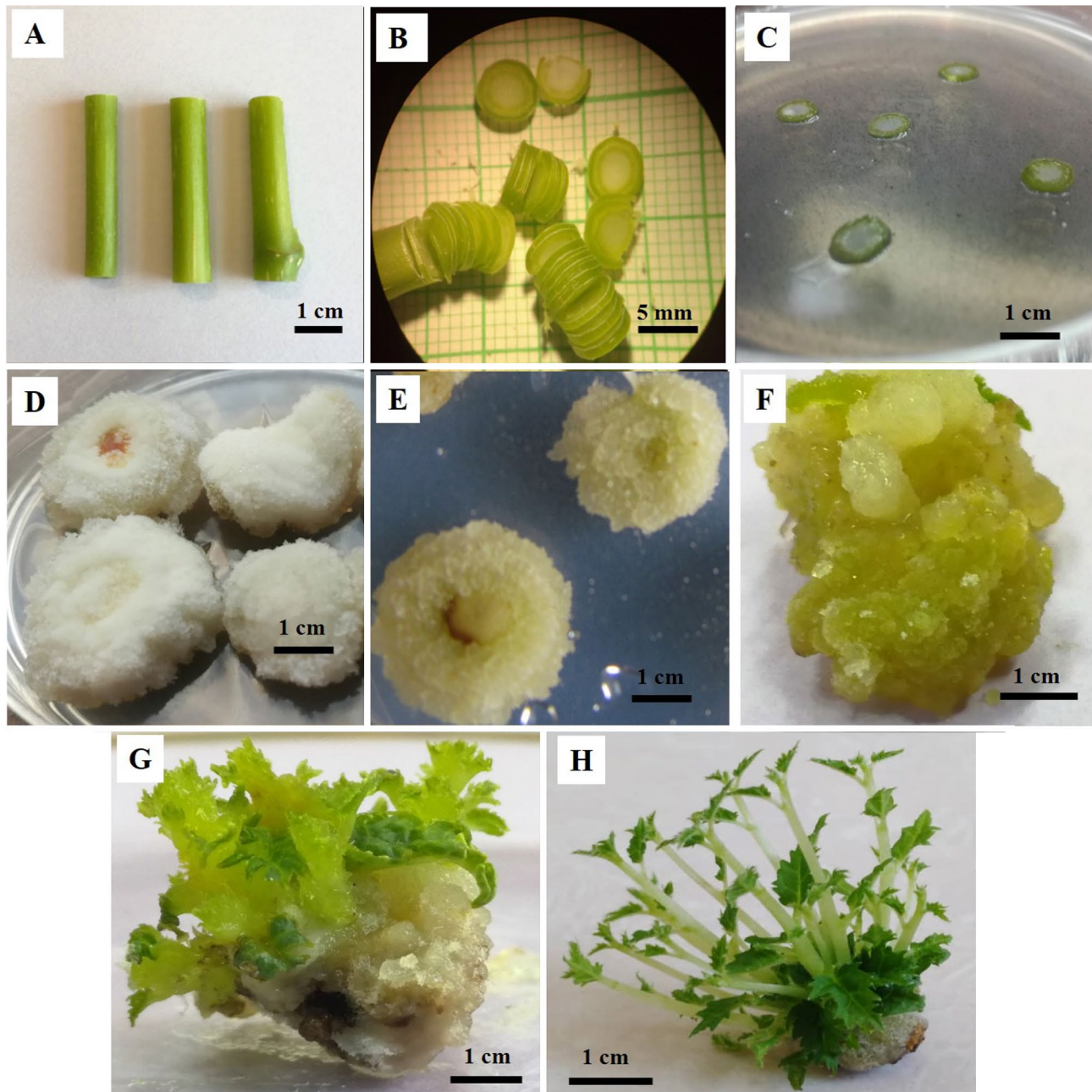
### Transverse TCL explant preparation

To obtain stem tTCL explants, newly grown stem samples almost 20 cm in length and 10 mm in diameter were taken from the 5-years-old *F. carica* L. cultivars 'Sabz' and 'Torsh' grown in the research greenhouse of the department of Horticultural Science at Shiraz University, Iran. The collected shoots were defoliated and divided into 5 cm segments. The stem segments were washed in a 1% commercial detergent solution and transferred to a laminar air flow cabinet. Surface sterilization was performed by immersion of the

stem segments in 70% ethanol for 1 min followed by soaking in 15% commercial bleach solution (containing 5% active chlorine) for 20 min and finally washing 3 times with sterilized distilled water. The tTCL explants were cut at a size of about 0.5–0.8 mm in thickness and 10 mm in diameter using a sharp razor blade under a binocular (Fig. 1a and b).

## Callus induction

According to our preliminary test with three culture media: MS (Murashige and Skoog 1962), MT (Murashige and Tucker 1969), and WPM (Lloyd and McCown 1981) and using different PGR combinations (BAP, TDZ, 2ip, Kin,



**Fig. 1** Morphogenic callus induction and shoot regeneration in *F. carica* L. from stem tTCL explant culture. **a** newly grown stem segments from mature mother plants, **b** tTCL explants (0.5–0.8 mm thickness) cut under a binocular, **c** explants cultured on callus induction media, **d** formation of non-morphogenic callus (white color and flaky structure) on IM5 medium (2.27  $\mu$ M TDZ + 10.74  $\mu$ M NAA), **e**

morphogenic callus formation (creamy-green color and nodular structure) on IM3 medium (9.08  $\mu$ M TDZ + 9.8  $\mu$ M IBA), **f** a morphogenic callus in meristematic domes formation phase on RM2 medium (17.68  $\mu$ M BAP + 4.54  $\mu$ M TDZ + 1.07  $\mu$ M NAA), **g** and **h** multiple shoot regeneration and leaves development on RM2 medium. (Color figure online)

2,4-D, IBA, NAA), the MT medium with TDZ in combination with either IBA or NAA recognized as the best treatment for callus induction. The subsequent experiment was conducted with MT medium as follow: 5 stem tTCL explants of both cultivars were cultured in Petri dishes (6 cm in diameter) containing 12 ml of induction media (IM media), which consisted of MT medium and vitamins supplemented with 87.64 mM sucrose, 83.3 mM activated charcoal and 19.32 mM agar (Fig. 1c). In order to induce morphogenic callus, an experiment with different concentrations of TDZ (2.27  $\mu\text{M}$ , 4.54  $\mu\text{M}$ , 9.08  $\mu\text{M}$ , 18.16  $\mu\text{M}$ ) in combination with constant concentrations of either IBA (9.8  $\mu\text{M}$ ) or NAA (10.74  $\mu\text{M}$ ) was performed as follows:

IM0: Control (PGR-free MT medium); IM1: 2.27  $\mu\text{M}$  TDZ + 9.8  $\mu\text{M}$  IBA; IM2: 4.54  $\mu\text{M}$  TDZ + 9.8  $\mu\text{M}$  IBA; IM3: 9.08  $\mu\text{M}$  TDZ + 9.8  $\mu\text{M}$  IBA; IM4: 18.16  $\mu\text{M}$  TDZ + 9.8  $\mu\text{M}$

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Regeneration percentage = (Number of calli with regenerated shoots)/(Total number of incubated calli)  $\times$  100%

Mean number of regenerated shoots = (Number of regenerated shoots)/(Total number of incubated calli)

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IBA; IM5: 2.27  $\mu\text{M}$  TDZ + 10.74  $\mu\text{M}$  NAA; IM6: 4.54  $\mu\text{M}$  TDZ + 10.74  $\mu\text{M}$  NAA; IM7: 9.08  $\mu\text{M}$  TDZ + 10.74  $\mu\text{M}$  NAA; IM8: 18.16  $\mu\text{M}$  TDZ + 10.74  $\mu\text{M}$  NAA. The pH of media was adjusted to 5.8 before autoclaving at 121  $^{\circ}\text{C}$  for 20 min. Subsequently, Petri dishes containing explants were incubated at  $25 \pm 1$   $^{\circ}\text{C}$  in darkness for 3 weeks. At the end of the third week two kinds of callus were recognized on different induction media. Non-morphogenic callus was defined as white colored appearance and having a flaky structure (Fig. 1d), while morphogenic callus was defined as having creamy-light green color with a nodular structure (Fig. 1e). Parameters of survival rate (%), callus induction (%), and morphogenic callus induction (%) in each treatment were calculated using the following formula:

Survival rate percent = (Number of survived explants)/(Total number of incubated explants)  $\times$  100

Callus induction percent = (Total number of explants producing callus)/(Total number of incubated explants)  $\times$  100

Morphogenic callus induction percent = (Total number of explants producing morphogenic callus)/  
(Total number of incubated explants)  $\times$  100

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### Shoot regeneration

For shoot regeneration, about 50 mg of morphogenic callus were transferred to 250 ml glass jars containing 40 ml of regeneration media (RM media) including MS medium and vitamins, 87.64 mM sucrose, 851.6  $\mu\text{M}$  ascorbic acid,

1301.2  $\mu\text{M}$  citric acid, 20.21 mM agar along with different PGR concentrations as follows:

RM0: Control (PGR-free MS medium); RM1: 8.84  $\mu\text{M}$  BAP + 4.54  $\mu\text{M}$  TDZ + 1.07  $\mu\text{M}$  NAA; RM2: 17.68  $\mu\text{M}$  BAP + 4.54  $\mu\text{M}$  TDZ + 1.07  $\mu\text{M}$  NAA; RM3: 26.63  $\mu\text{M}$  BAP + 4.54  $\mu\text{M}$  TDZ + 1.07  $\mu\text{M}$  NAA; RM4: 8.84  $\mu\text{M}$  BAP + 9.08  $\mu\text{M}$  TDZ + 1.07  $\mu\text{M}$  NAA; RM5: 17.68  $\mu\text{M}$  BAP + 9.08  $\mu\text{M}$  TDZ + 1.07  $\mu\text{M}$  NAA; RM6: 26.63  $\mu\text{M}$  BAP + 9.08  $\mu\text{M}$  TDZ + 1.07  $\mu\text{M}$  NAA.

The cultures were placed in a growth chamber under a 16/8 h (light/darkness) photoperiod (light intensity of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), provided by cool-white fluorescent tubes and  $25 \pm 1$   $^{\circ}\text{C}$  temperature for 4 weeks. The regeneration percentage, mean number of shoots per explant in each treatment were calculated according to the following formula and shoot length were measured using a ruler.

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### Elongation, rooting and acclimatization

Regenerated shoots (0.5–2 cm length) were cultured on elongation medium (MS medium supplemented with 2.21  $\mu\text{M}$  BAP + 0.28  $\mu\text{M}$  GA<sub>3</sub>) for further growth. After 3 weeks, shoots with more than 2 cm length were put in half-strength MS medium (58.42 mM sucrose, 2.46  $\mu\text{M}$  IBA and 20.81 mM agar) for root development. Plantlets with well-developed roots were transferred for acclimatization to 400 ml plastic cups containing an equal ratio of coconut peat and fine perlite in a greenhouse with  $25/18 \pm 2$   $^{\circ}\text{C}$  (day/night) temperature and 85% relative humidity.

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### Flow cytometry analysis

To determine the fidelity of regenerated plants via the tTCL technique, after 6 months of acclimatization 10 plants of each cultivar were randomly selected along with their parents for flow cytometry analysis. According to Jowkar et al. (2009), about 1  $\text{cm}^2$  of young leaves of growing apical

buds from both cultivars were selected. The samples were chopped after adding 400  $\mu\text{l}$  of nuclei extraction buffer (CyStain Ultraviolet Precise P Nuclei Extraction Buffer; Sysmex Partec, Germany) containing 1% (w/v) of polyvinyl pyrrolidone (PVP) by a sharp razor blade for 30 s. Afterwards, 1600  $\mu\text{l}$  of staining buffer (CyStain Ultraviolet Precision P Staining Buffer, Sysmex Partec, Germany) were added to the mixtures and subsequently filtered by 50  $\mu\text{m}$  and 30  $\mu\text{m}$  nylon mesh (CellTrics, Sysmex Partec, Germany) respectively. The mixtures were kept on ice and darkness for 2 h. Ultimately, all mixtures were analyzed using a flow cytometry device (Partec PA I, Sysmex Partec, Germany) with at least three replications to detect the mean fluorescence intensity of the DNA.

### DNA extraction and genetic fidelity analysis using ISSR molecular markers

To investigate the genetic identity, 10 acclimated plants of each cultivar were randomly selected along with their parental plants. Total genomic DNA was isolated from 100 mg of fully expanded young fresh leaves by the CTAB method according to Japelaghi et al. (2011) with slight modifications. The isolated DNA was analyzed for its quality and quantity parameters by using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop™ 1000 Spectrophotometer, U.S.A) and performing electrophoresis on 1% agarose gel as well. A total of 15 ISSR-anchored primers (Metabion, Germany) were selected to carry out DNA analysis (Table 1). The PCR amplification was performed using a total reaction volume of 20  $\mu\text{l}$  containing 7  $\mu\text{l}$  sterile double distilled water, 10  $\mu\text{l}$  master mix (AMPLIQON, Denmark), 1  $\mu\text{l}$  of

ISSR primer (33.3  $\mu\text{M}$ ), and 2  $\mu\text{l}$  of template DNA (16 ng/ $\mu\text{l}$ ). The amplification reactions were carried out in a thermal cycler (Bio-Rad, USA) with an initial denaturation of DNA at 94  $^{\circ}\text{C}$  for 5 min, followed by 30 cycles of: 60 s denaturation at 94  $^{\circ}\text{C}$ , and 45 s annealing and 2 min extension at 72  $^{\circ}\text{C}$ ; and the final extension was set as 72  $^{\circ}\text{C}$  for 5 min. The PCR products were electrophoresed on a 2% agarose gel using 1  $\times$  TBE (Tris HCL, Boric acid, EDTA) buffer. The size of amplicons was estimated using 100–3000 bp DNA size marker (SMOBIO, Taiwan). All amplification reactions were repeated at least three times to check for reproducibility. The gels were photographed using a gel documentation system (Bio-Rad, USA).

### Experimental design and statistical analysis

Both callus induction and shoot regeneration phases were set up as a factorial experiment (with two factors including cultivar and PGR treatments) in a completely randomized design with 10 replications and 5 explants per replicate. Each experiment was repeated twice. Data were analyzed using SAS software version 9.4 (SAS institute, USA) and the means were compared by Duncan's multiple range test ( $p \leq 0.05$ ). All means are presented with  $\pm$  Standard Error (SE). Log transformation was performed, where zero was observed in the data.

Flow cytometry data were also analyzed using SAS software version 9.4 and the mean values were compared by Duncan's multiple range test ( $p \leq 0.05$ ).

For molecular analysis, only consistently reproducible and sharp bands were manually scored with regard to their

**Table 1** List of primers, number of amplicons and polymorphism percentage for ISSR analysis

Primer code	Primer Sequence (5'-3')	Annealing Temperature	No. of amplicons	No. of Loci	No. of Polymorphic loci	Polymorphism (%)
UBC807	(AG)8 T	52.5	88	5	2	40
UBC808	(AG)8C	54.5	154	8	2	25
UBC810	(GA)8 T	50	–	–	–	–
UBC811	(GA)8C	52	–	–	–	–
UBC812	(GA)8A	50.6	55	3	1	33.33
UBC814	(CT)8A	52.5	94	5	2	40
UBC815	(CT)8G	52	–	–	–	–
UBC816	(CA)8 T	50	–	–	–	–
UBC817	(CA)8A	50	–	–	–	–
UBC823	(TC)8C	52.6	106	6	2	33.33
UBC861	(ACC)6	58	176	8	0	0
IMA-5-Z	(CA)8GT	54	–	–	–	–
IMA-5-3	(CA)8TG	55	88	5	2	40
HB15	(GTG)3GC	36.8	66	3	0	0
<b>Total</b>			<b>827</b>	<b>43</b>	<b>11</b>	

presence (“1”) or absence (“0”) in the gel. All data were assembled as a binary character matrix and to minimize errors, faint and ambiguous bands were excluded from the statistical analysis and manual scoring was triple-checked. The data were analyzed using the NTSYS-pc software version 2.02. Genetic similarity coefficient and constructing the UPGMA dendrogram were performed by the SIMQUAL procedure of the software to determine genetic similarity among the regenerants and their corresponding parental plants.

## Results

### Callus induction from tTCL explants

Callus formation started from the eighth day in both cultivars and all induction media (IM) treatments, except for the control. Analysis of variance revealed that there were no significant differences in the interactions (at  $p < 0.05$ ) between cultivars and PGR treatments for the survival rate and callus induction, whilst a significant difference (at  $p < 0.05$ ) was observed between two cultivars and also among PGR treatments. The interactions between cultivar and PGR treatments was statistically significant (at  $p < 0.05$ ) only for the morphogenic callus induction parameter. Data showed that ‘Sabz’ had a higher survival rate (65.3%) compared to ‘Torsh’ cultivar (58.4%) (Fig. 2a). The highest survival rate was observed in IM3 (induction medium containing  $9.08 \mu\text{M}$  TDZ +  $9.8 \mu\text{M}$  IBA) and the lowest one was recorded in IM5 ( $2.27 \mu\text{M}$  TDZ +  $10.74 \mu\text{M}$  NAA) (Fig. 2b). The results also showed that ‘Sabz’ achieved a significantly higher rate of callus induction (55.7%) than the ‘Torsh’ cultivar (50.2%) (Fig. 2c). The highest value of callus induction (86%) was seen in IM3 treatment and the lowest callus induction was detected in IM5 (40%) and IM8 (41%) treatments (Fig. 2d). The morphogenic callus was only induced in two induction media, i.e. IM2 and IM3. In both cultivars, IM3 produced the highest rate of morphogenic callus compared to IM2. Furthermore, the Sabz cultivar stimulated by IM3 produced the highest rate of morphogenic callus (50%). (Fig. 2e).

### Shoot regeneration

Calli from the most responsive morphogenic callus induction medium (IM3 treatment) were used for assessing their regeneration capacity. Analysis of variance revealed that the interaction effects between cultivars and PGR treatments were not significant for all measured indices including percentage of shoot regeneration, number of shoots per explant and shoot length. Among the different regeneration media

(RMs) containing various PGR combinations, no shoot growth was observed in RM6 ( $26.63 \mu\text{M}$  BAP +  $9.08 \mu\text{M}$  TDZ +  $1.07 \mu\text{M}$  NAA). Between all PGR treatments, maximum shoot regeneration percentage (45%), number of shoots per explant (6.9) and shoot length (1.83 cm) were observed in RM2)  $17.68 \mu\text{M}$  BAP +  $4.54 \mu\text{M}$  TDZ +  $1.07 \mu\text{M}$  NAA (which was significantly higher than those of other regeneration media (Fig. 3b, d, f).

### Elongation, rooting and acclimatization

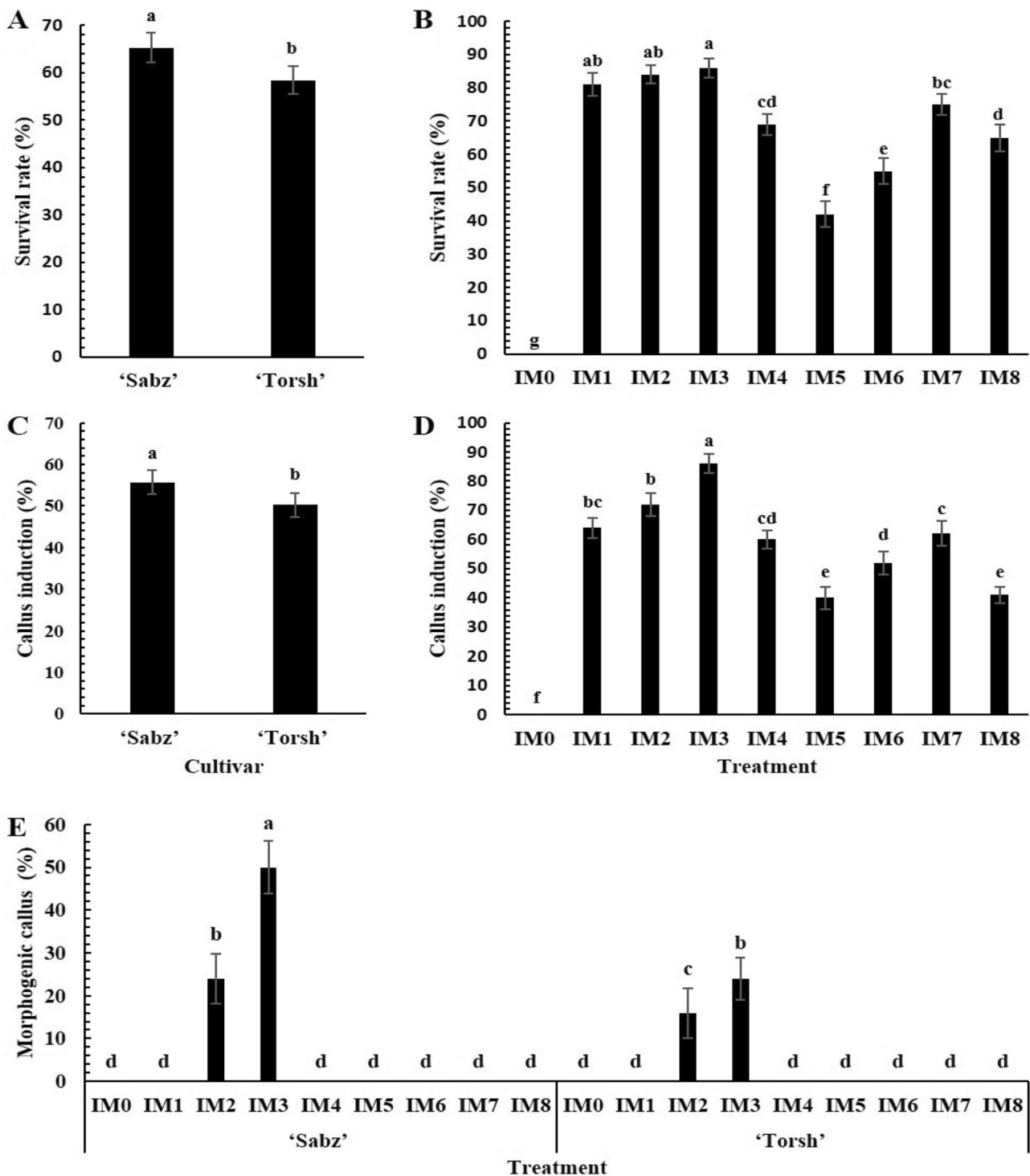
A total of 377 regenerated shoots of both cultivars (from RM media) put in elongation medium (MS supplemented with  $2.21 \mu\text{M}$  BAP plus  $0.28 \mu\text{M}$  GA<sub>3</sub>) started to grow long and after 3 weeks reached 3–5 cm in length (Fig. 4a and b). Afterwards, the shoots transferred to rooting medium (MS supplemented with  $2.46 \mu\text{M}$  IBA) and 339 of them (90%) developed their roots (Fig. 4c and d). Finally, 271 plantlets (80% of rooted plantlets) were successfully acclimatized in the greenhouse after they were taken to the plastic cups containing an equal ratio of coco peat and fine perlite. (Fig. 4e and f).

### Flow cytometry analysis

The ploidy level of in vitro regenerated plants via tTCL system and their corresponding parental plants of both cultivars ‘Sabz’ and ‘Torsh’ were assessed by the flow cytometry technique. The comparison of DNA peaks in the histograms showed an identical ploidy level between the in vitro regenerants and their corresponding parental plants in both cultivars (Fig. 5).

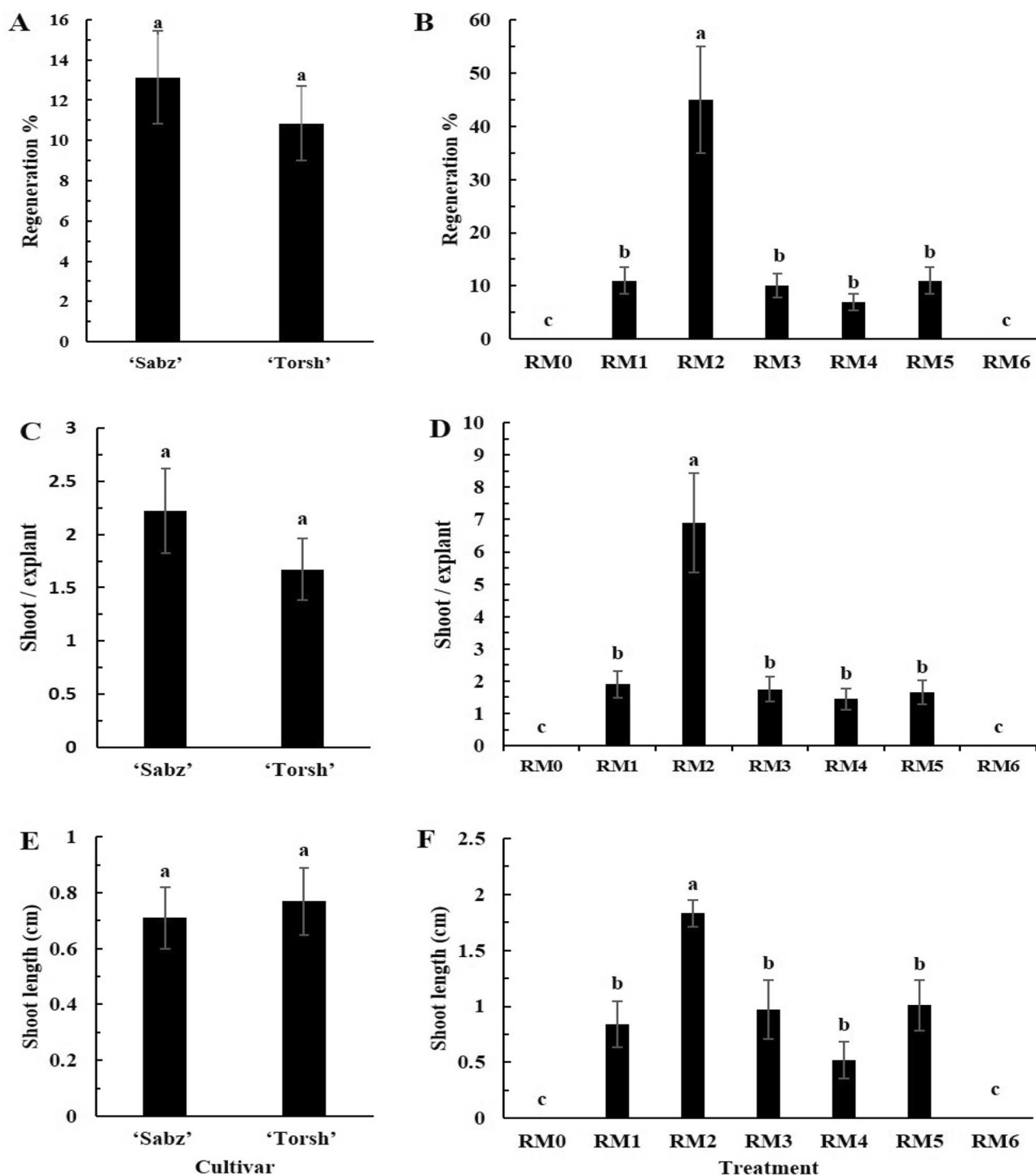
### ISSR molecular marker analysis

Among 15 ISSR primers analyzed in this study (Table 1), 8 primers including: UBC807, UBC808, UBC812, UBC814, UBC823, UBC861, IMA-5-3, and HB15 have shown desirable amplifications with clear, distinct and scorable DNA bands in both cultivars. A total of 827 bands were recorded from 20 random indirectly regenerated plants along with their corresponding parental plants (10 regenerated plants for each cultivar) (Table 1). The total number of amplified bands per primer varied from 55 to 176 and the amplicons appeared in a range of 250–3000 bp in size (Fig. 6). Six out of 8 primers (UBC807, UBC808, UBC812, UBC814, UBC823, IMA-5-3) showed polymorphism that resulted in a genetic distance of 33% between the two cultivars. A high percentage of genetic uniformity (96% in cultivar ‘Sabz’ and 97.4% in cultivar ‘Torsh’) was obtained when regenerants of each cultivar were compared to their corresponding parental plant (Fig. 7). Therefore, the high genetic uniformity percentage between the regenerants of each cultivar and their corresponding parental plant confirmed that no somaclonal



**Fig. 2** a Effect of cultivars on survival rate (%) of *F. carica* L., b Effect of different induction media on survival rate (%) of *F. carica* L., c Effect of cultivars on callus induction (%), d Effect of different induction media on callus induction (%), e Interaction of cultivars and different induction media on morphogenic callus induction (%). Treatments for induction media included: IM0 (control); IM1 (2.27  $\mu$ M TDZ+9.8  $\mu$ M IBA); IM2 (4.54  $\mu$ M TDZ+9.8  $\mu$ M IBA);

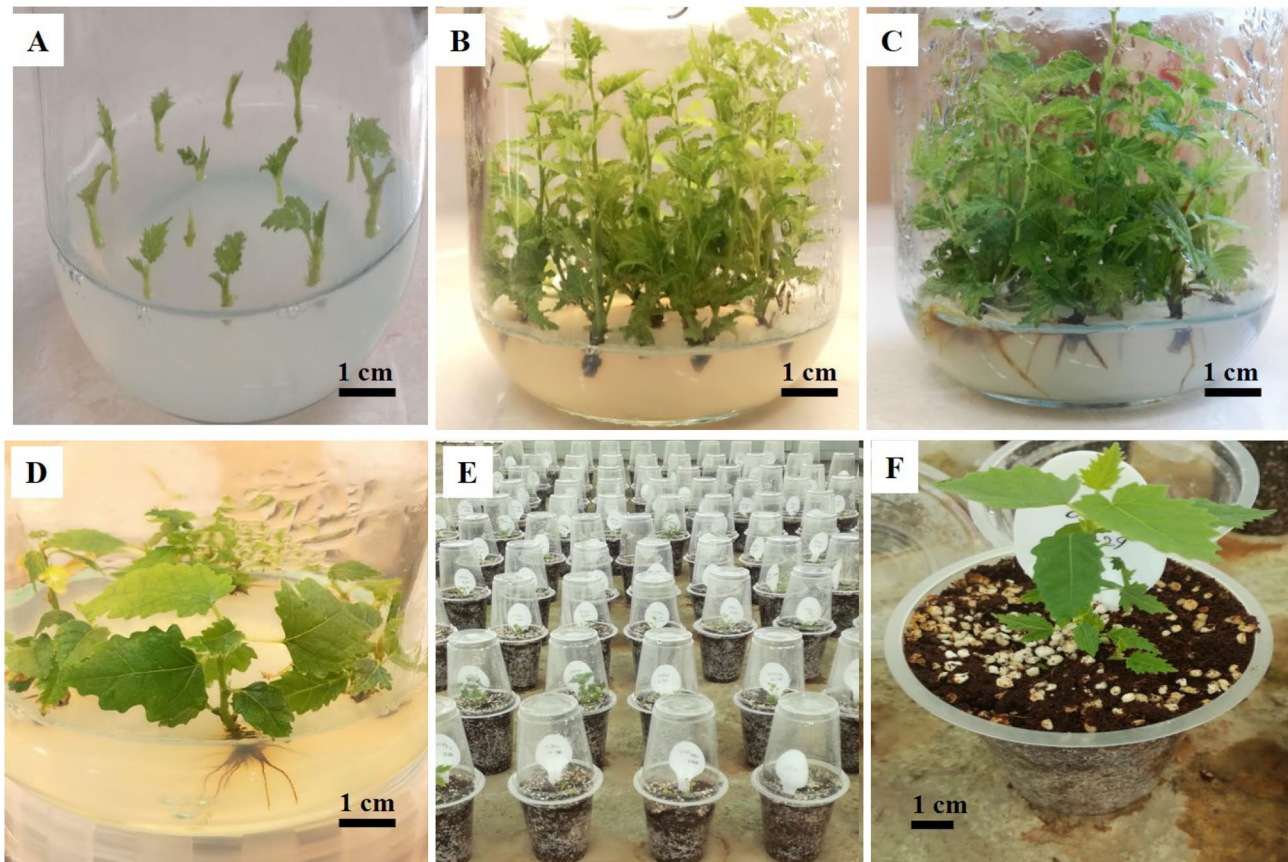
IM3 (9.08  $\mu$ M TDZ+9.8  $\mu$ M IBA); IM4 (18.16  $\mu$ M TDZ+9.8  $\mu$ M IBA); IM5 (2.27  $\mu$ M TDZ+10.74  $\mu$ M NAA); IM6 (4.54  $\mu$ M TDZ+10.74  $\mu$ M NAA); IM7 (9.08  $\mu$ M TDZ+10.74  $\mu$ M NAA); IM8 (18.16  $\mu$ M TDZ+10.74  $\mu$ M NAA). Means with different letters are significantly different at  $p < 0.05$  according to Duncan's multiple range tests



**Fig. 3** Effect of cultivars and different regeneration media on shoot regeneration percentage (%) of *F. carica* L. (a and b), number of shoots per explant (c and d) and shoot length (cm) (e and f). Regeneration media treatments included: RM0 (control); RM1 (8.84  $\mu$ M BAP+4.54  $\mu$ M TDZ+1.07  $\mu$ M NAA); RM2 (17.68  $\mu$ M BAP+4.54  $\mu$ M TDZ+1.07  $\mu$ M NAA);

RM3 (26.63  $\mu$ M BAP+4.54  $\mu$ M TDZ+1.07  $\mu$ M NAA); RM4 (8.84  $\mu$ M BAP+9.08  $\mu$ M TDZ+1.07  $\mu$ M NAA); RM5 (17.68  $\mu$ M BAP+9.08  $\mu$ M TDZ+1.07  $\mu$ M NAA); RM6 (26.63  $\mu$ M BAP+9.08  $\mu$ M TDZ+1.07  $\mu$ M NAA). Means with different letters are significantly different at  $p < 0.05$  according to Duncan's multiple range tests





**Fig. 4** In vitro elongation, rooting and acclimatization stages of the regenerated plantlets of *F. carica* L. **a** separated plantlets (less than 2 cm) cultured on elongation medium (MS medium supplemented with  $2.21 \mu\text{M}$  BAP +  $0.28 \mu\text{M}$  GA<sub>3</sub>), **b** elongated shoots after 21 days on elongation medium (almost 5 cm in length), **c** and **d** root devel-

opment 14 days after culture on rooting medium (MS medium supplemented with  $2.46 \mu\text{M}$  IBA), **e** rooted plantlets transferred to the plastic cups containing equal ratio of coco peat and fine perlite for acclimatization in the greenhouse, **f** successful acclimatized fig plant (6 weeks old)

variation had taken place from the indirect regeneration of figs using the tTCL technique.

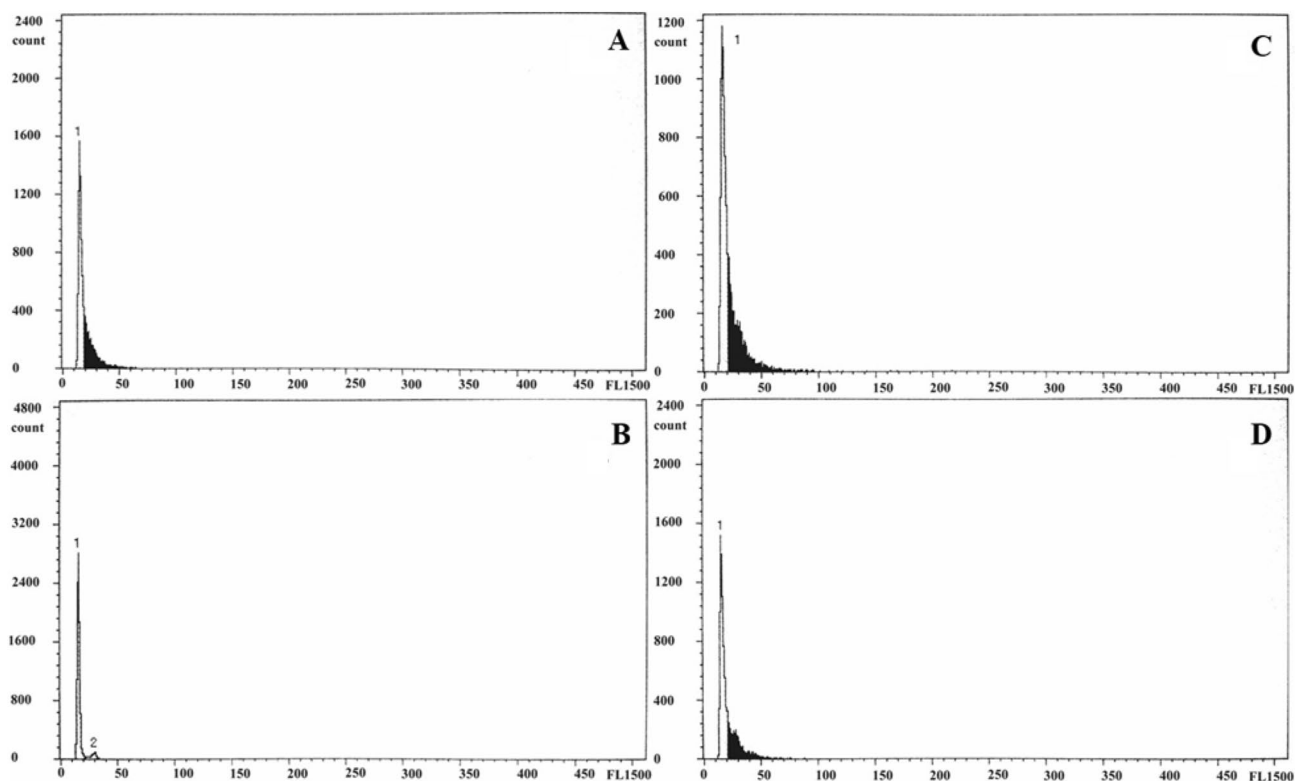
## Discussion

*F. carica* L. is known as a recalcitrant woody perennial regarding ability for in vitro morphogenesis (Kim et al. 2007a, b). Thus, the success of any biotechnological program linked to the selection of elite figs through in vitro techniques, largely depends on the establishment of a high-efficient morphogenesis protocol (Yakushiji et al. 2003). In this regard, the choice of a suitable explant and optimizing of the correct combination of PGRs in the culture media are the critical factors for establishing an efficient regeneration protocol (George et al. 2008; Kole et al. 2010).

The transfer rate of ingredients including water, minerals, energy, vitamins, and PGRs from the medium to the explant cells has a great influence on the morphogenesis efficiency. Therefore, TCL system has a great morphogenesis capacity

due to the fact that a large number of cells of explant are in connection with the culture medium (Nhut et al. 2006; Teixeira Da Silva and Dobránszki 2019).

Indirect morphogenesis studies on *F. carica* L. are limited to certain researches which have all been carried out on the basis of using in vitro derived leaf explants (Yakushiji et al. 2003; Yancheva et al. 2005; Kim et al. 2007a, b; Soliman et al. 2010; Dhage et al. 2012; Sharma et al. 2015). In all of the aforementioned studies, TDZ has played a key role in the stimulation of morphogenic callus induction and the development of adventitious shoots. This phenyl urea derivative cytokinin-like, is well known as an effective PGR for promoting callus induction and stimulating shoot organogenesis in many difficult to propagate species (Huetteman and Preece 1993). In fig morphogenesis studies it has been argued that combination of TDZ with an auxin, mostly IBA, can strongly induce a high morphogenic potential from leaf explants in *F. carica* L. (Yancheva et al. 2005; Kim et al. 2007a, b). Similar results about the stimulatory effect of TDZ and



**Fig. 5** Flow cytometry histograms of the fluorescent nuclei isolated from two *F. carica* L. cultivars. **a** mother plant of the ‘Sabz’ cultivar, **b** indirect regenerated plant of the ‘Sabz’ cultivar, **c** mother plant of the ‘Torsh’ cultivar, **d** indirect regenerated plant of the ‘Torsh’ cultivar

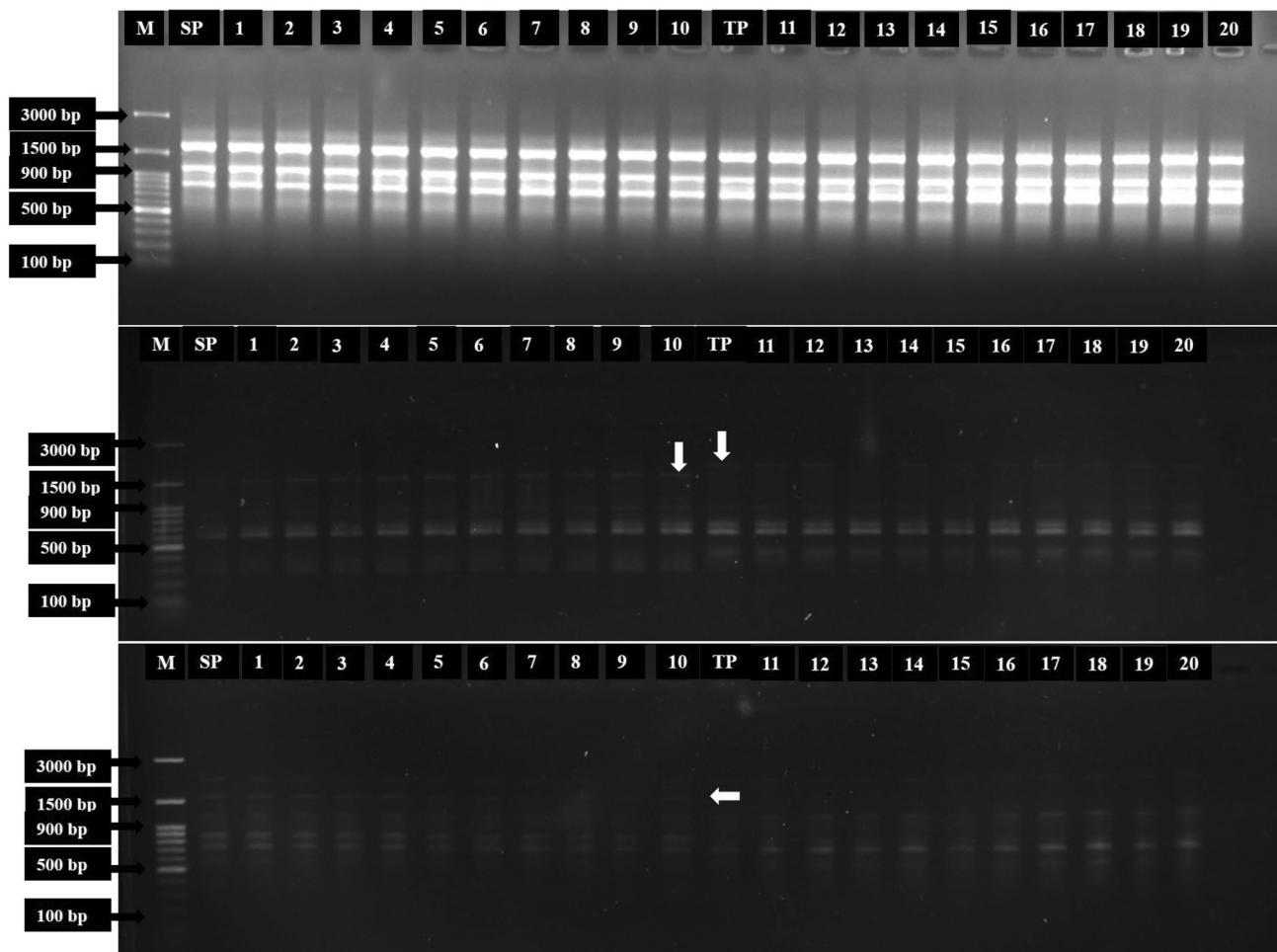
IBA were achieved in the present study, by using stem tTCL explants. It produced morphogenic callus (with creamy-green color and nodular structure) on IM3 containing  $9.08 \mu\text{M}$  TDZ +  $9.8 \mu\text{M}$  IBA which recorded as the best induction medium (Fig. 1e). Generally, an auxin is required in culture medium particularly in micropropagation of recalcitrant species via callus mediated regeneration. Selecting the type of auxin and its administered concentration is largely depended on genotype, type of explant and interactions between applied auxin and the natural endogenous substances as well. The capability of IBA as a synthetic auxin for in vitro callus induction refers to its ability for dedifferentiation of genetically programmed tissues and promoting of the cell division process (George et al. 2008).

Despite the beneficial effects of TDZ on in vitro morphogenesis, Ahmad and Faisal (2018) reported that in shoot regeneration and somatic embryogenesis, prolonged exposure of explants to TDZ can lead to callus necrosis or reshaping of formed buds or somatic embryos back to callus. Accordingly, we observed that when the stem tTCL explants were exposed to higher ( $18.16 \mu\text{M}$ ) and lower ( $2.27 \mu\text{M}$ ) concentrations of TDZ. In this case, non-morphogenic callus in white color and flaky structure was formed. Moreover, when the tTCL explants were placed on IM5 to IM8

containing various concentrations of TDZ and NAA, non-morphogenic calli were produced as well (Fig. 1d).

The secretion and oxidation of phenolic compounds from explants have always been the greatest obstacle to the in vitro morphogenesis of *F. carica* L. which leads to browning or death of the explants and dramatically reduces the micropropagation efficiency (Kim et al. 2007a, b; Dhage et al. 2015). According to our experiences on single node and shoot tip cultures and our observation on phenolic compounds secretion by explants, the exudation of phenolic compounds through the TCL method was extremely low compared to the other aforementioned methods.

Development of the meristematic domes occurred from the periphery of morphogenic calli 10 days after culturing on regeneration media (RM media), characterizing the indirect pattern of shoot regeneration with a multicellular structure (Fig. 1f). Induced calli from an individual explant can differ in terms of morphological and morphogenetic capacity, suggesting that cell layers or tissues vary with each other in their morphogenetic competence therefore, this ability is induced only in certain cells (George et al. 2008). The combination of BAP and TDZ had a great positive effect on growing of shoots from morphogenic calli. TDZ (at moderate concentration), BAP (at relatively high



**Fig. 6** PCR amplification products from 20 indirect regenerants and their corresponding parental plants of the two *F. carica* L. cultivars generated by ISSR-PCR with different primers (HB15, UBC807, UBC823 from up to down respectively). Lane M: DNA size marker (3000–100 bp), Lane SP: represents parental plant of the ‘Sabz’ culti-

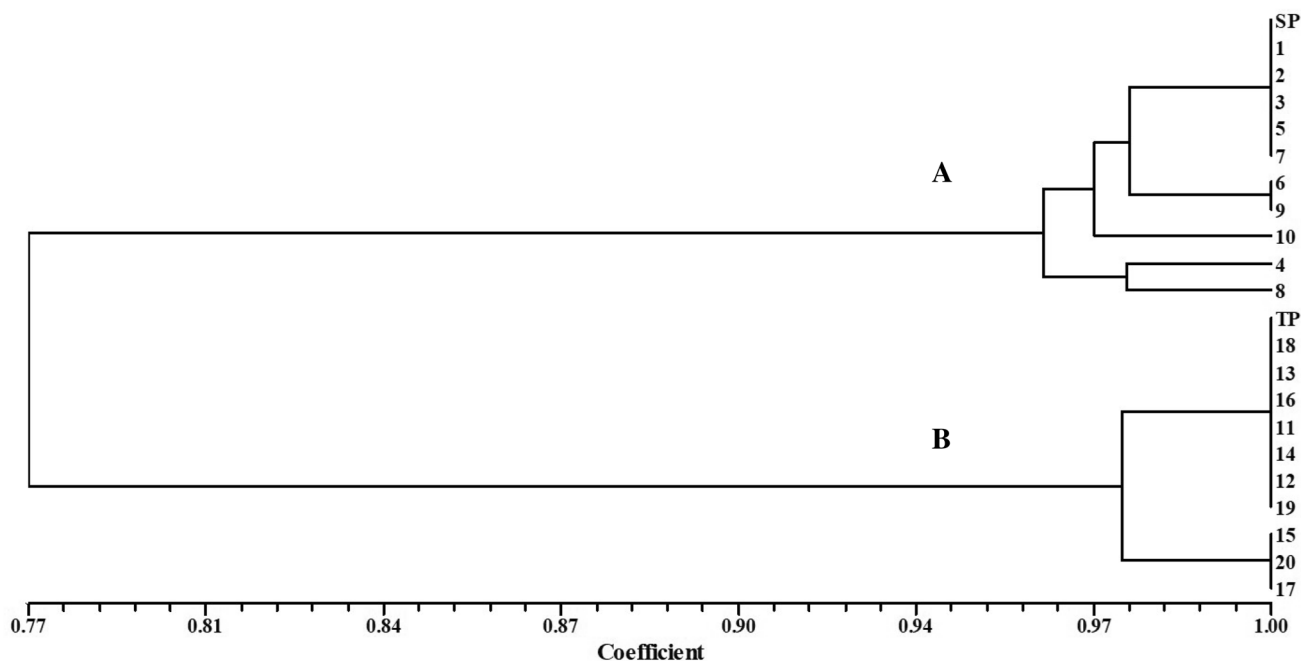
var; Lane 1–10 indirect regenerated plants via tTCL technique of the ‘Sabz’ cultivar; Lane TP: represents parental plant of the ‘Torsh’ cultivar and Lane 11–20 indirect regenerated plants via tTCL technique of the ‘Torsh’ cultivar. The white arrows show the polymorphic DNA band

concentration) along with NAA (at low concentration) could well convert the morphogenic calli to bud primordia and rising of shoots (Fig. 1g and h). Similarly, Deore and Johnson (2008), achieved the maximum adventitious shoot buds from leaf disc explants of *Jatropha curcas* L. when BAP (2.22  $\mu\text{M}$ ) + TDZ (2.27  $\mu\text{M}$ ) + IBA (0.49  $\mu\text{M}$ ) were added to the regeneration medium. They reported that the elimination of TDZ from the medium drastically reduced the shoot bud formation and resulted in higher callus induction.

It is well believed that cytokinins are essential in stimulating indirect and direct shoot organogenesis. The type of cytokinin and also the auxin/cytokinin ratio are the most determining factors in the efficiency of the plant’s morphogenesis. Two possible hypotheses could explain why an adenine-based cytokinin like BAP may promote shoot morphogenesis: (1) an extra concentration of BAP in the tissue can hinder the degradation of natural cytokinins through

feed-back inhibition, or by competing for the enzyme systems which are involved in the cytokinin metabolism and (2) produced-adenines from synthetic cytokinins (BAP) degradation can be used as a substance for the synthesis of natural cytokinins like zeatin (George et al. 2008).

In vitro plant regeneration through callus mediated protocols may accompany the risk of somaclonal variation. This phenomenon might be traceable by some traditional and modern techniques (Zafar et al. 2019). Cytological techniques and genetic molecular markers are the most common tools that are widely used in many studies for confirming the genetic fidelity of in vitro regenerants and possibly detecting somaclonal variations (Raji et al. 2018). The results of the flow cytometry analysis demonstrated that the histogram peaks from nuclei of regenerated plants in both cultivars are identical to the DNA peaks of their corresponding parental plants (Fig. 5). It could be inferred that their ploidy level was



**Fig. 7** Dendrogram based on UPGMA of Jaccard's similarity matrix generated using ISSR molecular markers analysis, showing a high genetic similarity in regenerated plants via stem tTCL culture and their corresponding parental plants of two *F. carica* cultivars. (A) cultivar 'Sabz' and (B) cultivar 'Torsh'. SP: represents parental plant of

cultivar 'Sabz'; Numbers 1–10 indirect regenerated plants via tTCL technique of cultivar 'Sabz'; TP: represents parental plant of cultivar 'Torsh' and numbers 11–20 indirect regenerated plants via tTCL technique of cultivar 'Torsh'

not changed, so the genetic stability remained preserved in the regenerated plants of both cultivars. Many reports have also confirmed that the genetic uniformity of regenerated plants via callus culture can be evaluated by flow cytometry analysis (Ali et al. 2017; Carloni et al. 2018; Konar et al. 2018; Raji et al. 2018).

Alternatively, ISSR markers could also be used to examine the regenerants because they hold the reproducibility advantage like AFLP and microsatellite markers and have data abundance similar to RAPD markers. These features make ISSR analysis as a powerful tool for assessing genetic fidelity among plants. Thanks to the long primer size (16–25 bp) and relatively high annealing temperature (45–60 °C), ISSR markers have a high reproducibility characteristic (Alizadeh et al. 2015). Leroy and Leon (2000) reported that general genetic occurrences underlying plant genomic instability such as deletions, amplifications, translocations, insertions, recombination or chemical alterations can be properly detectable using ISSR markers. Earlier reports have well documented the efficacy of ISSR molecular markers for fidelity check and in vitro selection of *F. carica* L. (Dessoky et al. 2016; Metwali et al. 2016). The results of ISSR analysis in the current study displayed a high genetic uniformity between the in vitro regenerants and their corresponding parental plants in both *F. carica* L.

cultivars (Fig. 7). Therefore, it can be stated that the developed regeneration protocol is suitable for mass-propagation of this species and ISSR markers are reliable for the stability analysis of the germplasm of the propagated plants.

## Conclusion

In conclusion, we attained an effective, rapid and high-efficient in vitro propagation method for mass production of genetically identical plants in *F. carica* L. using the TCL procedure. Providing highly efficient callus production and shoot regeneration from callus (6.9 shoots per explant) and easy control of the phenolic compounds are the most important advantages of using stem tTCL explants in fig. The flow cytometry and the ISSR analysis also confirmed the clonal identity (about 97%) of the in vitro regenerated plants. This protocol is suggested as a powerful method for commercial micropropagation of fig, in vitro breeding programs, ploidy studies, as well as mass production of medicinal compounds from callus culture in bioreactors.

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