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Germination of Persian Walnut Somatic Embryos and Evaluation of their Genetic Stability by ISSR Fingerprinting and Flow Cytometry

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***Abstract*. Somatic embryos (SEs) can play important roles in genetic manipulation and breeding. They can be used as targets for induced mutagenesis, as material for cryopreservation and germplasm conservation, and for transformation or gene editing in support of plant improvement and proof of gene function. However, germination rates of walnut (*Juglans regia*) SEs are low, and the genetic stability of plantlets regenerated from them has not been explored. Here, we studied first the effects of gibberellic acid (GA_3) and low temperature storage (LTS) on germination of walnut somatic embryos. Second, we assessed the genetic fidelity of plantlets regenerated from these SEs by comparing them to each other and to their cultivar of origin. Results showed that GA_3 and LTS increased the walnut SE germination rate. The best rate was observed when SEs were subjected to LTS for 60 d followed by culture on a medium with either 1 or 3 mg·L^{-1} GA_3 (56.6% and 46.6% germination respectively). Genetic stability was evaluated, using flow cytometry and 15 sets of ISSR primers. Flow cytometry indicated that all samples (i.e., regenerated and parental counterpart) showed the same peak. Amplified fragments of inter simple sequence repeats (ISSR) primers ranged in size from ≈200 to 1800 bp. All ISSR profiles of regenerants were monomorphic. Results did not show any genetic differences among plantlets regenerated from SEs or from their parental counterpart. Due to this apparent genetic stability, walnut SEs can be useful for genetic transformation and germplasm conservation.**

Based on historical evidence, walnuts have been used as a food source since at least 7000 BC (Dreher et al., 1996). The high content of oil (unsaturated fatty acids), protein, antioxidants, phenolic acids, minerals, vitamins, and tocopherol in walnut kernels have encouraged people worldwide to include walnuts in their diet (Rodushkin et al., 2011). Furthermore, according to ethnopharmacological studies, walnut leaves have been used by indigenous people globally to treat ailments, including diarrhea, sinusitis, and diabetes (de Souza et al., 2017; Mouhajir et al., 2001; Nasab and Khosravi, 2014). The wood of walnut trees is of high quality and is used for making durable furniture. The immature fruits can be pickled in vinegar (Facciola, 1990).

Somatic embryogenesis plays an important role in micropropagation, genetic manipulation, cryopreservation, induced mutagenesis, and germplasm conservation in plants (Sharma et al., 2012; Vahdati et al., 2008). In general, somatic embryogenesis can provide quality materials for biotechnology development in plants, including walnuts Tulecke and McGranahan (1985) reported the first successful somatic embryogenesis in walnut. During the past three decades, additional researchers have studied the induction, proliferation, maturation, and germination of SEs (Cornu and Jay-Allemand, 1989; Tulecke et al., 1988; Vahdati et al., 2006). Despite all the benefits that somatic embryos have for biotechnological advancements, plantlets regenerated from somatic embryos could exhibit somaclonal variation (Bradaï et al., 2016). Heritable adverse changes due to somaclonal variation can be a problematic in tree-breeding programs, which typically encounter long juvenile phases before phenotypic selection of mature elite genotypes, a delay period requiring substantial investment of time and money (Fourré et al., 1997). Various factors, such as explant source, ploidy level, genotype, regeneration system, plant growth regulators, and culture conditions, can contribute to somaclonal variation (Brar and Jain, 1998). Therefore, homogeneity and trueness-to-type of regenerated plantlets should be checked. Several methods are available for this analysis, including chromosome determination, flow cytometry, molecular markers, and biochemical and phenotypic analyses (Bradaï et al., 2016; Harding, 2004; Sadat-Hosseini et al., 2011). Molecular analyses and flow cytometry have been used in many species to evaluate the trueness-to-type of plantlets regenerated via somatic embryogenesis. These species include olives (Bradaï et al., 2016), muskmelon (Raji et al., 2018), chrysanthemum (Lema-Rumińska and Śliwińska, 2015), ajowan (Niazian et al., 2017), Italian grapevine (Carra et al., 2016), *Anthurium andraeanum* (Bhattacharya et al., 2016), citrus species (Meziane et al., 2017), and *Cassia occidentalis* (Naz et al., 2016). In walnut, RFLP and isozyme analysis were used to determine the origin of SEs by Aly et al. (1992). Molecular markers commonly used for the evaluation of somaclonal variation include randomly amplified polymorphic DNA (RAPD), restricted fragment length polymorphism (RFLP), simple sequence repeats (SSRs), and ISSR. Of these, ISSR is the fastest, simplest, and most powerful (Raji et al., 2018).

Persian walnut somatic embryos were first obtained in the 1980s, but the homogeneity of

Table 1. Effect of GA_3 and LTS on walnut SE development. Values shown are means of three replicates of ten embryos each. Those with the same letter in each row do not differ significantly by Tukey's test ($P < 0.05$).

	Days of LTS								
	20			40			60		
	GA_3 mg·L^{-1}								
	0	1	3	0	1	3	0	1	3
Shoot formation only (%)	6.6 b	16.6 ab	16.6 ab	20 ab	20 ab	23.3 ab	23.3 ab	33.3 a	26.6 a
Root formation only (%)	3.3 b	10 ab	10 ab	13.3 ab	20 ab	20 ab	10 ab	23.3 ab	26.6 a
Both roots and shoots (%)	3.3 c	6.6 c	10 bc	33.3 b	36.6 b	33.3 b	43.3 ab	56.6 a	46.6 a

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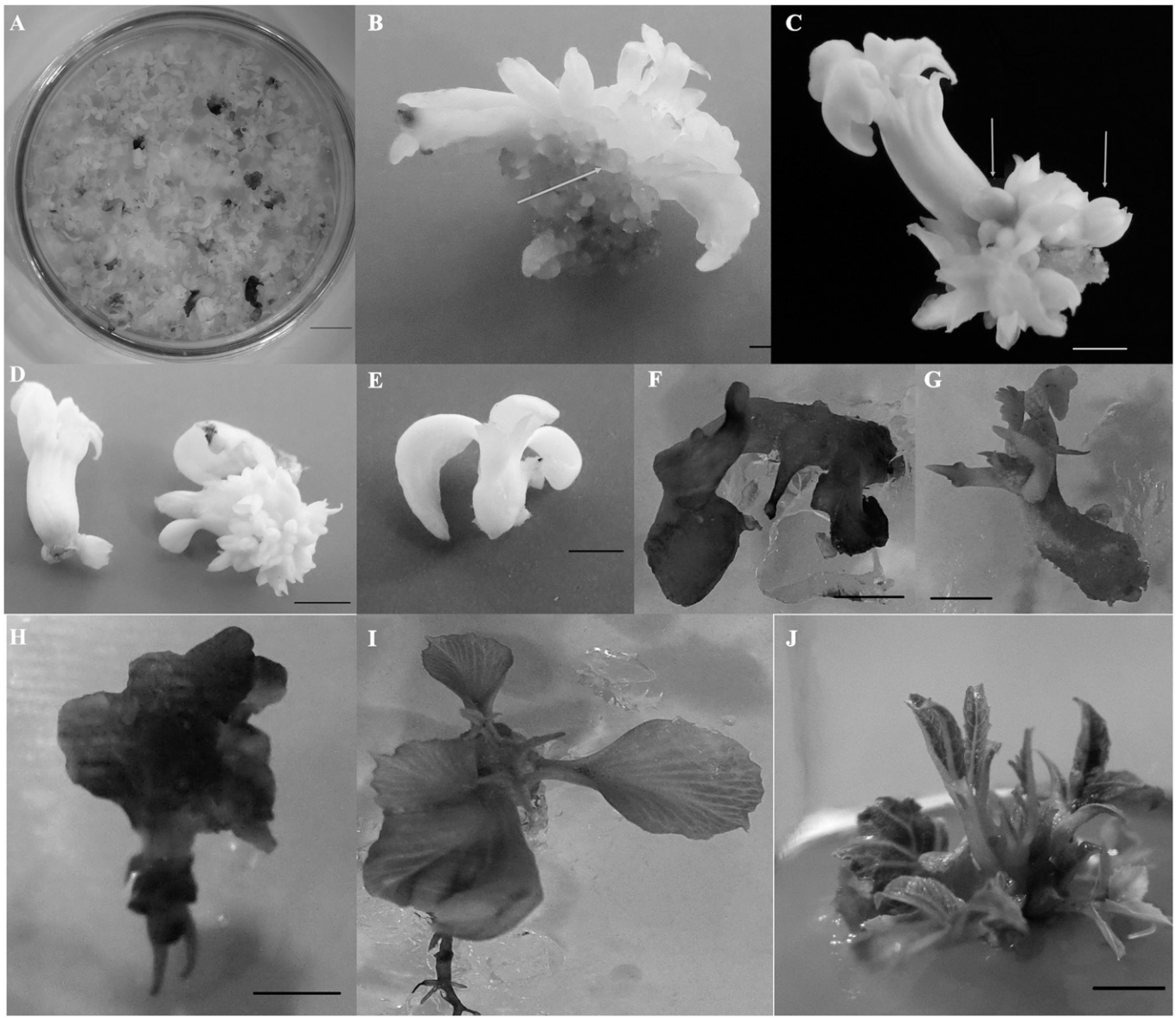

Fig. 1. Somatic embryos and plant regeneration of *J. regia*. (**A**) Somatic embryos 12 d after subculture. (**B**) Globular embryos formed after 2 weeks. (**C**) and (**D**) mature and torpedo somatic embryos. (**E**) Somatic embryo before transferring to germination medium (bar = 3 mm). (**F**) Necrotic somatic embryos on the germination medium. (**G**) SE with only shoot formation. (**H**) SE with only root formation. (**I**) SE with shoot and root meristem. (**J**) Growing regenerated shoots developed from a somatic embryo on DKW medium under in vitro conditions (bar = 6 mm).

plantlets regenerated from these has not been examined to date. The aim of this study was to evaluate the genetic stability of plants derived from walnut somatic embryos. To this end, the ploidy level of regenerated plants was compared with their parents by using flow cytometry. Genetic stability among plantlets obtained by somatic embryogenesis was evaluated by ISSR. Finally, we report the first examination of the genetic stability of plants regenerated from somatic embryos of walnut. This information can be useful in evaluating the future application and reliability of somatic embryogenesis in breeding programs and for genetic modification.

Materials and Methods

Source of somatic embryos and culture conditions. SEs of Persian walnut (*Juglans regia* L.) were regenerated from immature walnut catkins of the cultivar Chandler (Mendum and McGranahan, 1995). This embryogenic line had been maintained for 23 years by means of secondary embryogenesis on hormone-free Driver and Kuniyaki Walnut (DKW) medium (Driver and Kuniyuki, 1984), with subculturing every 1 to 2 weeks. Embryos for this work were selected at the globular stage and cultured on a maturation medium (DKW) with 30 $g \cdot L^{-1}$ sucrose and 2 $mg \cdot L^{-1}$ abscisic acid (ABA) for 6 weeks, with weekly transfer to fresh medium (Vahdati et al., 2008). All cultures were maintained at 25 ± 1 °C in the dark.

Germination of somatic embryos. Mature, translucent-white embryos were selected for germination and chilled at 4 °C for 20, 40, or 60 d to overcome dormancy (without transfer to a new medium). These embryos were then transferred to sterile, empty petri dishes and placed over a saturated solution of $ZnSO_4$ in a desiccator at 25 °C for 4 d in the dark. When the embryos turned opaque white, they were transferred to a solid DKW medium with 0, 1, or 3 $mg \cdot L^{-1}$ GA_3 and kept in a growth chamber for 15 d at 26 ± 1 °C with a 16/8-h photoperiod. In this study, the embryos that showed both a green shoot and a root were classified as germinated.

DNA extraction. Leaf tissue samples (150 mg) were ground in 1.5-mL Eppendorf tubes containing liquid nitrogen. Then 700 mL of extraction buffer [2% Cetyl trimethylammonium bromide (CTAB), 1.4 M NaCl, 100 mM tris(hydroxymethyl)aminomethane (Tris), and 20 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.3] was added to the powdered leaves. Total DNA was extracted according

to the CTAB method (Doyle and Doyle, 1987) with minor modifications. DNA quality and concentration were determined by a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA).

Molecular analysis. Assessment of genetic stability between the plantlets regenerated from SEs and their parental counterpart was performed using 15 sets of ISSR primers (Table 2). Seven plantlets were randomly selected for analysis, and the total DNA of each was amplified by polymerase chain reaction (PCR). PCR was performed in a 25-µL reaction volume consisting of 12.5 µL Ampliqon (Taq DNA Polymerase Master Mix RED, Odense, Denmark), 10.5 µL double-distilled water, 1 µL DNA (50 ng/µL), and 1 µL primer (10 pmol). Amplification was performed using initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 60 s (denaturation), annealing (49.5 to 57.3 °C) for 60 s, and extension at 72 °C for 60 s, and a single final extension step at 72 °C for 5 min. PCR was performed on a BioRad thermocycler (Bio-Rad, Hercules, CA).

Flow cytometry analysis. The genome size of seven plants regenerated from SEs and their parental source, 'Chandler', were evaluated by flow cytometry. For this purpose, 0.5 cm^2 of leaf tissue was chopped with a sharp scalpel in a glass petri dish containing 500 µL of nuclei extraction buffer. Then, 1600 µL of a nuclei-staining solution (CyStain PI Absolute P; Sysmex America, Lincolnshire, IL) was added to the crude mixture. To eliminate cell debris, the suspension was then passed through a 50-µm filter. The genome size of each sample was calculated using a flow cytometry machine (Partec, Münster, Germany).

Data analysis. Germination of SEs was analyzed using a complete randomized design with two factors and three replications (individual petri dishes), consisting of ten mature SEs per dish. Analysis of variance was carried out using the Statistical Analysis System (SAS Institute, Inc., 1991), and mean values were compared using Tukey's test ($P < 0.05$). For molecular analysis, only amplified bands in the range of 200 to 2000 bp were considered. Polymorphic ISSR markers were scored for presence (1) or absence (0) of bands.

Results and Discussion

Somatic embryo germination. The effects of GA_3 and low temperature storage (LTS) (4 °C) on the germination rates of somatic walnut embryos were examined (Table 1). GA_3 application and LTS both increased the germination of somatic embryos. Germination rate increased with an increasing period of LTS. The best germination was produced by LTS for 60 d followed by culture on medium with 1 or 3 mg l^{-1} GA_3 (56.6% and 46.6% germination). The lowest germination rate occurred following the shortest LTS period tested (20 d) and culture in the absence of GA_3 (Fig. 1). The effect of LTS and GA_3 on germination was consistent with the earlier results of Tulecke and McGranahan (1985), and Deng and Cornu (1992). Previous walnut SE germination studies found germination rates of 45% (Deng and Cornu, 1992), 41% (Vahdati et al., 2008), and 6.05% (Jalali et al., 2017). Lack of shoot or root meristems is a common abnormality in walnut somatic embryos (Jalali et al., 2017; Vahdati et al., 2008). Studies of somatic embryo germination in other species, including *Pinus radiata* (Montalbán et al., 2015), *Picea morrisonicola* (Liao and Juan, 2015), *Phoenix dactylifera* (Shareef et al., 2016), *Pyrus communis* (Ameri et al., 2018), and *Picea abies* (Tikkinen et al., 2018) have also found pretreatment by LTS followed by transfer to a medium containing plant growth regulators to be beneficial.

Flow cytometry analysis. Flow cytometry is an appropriate method for aneuploid/ploidy identification and genome size analysis in plants (Khorami et al., 2018; Sliwinska and Thiem, 2007). Ploidy stability among walnut plantlets regenerated from SEs and their parental counterpart was assessed using flow cytometry. Total DNA of 20 regenerants was analyzed and compared. The results showed no differences in ploidy level among these plantlets or relative to Chandler, their cultivar of origin (Fig. 2). In addition, the histogram developed from flow cytometry analysis showed a peak similar to the nuclear 2C DNA content (i.e., peak 2) at channel 100 (Fig. 2A and B), confirming that the genome size of plants regenerated from SEs and their parental counterpart, 'Chandler', are similar. Some factors affecting the genetic stability of regenerated plants in vitro may include explant source, genotype, ploidy level, plant growth regulators, regeneration system, and culture conditions (Brar and Jain, 1998). Our flow cytometry results did not identify any problems resulting from the use of plant growth regulators, culture conditions, or the regeneration system used in producing

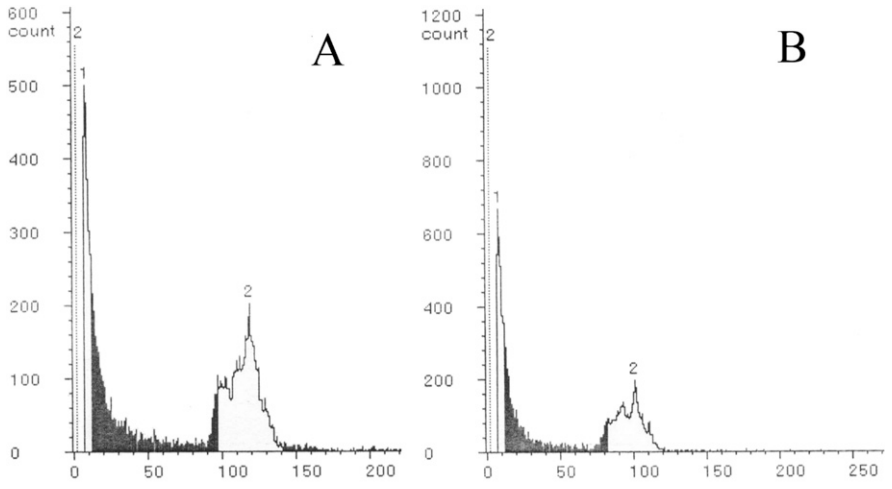

Fig. 2. Flow cytometry analysis of *J. regia*. (**A**) Typical flow cytometry profile of plants regenerated by SEs (all samples showed 2 at channel 100). (**B**) Flow cytometry of the parental counterpart, the cultivar Chandler.

Table 2. Summary of ISSR primers for genetic analysis of plantlets derived from walnut somatic embryos.

Sr. no.	Primer code	Sequence (5′-3′)	Tm (°C)	Scorable bands per primer	Size range of amplified product (bp)
1	(GA)8-G	GAGAGAGAGAGAGAGAG	53.5	6	400–1800
2	(AC)8-TG	ACACACACACACACACTG	51.5	7	300–1800
3	(AGTG)4	AGTGAGTGAGTGAGTG	52	6	400–1800
4	(GATA)5	GATAGATAGATAGATAGATA	49.5	5	500–1500
5	(TCT)6	TCTTCTTCTTCTTCTTCT	51	6	400–1800
6	(GA)8-TC	GAGAGAGAGAGAGAGATC	52.5	9	300–1500
7	TGT-(CAC)5	TGTCACCACCACCACCAC	57.3	5	300–1500
8	CAC-(TGT)5	CACTGTTGTTGTTGTTGT	50.8	8	300–1800
9	ACA-(CT)7	ACACTCTCTCTCTCTCT	53	9	500–1800
10	AGA-(AC)7	AGAACACACACACACAC	54	6	300–1800
Total				67	

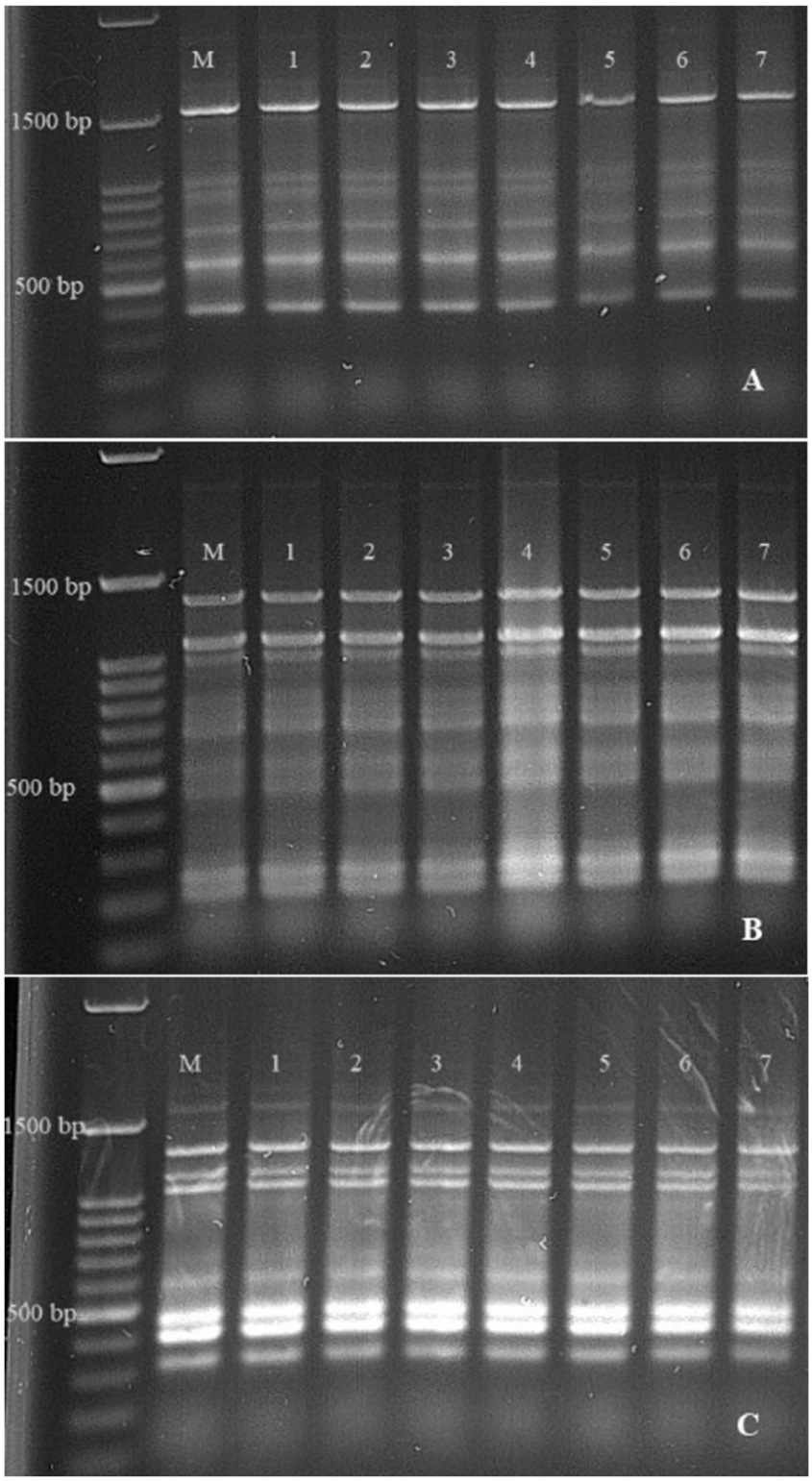

Fig. 3. ISSR fingerprint profile obtained with primer (AGTG)4 (**A**), (GA)8-TC (**B**), and CAC-(TGT)5 (**C**), respectively. M = mother plant (the cultivar Chandler); 1–7 regenerants from 'Chandler' somatic embryos.

walnut SEs. Genome size similarity and fidelity were also reported in previous studies of regeneration from somatic embryos of other species (Niazian et al., 2017; Pinto et al., 2004; Viehmannova et al., 2016).

Molecular analysis. Genetic fidelity of plantlets regenerated from SEs and their parental counterpart were also compared using ISSRs primers. Only 10 of the 15 primers tested produced scorable and distinct bands. For each primer, the number of scorable bands varied from 5 (TGT-(CAC) 5) to 9 (GA)8-TC and ACA-(CT)7, with an average value of 6.7 bands per primer (Table 2). The amplified fragments ranged in size from ≈200 to 1800 bp (Table 2, Fig. 3A–C). All ISSR profiles of regenerants were monomorphic, and all 10 ISSR primers used produced similar banding patterns for all regenerated plantlets and their parental counterpart. The ISSR profiles obtained using primers (AGTG)4, (GA)8-TC, and CAC-(TGT)5 are shown in Fig. 3A–C, respectively. Somaclonal variation or genetic instability in plants could potentially be caused by long-term in vitro storage of plant germplasm (Sherif et al., 2018). Molecular markers such as amplified fragment length polymorphism (AFLP), RAPD, ISSRs, and start codon targeted (SCoT) all have been used to assess the genetic stability of in vitro versus in vivo plants (Bhattacharyya and Kumaria, 2015; Khilwani et al., 2016; Milella et al., 2011; Seth et al., 2017; Sherif et al., 2018). Some previous studies have reported 100% homogeneity among plantlets regenerated from somatic embryos. These types include *Bacopa monnieri* (Khilwani et al., 2016), *Citrullus lanatus* (Vinoth and Ravindhran, 2016), and *Abutilon indicum* (Seth et al., 2017). Even though the walnut somatic embryos used in this study have been subcultured for more than 20 years, surprisingly, the ISSR profiles showed 100% genetic stability when the regenerated plantlets were compared with the cultivar of origin. This finding could be partly a result of not using any plant growth regulators in the culture medium during this time.

Conclusion

In this study, we report a reliable method for regenerating walnut plants from SEs and show SE germination is improved by cold storage and GA_3 application. Furthermore, we evaluated the genetic fidelity of plants regenerated from walnut SEs that had been subcultured for more than 20 years (since 1995). Flow cytometry analysis and ISSR profiles did not identify any genetic variation among the SE-derived plantlets or deviation from the cultivar of origin. The apparent genetic stability supports use of walnut SEs for clonal plant production, genetic transformation, gene editing, proof of gene function, and other genomic applications, collectively facilitating the progress of walnut breeding programs.

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