GENETIC STABILITY MONITORING IN MICROPROPAGATED TOMATO CULTIVARS

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Abstract. The effect of five different hormonal combinations on genetic stability of micropropagated shoots from five tomato (*Lycopersicon esculentum* Mill.) cultivars was investigated by amplified fragment length polymorphism (AFLP) and sequencing of lycopene β-cyclase gene (LCY-B). Using four primer sets, a number of 107 highly reproducible fragments were generated through AFLP. The banding patterns for each primer were highly uniform in all cultivars comparable to mother plants. Sequence analysis of LCY-B gene (1344 bp) revealedno genetic differences between *in vitro*-derived tomato regenerants under different hormonal treatments and their mother plants. These results indicate that the developed *in vitro* tomato plants were true-to-type after short term exposure to different hormonal treatments.

Keywords: micropropagation, genetic stability, AFLP, LCY-B gene.

Rezumat. Monitorizarea stabilităţii genetice la soiuri de tomate micropropagate. Efectul diferitelor combinaţii hormonale asupra stabilităţii genetice la cinci soiuri de tomate (*Lycopersicon esculentum* Mill.) micropropagate a fost studiat atât prin tehnica AFLP cât şi prin secvenţierea genei licopen β-ciclază (LCY-B). Prin utilizarea a patru seturi de amorse, un număr de107 de fragmente reproductibile au fost generate prin AFLP. Patternurile pentru fiecare amorsă utilizată au fost uniforme la toate soiurile fiind comparabile cu cele de la plantele mamă. Secvenţierea gene LCY-B (1344 bp), nu a relevat diferenţe genetice între tomatele regenerate *in vitro* sub influenţa diferitelor tratamente hormonale şi plantele mamă. Rezultate obţinute indică faptul că, plantele de tomate crescute *in vitro* au fost identice cu plantele mamă după expunerea pe termen scurt la diferite tratamente hormonale.

Cuvinte cheie: micropropagare, stabilitate genetică, AFLP, gena LCY-B.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important *Solanaceae* crop species in the world, not only due to its economic value, as it possesses appreciable quantities of vitamins and minerals, but also to its relevance as a model system for genetic, molecular and physiological analyses (GUBIŠ et al., 2004). Tomatoes are the major source of lycopene (20-50 mg of lycopene/100 g of fruit weight) (KALLOO, 1991), which is one of the most powerful antioxidant in the carotenoid family, and it is also known to prevent cancer (BLOCK et al., 1992; GERSTER, 1997; RAO & AGARWAL, 2000). Thus, numerous studies reported the use of plant tissue culture techniques in tomato breeding programs (COMPTON & VEILLEUX, 1991; EL-BAKRY, 2002; GUBIŠ et al., 2003, 2004, 2005; BHATIA et al., 2004, 2005; ISHAG et al., 2009; RASHID & BAL, 2010; ASHAKIRAN et al., 2011; MAMIDALA & NANNA, 2011; VINOTH et al., 2012).

Plant tissue culture has the potential to produce mass quantities of identical, disease free planting material for commercial use. However, this technique is often associated with somaclonal variations andrequires periodic monitoring of the genetic stability of *in vitro* grown plantlets (PANDA et al., 2007).Somaclonal variations may occur due to modifications in the chromosome number, chromosome breaks, transposon activations, deletions, genome rearrangements, polyploidy, nucleotide substitutions or methylation patterns (LARKIN & SCOWCROFT, 1981; DEVERNO et al., 1994; JALIGOT et al., 2000). The factors influencing the genetic stability of tissue culture (TC) regenerants, include the composition of the culture medium, especially the concentration and type of plant growth regulators (PGRs) used, physical conditions and duration between successive subcultures (VASIL, 1979). Several strategies were employed to detect somaclonal variations in TC-derived tomato plants, including cytogenetic analysis (ASAKURA et al., 1995), molecular markers such as RAPD (SONIYA et al., 2001) and AFLP (SINGH et al., 2002; BHATIA et al., 2005) and methylation patterns analysis (SMULDERS et al., 1995). Overall, DNA-based markers appeared to be more efficient for screening the genetic integrity of TC-raised plants, since they are not affected by environmental factors and generate reproducible results (SINGH et al., 2002). Among molecular techniques, AFLP has a wider genome coverage compared to other DNA-based markers (BREYNE et al., 1997), it is characterized by high reproducibility and does not require prior sequence information (BHATIA et al., 2005). Therefore, AFLP proved to be the most reliable tool in establishing the clonal fidelity of TC-derived plants (TURNER et al., 2001; POPESCU et al., 2002; SINGH et al., 2002; BHATIA et al., 2005).

In the present study, in order to establish if there are any genetic changes induced in micropropagated tomato plants under the influence of different PGRs comparing with *in vitro* seed-derived mother plants cultured on hormone free medium, we have employed two different approaches: AFLP, for entire genome screening and a candidate gene approach by sequencing the lycopene β-cyclase gene (LCY-B) from leaves.

MATERIAL AND METHODS

Plant material, culture medium and growth condition. In vitro cultures were established from mature tomato (*Lycopersicon esculentum* Mill. cultivars 'Capriciu', 'Darsirius', 'Kristin', 'Pontica' and'Siriana') seeds provided by the

Research Institute for Vegetable and Flower Gardening, Vidra, Romania. Seeds were surface sterilized with 1% sodium hypochlorite for 15 min, rinsed three times with sterile distilled water before transferred to 100 ml Erlenmeyer flaskscontaining30 ml half-strength (1/2) MURASHIGE & SKOOG (1962) (MS) medium supplemented with 30 g/l sucrose and solidified with 7.6gl^{-1}agar. The pH of the medium was adjusted to 5.7 before autoclaving (20 min at 121^{o}C). The cultures were maintained in the dark for four days and then transferred to 16 h light/8 h dark photoperiod under white fluorescent light (36 µmol$m^{2}s^{-1}$ photosynthetic active radiation - PAR) at 24±1°C. Nodal explants (1-1.5 cm in length) and shoot apices, excised from one individual (mother plant represented by 1 month-old seedlings) of each cultivar, were inoculated on MS medium supplemented with 1.5 mg l^{-1}Zeatin (Z), 0.1 mg l^{-1}Indole-3-acetic acid (IAA), 30 g l^{-1} sucrose and 7.5 g l^{-1} agar for rapid *in vitro* multiplication and regeneration. These stock cultures were further used for testing the effects of various PGRs on genetic stability of TC derived tomato regenerants. For this purpose single node explants (1-1.5 cm in length) excised from stock culture regenerants were inoculated in 150 ml Erlenmeyer flasks containing 40 ml MS medium supplemented with the following hormonal combinations: 1) thidiazuron (TDZ) (1.5 mg l^{-1}) + β-Indolilbutiric acid (IBA) (0.2 mg l^{-1}); 2) Z (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 3) Kinetin (K) (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 4) N^{6}-Benzyladenine (BA) (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 5) BA (0.5 mg l^{-1}) + K (0.3 mg l^{-1}) + IBA (0.2 mg l^{-1}). Regenerated plantlets were subcultured four times, with 45 days intervals, on the same hormonal variant. The growth conditions mentioned above were maintained throughout the experiments.

Genomic DNA isolation. Total genomic DNA was isolated from silica gel dried leaf tissue (12mg) using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Purified total DNA was quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific) and wasstored at -80^{o}C until use.

AFLP assay was carried out according to VOS et al. (1995) with minor modifications.

DNA restriction. The digestion mixture was prepared on ice by mixing the following constituents: 2X Tango Buffer (Fermentas), 10 µg/ml Bovine Serum Albumin (BSA) (Applied Biosystems), 0.1 U *Mse*I (Fermentas), 0.25 U*Eco*RI (Fermentas). This mixture was brought to a final volume of 15 µl with ultrapure (UP) water and was distributed on a 96 well microplate. For each sample 5 µl of genomic DNA was added to the digestion mixture. The sampleswereincubated for 2 hours at 37ºC.

*Adaptors ligation.*The ligation mixture was prepared on ice and included: 2XT4 Buffer, 0.36 µM of each *Mse*I adaptor, 0.36 µM of each *Eco*RI adaptor, 0.05 UT4 ligase (Fermentas) and UP water was added to the final volume of 20 µl. The ligation mixture (20 µl) was added to the previously digested DNA and was incubated for 2 hours at 37ºC. This mixture was further diluted 1:10 with UP water.

Pre-selective amplification. The ligated DNA was pre-amplified using *Eco*RI and *Mse*I primers, with one selective nucleotide at the 3'end of each primer. The following cycling parameters were used: 1 cycle of 72°C for 2 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min and 1 cycle of final elongation at 72°C for 10 min.

Selective amplification. Four primer combinations: *Eco*RI-AAC/*Mse*I-CAA, *Eco*RI-AAC/*Mse*I-CAG, *Eco*RI-AGC/*Mse*I-CTT, *Eco*RI-AGC/*Mse*I-CAC were chosen based on literature (Miao et al. 2009) (Table 1). The cycling parameters were as it follows: 1 cycle at 95°C for 10 min, 13 cycles at 94°C for 30 s, 65→55.9ºC (ΔT = -0.7ºC/cycle) for 1 min, 72°C for 1 min, 23 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and 1 cycle of final elongation at 72°C for 10 min.

Table 1. Sequences of primers and adaptors used for AFLP assay.

Name	**Sequence 5'→3'**
*Mse*I- adapter 1	GACGATGAGTCCGAG
*Mse*I- adapter 2	TACTCAGGACTCAT
*Eco*RI- adapter 1	CTCGTAGACTGCGTACC
*Eco*RI- adapter 2	AATTGGTACGCAGTCTAC
*Mse*I-C preselective primer	GATGAGTCCTGAGTAA**C**
*Eco*RI-A preselective primer	GACTGCGTACAATTC**A**
*Eco*RI-AAC selective primer	GACTGCGTACCAATTC**AAC**
*Eco*RI-AGC selective primer	GACTGCGTACCAATTC**AGC**
*Mse*I-CAA selective primer	GATGAGTCCTGAGTAA**CAA**
*Mse*I-CAG selective primer	GATGAGTCCTGAGTAA**CAG**
*Mse*I-CTT selective primer	GATGAGTCCTGAGTAA**CTT**
*Mse*I-CAC selective primer	GATGAGTCCTGAGTAA**CAC**

PCR products purification was performed by using a mixture of Sephadex and Sephacryl (1:1) (GE Healthcare Bio-Sciences AB) on Millipore plates. The purified products were diluted 1:50 before further use. The PCR products were prepared for capillary electrophoresis by adding 0.1 µl of GeneScan™ 500 ROX Size Standard (Applied Biosystems) and 10 µl of HiDi formamide to 1.5 µl. of sample.

*AFLP fragments detection.*The AFLP samples were migrated on ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems) using a 36 cm capillary and POP 7™ polymer. The reproducibility of the method was tested by using replicates and one negative control for each primer combination (BONIN et al., 2004).

Sequencing of lycopene β-cyclase gene (LCY-B)

Total RNA isolation. RNA was isolated from fresh leaves (50 mg) by using a commercial kit: Direct-Zol RNA Mini Prep from Zymo Research. RNA quantification was performed by spectrophotometric evaluation (NanoDrop 2000 Spectrophotometer, Thermo Scientific) and the resulting values ranged from 270 to 1650 ng $µl^{-1}$.

RNA reverse transcription was performed by using a commercial kit SuperScript™ II RT (Invitrogen). The gene specific primers used to amplify LCY-B from the obtained cDNA were design with the means of Pearl Primer software v. 1.1.21 (MARSHALL, 2004) based on existing Gen Bank submissions of the gene. One internal primer combination (Table 2) was needed for full sequencing of the gene.

Table 2. Primers sequence for lycopene β-cyclase amplification and sequencing.

	Primer	**Sequence 5'→3'**
External primers	*Lyc1503F*	AACCTTGAATTTCTGAACCCAC
	Lyc1503R	CTAATGGAACAGTTCCCTTTGTC
Internal primers	*LycIF*	GTTAGATTGTCTAGATGCTACCTG
	LycIR	TGTCCTTGCCACCATATAACC

PCR purification. Wizard R SV Gel and PCR Clean-Up System (Promega) was used to purify PCR products. The quality of the purified products was checked by agarose gel electrophoresis 1%.

LCY-B sequencing. Sequencing of LCY-B gene was performed with Big Dye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.1 (Applied Biosystems), using an Eppendorf Mastercyclerep Gradient S. PCR products were purified by Sephadex and Sephacryl (1:1). Samples (20 µl) were mixed with 10 µl HiDi formamide and migrated on ABI PRISM® 3130 Genetic Analyzer using a 36 cm capillary and POP 7™ polymer.

Assessment of genetic integrity and data analysis

AFLPand sequencing of lycopene β-cyclase genewere performed on *in vitro* regenerated tomato plants after short-term exposure (about 6 months with regular subcultures at 45 days of intervals) to five different hormonal treatments compared to *in vitro* seed-derived donor (mother) plants, grown on hormone free medium, used as controls (0). Leaves from *in vitro* regenerants of each cultivar were collected and subjected to genomic DNA and total RNA extraction. A total of 30 regenerants (1 individual x 5 cultivars x 5 hormonal variants + 1 control sample x 5 cultivars)were analysed by AFLP and for sequencing of LCY-B gene.

For AFLP, Gene Mapper v.4.0 (Applied Biosystems) was used for scoring. Fragments within the 50–500 bp range were scored to produce a present (1)/absence (0) matrix. Based on the final AFLP matrix, a Neighbor Joining tree was generated by means of Splits Tree software v4.10 (HUSON & BRYANT, 2006). Bootstrap values were obtained with 1000 replicates (Fig. 1). Sequence alignment and correction of sequence errors were performed in Bio Edit v7.0.9.0 (HALL, 1999). A Neighbor Joining tree was constructed with Mega software v4.1 (TAMURA et al., 2007) based on Kimura's genetic distance (1980) (Fig. 2). The bootstrap values were obtained with 1000 replicates.

RESULTS AND DISCUSSIONS

AFLP analysis

The final AFLP matrix contained 107 fragments, with sizes ranging between 51-241 bp. All bands and the relative shape and size of the peaks in the electropherogram were highly reproducible. The reproducibility of the experiment was 100%. The average number of AFLP fragments was 100 in cultivar 'Capriciu', 98 in cultivars 'Darsirius', 'Kristin' and'Pontica' and 99 in cultivar 'Siriana'. For each cultivar, all DNA fingerprints were identical and the number of fragments remained constant indifferent of the hormonal treatment used. The Neighbor-Joining (NJ) analysis revealed that both treated and non-treated (mother)tomato plants were grouped together for each cultivar, thus providing a strong evidence of genetic uniformity (Fig. 1).

AFLP assay gave no indication of genetic variation in shoots analysed after short-term exposure (6months) to different hormonal combinations. This lack of polymorphism suggests that the genetic integrity of the plants was not altered indifferent of the hormonal treatment applied. There are several studies undertaken to check the genetic fidelity of TC-derived tomato plants. These studies were mainly performed atphenotypic and cytogenetic levels. While DELANGHE & DE BRUIJNE (1976) observed no phenotypic variations in recovered tomato plants derived from two-year-old callus tissue, SOMASUNDAR & GOSTIMSKY (1992) as well as MANDAL (1999) reported phenotypic differences in callus culture regenerated tomato plants. However, young callus and shoot tips were reported to deliver morphologically and cytogenetically normal tomato plants (NOVAK & MASKOVA, 1979). Therefore, shoot tips or meristems are considered the best choice as explant sources for regenerating morphologically stable plants (KARTHA et al., 1977). Molecular-based stability studies have been scarcely applied to tissue cultured tomato plants. Thus, SMULDERS et al. (1995) found no differences when studied the DNA methylation of tomato callus and compared it with the methylation of leaf DNA from regenerated plants. Moreover, SONIYA et al. (2001) based on RAPD analysis showed that ten regenerated tomato plants were more than 95% similar to the mother plant but one was found to be distinctly different. Similar to our results, BHATIA et al. (2005) failed to detect any differences in the AFLP patterns of 'Red Coat' cultivar. Tomato shoots derived from cotyledonary explants cultured on MS medium supplemented with 15 mM Z when comparing with parent cotyledonary explants.

Sequencing of LCY-B gene

LCY-B gene, in plants, codifies for lycopene β-cyclase, a crucial enzyme in carotenoid biosynthesis pathway. Tomato contains two types of encoding genes for lycopene β-cyclases: LCY-B expressed in leaves, flowers and fruits and CYC-B expressed exclusively in flowers and fruits (DALAL et al., 2010). The gene analysed in our cultivars was LCY-B expressed in leaves.

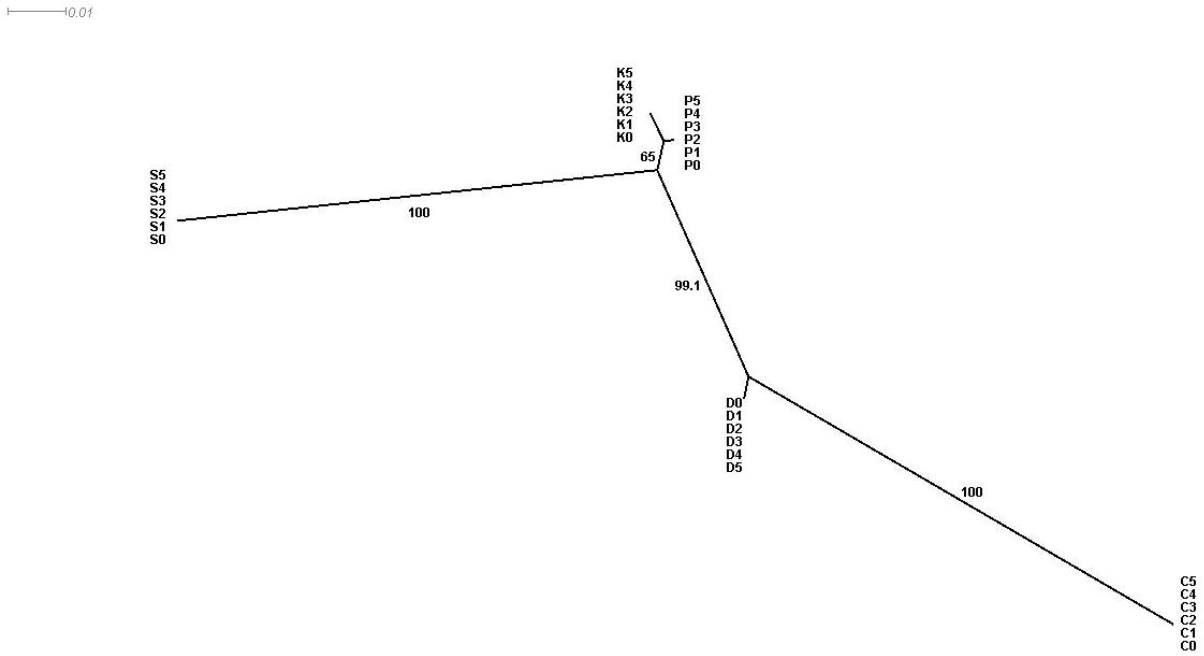
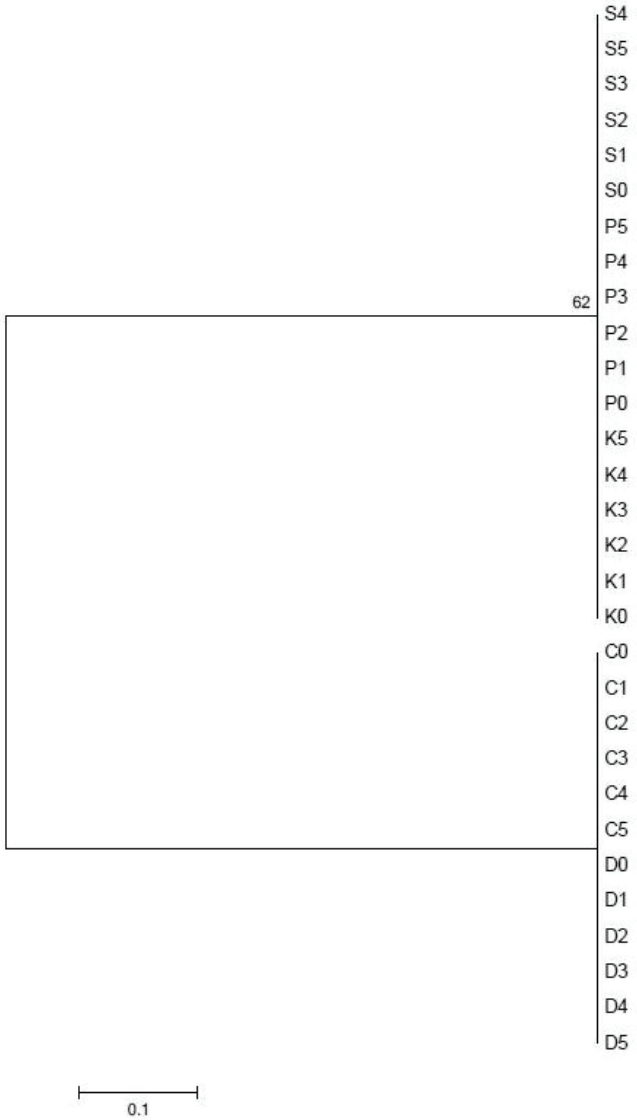


Figure 1. Neighbor Joining analysis based on AFLP data in five *Lycopersicon esculentum* cultivars: S – 'Siriana', K – 'Kristin', C – 'Capriciu', P – 'Pontica', D – 'Darsirius'. 0 - control (mother) seed-derived plants cultured on hormonal free, half-strength MS medium; 1 - MS + TDZ (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 2 - Z (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 3 - Kinetin (K) (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 4 - BA (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 5 - BA (0.5 mg l^{-1}) + K (0.3 mg l^{-1}) + IBA (0.2 mg l^{-1}).



Two Gen Bank (NCBI) published sequences of LCY-B gene: of 1503 bp (EF650013) and 1650 bp (X86452), were used to design the primer combinations for gene amplification. Stable and constant fragments were generated in all tested cultivars only for LCY-B of 1503 bp. After manual correction of sequence errors and sequence alignment, a 1344 bp sequence for LCY-B gene was obtained in all tested cultivars. Sequences of each cultivar were registered in Gen Bank database: KC140135-KC140139. A SNP (Single Nucleotide Polymorphism), consisting of a transversion (G→T) in position 1139 in cultivars. 'Capriciu' and 'Darsirius' was revealed in all TC tomato regenerants indifferent of the hormonal treatment or lack of it suggesting that these cultivars are mutants for LCY-B gene. This mutation is not dependent of the PGR treatment applied since all the obtained sequences were identical between the non-treated mother plants and the hormone treated variants.

This genetic uniformity is best illustrated by the NJ analysis (Fig. 2). Based on this observation we may conclude that all hormonal variants tested here may be safely used for micropropagation or tissue transformation purposes in all tested tomato cultivars.

Figure 2. Neighbor Joining tree based on sequences of lycopene β-cyclase gene.Cultivars designations are as follows: S – 'Siriana', K – 'Kristin', C – 'Capriciu', P – 'Pontica', D – 'Darsirius'. 0 - control (mother) seed-derived plants cultured on hormonal free, half-strength MS medium; 1 - MS + TDZ (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 2 - Z (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 3 - Kinetin (K) (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 4 - BA (1.5 mg l^{-1}) + IBA (0.2 mg l^{-1}); 5 - BA (0.5 mg l^{-1}) + K (0.3 mg l^{-1}) + IBA (0.2 mg l^{-1}).

However, single-gene mutations were reported in TC raised tomato plants (BUIATTI et al., 1985; BULK et al.,1990). Anyway, it was proven that these mutations were not dependent on the explant source or prolonged culture duration. DNA polymorphism was also detected in callus-derived tomato plants at the Cab locus in two of the 17 somaclones tested (NAMBISAN et al., 1992).

In our study, genetic stability of the regenerated tomato plants may be due to short term exposure to the employed hormonal treatments and lack of callus phase. Moreover, it is essential to apply more accurate genetic fidelity tests in order to verify the integrity of essential genes during micropropagation. Therefore the analysis of DNA sequences linked with particular genes would be more reliable for testing the genetic fidelity of TC-derived plant material. Moreover, molecular screening at the early stages of micropropagation are considered to meet the requirements for clonal uniformity of micropropagated plants, providing the opportunity to re-evaluate and modify the propagation protocols in order to achieve the genetic fidelity of the TC-raised plants (BHATIA et al., 2004; 2005).

CONCLUSION

No genetic changes were detected in the five tomato cultivars subjected to different hormonal treatments when analysed with AFLP or by sequencing of LCY-B gene. This study confirms that all hormonal variants tested here may be safely used for micropropagation or tissue transformation purposes in all tested tomato cultivars.

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