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In vitro Regeneration and Somatic Embryogenesis in Citrus

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Abstract

Better results were obtained when stigma explants of variegated lemon and citron were used. After ten months, somatic embryos developed into plantlets at a frequency ranged from 13.3 for lime to 66.7% for lemon. Virus presence was tested by ELISA and RT-PCR. The results indicated that the plantlets regenerated through somatic embryogenesis are CTV-free. RAPD analysis was used to asses the genetic stability of plantlets as compared to the mother plants. The results indicated that most plantlets belong to the respective mother plants and the polymorphism percentage was genotype and explant-dependant.

Introduction

Recent advances in tissue culture and genetic transformation offer new opportunities for the citrus genetic improvement. The development of efficient procedures for the regeneration *in vitro* of somatic embryos of many citrus species would be required as an intermediary for genetic transformation and improvement of genotypes. Therefore, the improvement of somatic embryo regeneration protocols of citrus is of great interest (Angela et al. 2006). The development of efficient tissue culture protocol is necessary for conservation and genetic improvement of citrus, an important fruit crop worldwide. In citrus, the production of embryogenic callus lines were reported from excised nucelli (Rangan et al. 1968), abortive ovules (Bitters et al. 1970), unfertilized ovules (Button and Bornman 1971, Pasquali and Biricolti 2004), undeveloped ovules (Starrantino and Russo 1980), juice vesicles (Nito and Iwamasa 1990), anthers (Hidaka et al. 1981 and Benelli et al. 2010), From immature seeds separated from immature fruits (Gholami 2013) styles and stigmas (Carimi et al. 1995, Angela et al. 2006 and Meziane et al. 2012) as well as from leaves, epicotyls, cotyledons (Kiong et al. 2008) and root segments (Gill et al. 1995). Moreover, tissue explants

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and embryogenic cells have, generally, a high capacity to regenerate plants and can be used in protoplast fusion and genetic transformation (Vardi and Galun 1989, Vardi and Spiegel-Rov 1992, Tavano et al. 2009). Although in the genus *Citrus* somatic embryogenesis was reported a long time ago (Maheshwari and Rang Swamy 1958), a number of difficulties have been encountered in establishing reliable protocols. In the abundant literature on *Citrus* somatic embryogenesis, it appears that results vary greatly depending on the genotypes (Mendes-da-Gloria et al. 2001, Tomaz et al. 2001, Niedz et al. 2002, Ramirez et al. 2003). The embryogenic potential of citrus varied with genotype and type of explant. Regeneration methods that involve the use of embryogenic callus of nucellar origin generally provide the best results. Unfortunately these systems provide only poor results with monoembryonic species. Kobayashi et al. (1981) cultured the ovules of 23 monoembryonic cultivars and never obtained nucellar embryos and in some genotypes characterized by low levels of polyembryos; for example, many cultivars of lemon. Somatic embryogenesis could be obtained through intact stigma and style culture (De pasquali et al. 1994, Carimi et al. 1995, 1998, 1999), which led to the question of whether such an embryogenic potential could be expressed in all the different parts of the pistil and in structures such as thin cell layer (TCL) explants. Although somatic embryogenesis, especially from ovule tissue and embryos developed from ovules, have been extensively investigated in citrus, there are only a few reports on somatic embryogenesis from somatic tissue that is neither nucellar nor ovular in origin. Little is known about the effect of tissue type on somatic embryogenesis in citrus. The use of stigma and style culture as explant for somatic embryogenesis was successfully applied in preliminary trials for sanitizing lemon affected by a mixture of graft-transmissible agents, amongst which CPsV may not have been present (D'Onghia et al. 1997, 2001). The main objective of this investigation was to produce citrus plants through somatic embryogenesis from stigma, style and ovary. Moreover, resulting plantlets were investigated for virus presence as well as genetic stability.

Materials and Methods

Flower buds of different citrus species and cultivars illustrated in Fig. 1A were harvested just before opening from mature trees of different species and cultivars of citrus as mentioned in Table 1 grown at, Moshtohor, Kalubia, Egypt.

Flower buds were washed carefully with running tap water and surface sterilized by immersing in 70% (v/v) ethanol for 1 min, then soaked in a 38% Clorox [commercial bleach (2% sodium hypochlorite solution)] for 15 min, followed by three 5 min rinses in sterile distilled water. Stigma, style and ovary

explants were excised from flower buds with a scalpel under aseptic conditions and placed vertically onto the culture medium, explants were initially cultured on MS basal salts and vitamins amended with 3 mg/l BA for callus initiation. For somatic embryogenesis callus drived from stigma, style and ovary were subcultured on MS supplemented with sucrose 50 g/l, malt extract 500 mg/l and 3.0 mg/l BA and solidified with 7 g/l Difco Bacto agar. The pH of the medium was adjusted to 5.8 autoclaved at 121ºC for 25 min. An average of 10 explants were cultured in each Petri dish (10 cm diam.) containing 20 ml of the culture medium and sealed with parafilm M.

Table 1. List of common and scientific name of different species and cultivars of citrus trees under investigation.

Sl. No.	Common name	Scientific name
1	Washington navel orange	*C. sinensis* (L.)
2	Satsuma mandarin	*C. unshiu* (Mak.)
3	Lemon	*C. limon* (L.)
4	Variegated lemon	*C. limon* (L.)
5	Lime	*C. aurantifolia* (Christm.)
6	Citron	*C. medica* (L.)
7	Pummelo	*C. grandis* (L.)
8	Rough lemon	*C. jambhiri* (Lush.)
9	Sour orange	*C. aurantium* (L.)
10	Volkamer lime	*C. volkameriana* (Ten. & Posg.)
11	Rangpur lime	*C. limonia* (Osb.)

Cultures were incubated at 25 ± 1ºC under a 16 hrs day length with illumination of 100 μmol/m^{-2}/s^{-1} Osram cool white 18 w fluorescent tubes. After four weeks, cultures were transferred to a fresh medium in 250 ml jars filled with 30 ml of the same medium. All culture explants were subcultured at four weeks intervals on the same fresh culture medium up to ten weeks.

Somatic embryos (1 - 2 mm in diam.) arising from embryogenic calli were isolated and cultured into test tube (25 × 150 ml) containing 20 ml of MS solid medium (7 g/l agar) supplemented with 50 g/l sucrose and 1 mg/l GA_3. Culture tubes were sealed with aluminium foil. The pH was adjusted to 5.8 ± 0.1 with 0.5 M sodium hydroxide before autoclaved at 121ºC for 25 min. The culture tubes were kept in the culture room at 27ºC and exposed to a 16 hrs day length, with illumination of 100 μmol/m^{-2}/s^{-1} Osram cool – white 18 w fluorescent lamps. Embryo germination was count for a period of ten months weekly.

All experiments were set up as a randomized complete design with three replicates for each treatment with at least 10 explants per replicate (n = 30). Data were subjected to ANOVA with mean separation (p = 0.05) by DMRT using SAS ver.6.12(SAS Institute, Cary, NC, USA).

Genomic DNA was isolated on a mini-prep scale as mentioned by Murray and Thomposon (1980). Small pieces (0.5 g) of young leaves of citrus species growing under field conditions and tissue culture-derived plantlets were frozen in liquid nitrogen in Eppendorf tubes and homogenized in 500 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl, pH 8.0, 0.1 M ß-mercaptoethanol). The extract was incubated at 60ºC for 20 min. To this 500 µl of phenol : chloroform : isoamyl alcohol (24 : 24 : 1) were added and mixed by vortexing for 30 sec followed by centrifugation at 10,000 xg for 5 min at room temperature. The aqueous phase was transferred to another tube. This was once again extracted with 500 µl of chloroform: isoamyl alcohol (24 : 1) in Eppendorf tube. To the aqueous phase, 0.6 volume of isopropanol were added, precipitated the genomic DNA and spooled the fibrous genomic DNA. Genomic DNA was then washed three times with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37ºC for 30 min, followed by extraction with phenol: chlorform: isoamyl alcohol and the aqueous phase was transferred to a fresh tube. Therafter, the genomic DNA was precipitated by adding 0.3 M sodium acetate, pH 5.2 (final concentration) and 2.5 vol of ethanol and collected by centrifugation at 10,000 xg for 20 min at 4ºC. The pellet was washed with 70% ethanol, vacuum dried and dissolved in TE.

Reactions for RAPD analysis were set up using seven random oligonucleotide (10 mer) primers (Operon technologies Inc., Alameda, California). The primers are OPK01 (5′ TGC CGA GCT G 3′), OPK02 (5′ GTG AGG CGT C 3′), OPK03 (5′ CCC TAC CGA C 3′), OPK04 (5′ TCG TTC CGC A 3′), OPK05 (5′ CAC CTT TCC C 3′), OPK06 (5′ GAG GGA AGA G 3′) and OPK07 (5′ CCA CAG CAG T 3′). The PCR reactions were carried out in 50 µl volumes containing 100 ng of genomic DNA, 1.0 µM primer, 200 µM of dATP, dDTP, dCTP, dGTP, 10 mM tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatine. The Taq DNA polymerase concentration was 1.5 units per assay. PCR reaction was conducted using a Perkin Elmer 2400 (Germany) thermocycler programmed as follows : 94ºC/5 min (1 cycle), 94ºC/30 sec, 36ºC/30 sec, 72ºC/2 min (45 cycle) and 72ºC/7 min (1 cycle), then held at 4ºC. The amplification products of PCR were size-separated by gel electrophoresis in 1% agrose gels with 1 x TBE buffer using a Pharmacia G N. 100 submarine gel electrophoresis apparatus and stained with ethidium bromide and visualized with UV transilluminator and photographed. A 100 bp DNA ladder (Promega) was used as a standard with molecular sizes of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. DICE computer package was used for the analysis of RAPD-PCR products (Yang and Quiros 1993).

CTV infection was assayed in somatic embryogenesis derived plantlets obtained by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) **(**Clark and Adams 1977). It was carried out according to the manufatcor instructions. The A_{405} nm value of each well was measured by an enzyme immunosorbent assay plate reader (Model 3550 microplate reader).

The two-step RT-PCR was performed and included two separate steps: reverse transcription and PCR amplification. Reverse transcription for the synthesis of the first strand cDNA was made by Superscript™ II RNase H-Reverse Transcriptase (Bioron). The total volume was 20 µI, which contained 50 units of Superscript™ II reverse transcriptase, 50 mM Tris-HCL (PH 8.3), 75 mM KCL, 3 mM $MgCl_2$, 10 mM DTT, 2 pmol reverse primer 5′ TCA ACG TGT GTT GAA TTT CCC AAG C 3′ , 0.5 mM each dATP , dTTP, dCTP, dGTP, and 200ng of nucleic acid preparations. The contents (RT mixture) were mixed gently and incubated at 42°C for 50 min. The PCR amplification was performed using 25 µl of reaction mixture containing 20 mM tris-HCl (pH 8.4), 50mM KCL, 1.5 mM $MgCl_2$, 0.2 mM each dATP , dTTP, dCTP, dGTP, 5 pmol forward primer 5′ AAC GCC CTT CGA GTC TGG GGT AGG A 3′, 5 pmol reverse primer 5′ TCA ACG TGT GTT GAA TTT CCC AAG C 3′ as reported by Rowhani et al. (1998), 1.25 units of Taq DNA polymerase, and 2 µI of RT mixture. The thermal cycle conditions were: one cycle at 94ºC for 5 min; 30 cycles at 94ºC for 30 s, 57ºC for 30 s, 72ºC for 1 min : followed by 72ºC extension for 10 min.

Results and Discussion

Friable-yellow callus (Fig. 1b,c) initiated from stigma, style and ovary explants could be observed after 2 - 3 weeks of incubation. (Table 2) indicated frequencies of callus initiation from different explants scored after four weeks of incubation. The callusing percentage was affected by genotype and type of explant, also different genotypes showed significant differences in callus initiation frequencies. Washington navel orange explants showed the highest callus formation mean percentage (59.5) whereas the lowest mean (1.7) was observed in Satsuma mandarin explants. The type of explant showed a significant effect on callus induction as shown in (Table 2). Maximum callusing percentage was observed with ovary explants as a mean of all genotypes (42.0) followed by stigma and style explants (33.5 and 20.9), respectively. The interaction between genotype and type of explant showed that style explants of Washington navel orange was the best responding explant (100%), followed significantly by ovary explants of Rangpur lime and stigma explants of Citron without significant difference (71.4 and 68.4%), respectively. Ovary explants for both Washington navel orange and Lime came next without significant difference between them

(57.10 and 57.12%, respectively). On the other hand, no response could be detected from style explants of Satsuma mandarin, Lime, Pummelo and Sour orange and stigma explants of Satsuma mandarin for callus initiation.

Table 2. Callusing percentage of different citrus genotypes and explants (after 4 weeks).

Genotype	Callusing (%)			
	Explant type			
	Style	Stigma	Ovary	Mean
Washington navel orange	100 a	21.4 l	57.10 c	59.5 A
Satsuma mandarin	0.0 p	0.0 p	5.0 o	1.7 H
Rough lemon	14.3 m	33.3 ij	50.0 d	32.5 DE
Lemon	13.3 m	33.3 ij	42.8 ef	29.8 F
Lime	0.0 p	46.7 de	57.12 c	34.6 D
Variegated Lemon	46.2 de	36.0 hi	30.8 jk	37.7 C
Pummelo	0.0 p	50.0 d	50.0 d	33.3 EF
Citron	38.1 gh	68.4 b	28.6 k	45.1 B
Rangpur lime	9.1 n	35.7 hi	71.4 b	38.7 C
Volkamer lime	9.4 n	36.1 hi	41.7 fg	29.1 F
Sour orange	0.0 p	7.2 no	28.0 k	11.7 G
Mean	20.9 C	33.5 B	42.0 A	

Different letters within a column indicate significant differences at $p < 0.05$ according to DMRT.

Somatic embryos appeared after 2 - 3 months of culture as shown in Fig. 1-d. (Table 3) indicated that somatic embryogenesis was significantly affected by genotype and type of explant. There were significant differences in somatic embryos frequencies among the genotypes. Variegated Lemon produced the highest significant mean percentage of somatic embryogenesis (44.1%) followed significantly by the somatic embryogenesis percentage of Citron (11.7%) and Sour orange (6.7%) with significant difference. Rough lemon and Lime showed the same percentage of somatic embryogenesis (6.1). On the other hand, all explants of Washington navel orange, Satsuma mandarin, Pummelo, Rangpur lime and Volkamer lime could not produce any somatic embryo assayed.

Somatic embryogenesis was also affected by the type of explant, somatic embryogenesis of stigma explants (13.0%) were significantly the highest than style (6.4%) or ovary explants (1.8%). Also, the best explant for producing somatic embryos varied according to the genotype where stigma explants were the best for Variegated lemon (80.0%) followed significantly by style explants for the same genotype (52.4%). Generally, stigma explants gave the highest results for the most citrus genotypes tested.

Number of produced somatic embryos is presented in (Table 3 and Fig. 1-e). In Table 3 it was clear that number of embryo was significantly affected by genotype and type of explant. Citron produced the highest significant mean number of embryos/explant derived embryogenic callus (11.0), whereas Sour orange produced the lowest number of embryos/explant (0.3) after ten months. Otherwise, in Washington navel orange, Satsuma mandarin, Pummelo, Rangpur lime and Volkamer lime, no embryos were induced from different explants-derived embryogenic callus. Belong to explant type, stigma explants produced an averaged 4.2 embryos/explant, higher significantly than style (2.8) or ovary (0.2).

Table 3. Somatic embryogenesis percentage of different citrus genotypes and explants (after 3 months).

Genotype	Somatic embryogenesis (%)			
	Explant type			
	Style	Stigma	Ovary	Mean
Washington navel orange	0.0 g	0.0 g	0.0 g	0.0 F
Satsuma mandarin	0.0 g	0.0 g	0.0 g	0.0 F
Rough lemon	0.0 g	18.2 e	0.0 g.	6.1 D
Lemon	10.0 f	0.0 g	0.0 g	3.3 E
Lime	0.0 g	18.2 e	0.0 g	6.1 D
Variegated Lemon	52.4 b	80.0 a	0.0 g	44.1 A
Pummelo	0.0 g	0.0 g	0.0 g	0.0 F
Citron	8.3 f	26.7 c	0.0 g	11.7 B
Rangpur lime	0.0 g	0.0 g	0.0 g	0.0 F
Volkamer lime	0.0 g	0.0 g	0.0 g	0.0 F
Sour orange	0.0 g	0.0 g	20.0 d	6.7 C
Mean	6.4 B	13.0 A	1.8 C	

Different letters within a column indicate significant differences at $p < 0.05$ according to DMRT.

Somatic embryos were transferred to MS supplemented with 50 g/l sucrose and 1 mg/l of GA_3 as shown in Fig. 1f and left to maturity and complete germination as shown in Fig. 1g. The highest germination percentage of embryos was 66.7 observed in Lemon followed by 48.2% for Citron with a significant difference between them. Embryos produced from stigma explants were superior in germination compared to drived from style or ovary explants (25.4, 20.0 and 18.2%, respectively) as shown in (Table 4). Finally, it can be conculaded that, ovary explants produced the highest percentage of callusing whereas stigma was the best explant for embryogenesis and number of embryos. Washington navel orange was superior to other genotypes for callus formation. But Variegated lemon and citron were the most producing genotypes of somatic embryos.

Table 4. Number of embryos of different citrus genotype and explants (after ten months).

Genotype	Number of embryo/explant			
	Explant type			
	Style	Stigma	Ovary	Mean
Washington navel orange	0.0 i	0.0 i	0.0 i	0.0 G
Satsuma mandarin	0.0 i	0.0 i	0.0 i	0.0 G
Rough lemon	0.0 i	2.7 g	0.0 i	0.9 E
Lemon	7.0 e	0.0 i	1.0 h	2.7 C
Lime	0.0 i	6.7 f	0.0 i	2.3 D
Variegated Lemon	11.2 d	16.4 b	0.0 i	9.3 B
Pummelo	0.0 i	0.0 i	0.0 i	0.0 G
Citron	12.5 c	20.5 a	0.0 i	11.0 A
Rangpur lime	0.0 i	0.0 i	0.0 i	0.0 G
Volkamer lime	0.0 i	0.0 i	0.0 i	0.0 G
Sour orange	0.0 i	0.0 i	1.0 h	0.3 F
Mean	2.8 B	4.2 A	0.2 C	

Different letters within a column indicate significant differences at $p < 0.05$ according to Duncan's New Multiple range test.

Table 5. Percentage of somatic embryo germination of different citrus genotypes and explants type (after ten months).

Genotype	Embryo germination (%)			
	Explant type			
	Style	Stigma	Ovary	Mean
Washington navel orange	0.0 g	0.0 g	0.0 g	0.0 F
Satsuma mandarin	0.0 g	0.0 g	0.0 g	0.0 F
Rough lemon	0.0 g	100 a	0.0 g	33.3 D
Lemon	100.0 a	0.0 g	100.0 a	66.7 A
Lime	0.0 g	40.0 f	0.0 g	13.3 E
Variegated Lemon	56.1 e	58.6 d	0.0 g	38.2 C
Pummelo	0.0 g	0.0 g	0.0 g	0.0 F
Citron	64.0 c	80.5 b	0.0 g	48.2 B
Rangpur lime	0.0 g	0.0 g	0.0 g	0.0 F
Volkamer lime	0.0 g	0.0 g	0.0 g	0.0 F
Sour orange	0.0 g	0.0 g	100.0 a	33.3 D
Mean	20.0 B	25.4 A	18.2 C	

Different letters within a column indicate significant differences at $p < 0.05$ according to Duncan's New Multiple range test.

Our results showed that the embryogenic potential of stigma explants are usually higher than style. However, De Pasquale et al. 1994 and Carimi et al. 1995, demonstrated that stigma never regenerated somatic embryos and rarely

produce callus when stigma and style were jointly cultured. In addition, the direct contact of explant with the culture medium may be an additional factor in the high frequency induction of somatic embryogenesis (Gill et al. 1993). The embryogenic potential has been demonstrated in different parts of the flower.In others report somatic embryos were regenerated from the immature inflorescences of *Sporoblus virginicus* (L.) Kunth (Straub et al. 1992).

Somatic embryogenesis from pistils cultures was obtained in *Dactylis glomerata* (L.) (Songstad and Conger 1986) and in a hybrid of *Cichorium* (Guerida et al. 1988), from ovary cultures in fuchsia (Dabin and Beguin 1987), *Vitis vinifera* (L.) (Goussard et al. 1991), *Allium cepa* (L.) (Campion et al. 1992) and *Caryratia japonica* **(**Zhou et al. 1994). However, somatic embryogenesis from styles or stigmas cultured separately has not been reported in the literature.

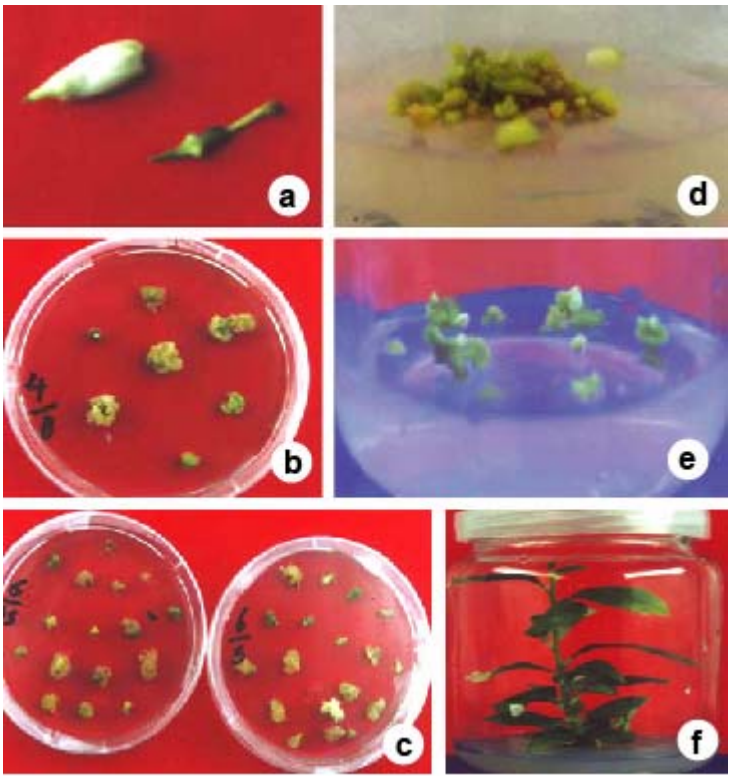

Fig. 1. (a) Citrus flower bud (just before opening) used as a source of explants (style-stigma-ovary). (b) Callus formation on ovary explants of lemon (*C. limon*) after 3 week of culture. (c) Callus formation on style(s) and stigma (ss) explants of lemon (*C. limon)* after 3 week of culture. (d) Somatic embryos on callus derived from stigma explants. (e) Embryo development at cotyledonary stage growing on germination medium. (f) Style-derived plantlet after 3 months on germination medium.

In our study, a procedure for culturing of stigma, style and ovary from different genotype of citrus has been established. These explants showed different embryogenic potentials on the same medium (MS salts + 50 g/l sucrose + 500 mg/l malt extract + 3 mg/l of BA). Our results indicated that lemon and citron are the best genotype to produce somatic embryogenesis. Somatic embryogenesis from citrus stigma and style culture has been descried as a new technique for the sanitation, conservation and safe exchange of citrus germplasm (D'Onghia et al. 2000). This improved method can be successfully applied to the propagation of plants free from graft transmissible pathogens (D 'Onghia et al. 1997, 2001).

The choice of plant growth regulators determines both the ability of the explant to respond and the mode of the morphogenic reaction and usually somatic embryogenesis is induced in the presence of auxin alone or in combination with cytokinins (Gaj 2004). In only few systems cytokinins supplemented alone were found to be effective in somatic embryogenesis induction (Carimi et al. 1999, Nanda and Rout 2003).

Standard DAS-ELISA test as described by Clark and Adams (1977) failed to detect CTV from regenerated plantlets of citrus in that the OD 504 values of all the samples tested were low to be differentiated from negative controls as shown in Table 6. Our data indicated that standard DAS-ELISA could be used successfully in detection of CTV. Although simpler and quicker the standard DAS-ELISA was shown to be ineffective.

Table 6. Detection of citrus tristeza virus in stigma, style and ovary-derived plantlets via DAS-ELISA.

Sl. No.	Genotype	Explant	$O.D._{504}$ value			Results
			R1	R2	Mean	
1	Lemon	Style	0.091	0.106	0.098	-
		Ovary	0.098	0.090	0.094	-
2	Lime	Stigma	0.113	0.089	0.101	-
3		Stigma	0.102	0.094	0.098	-
	Citron	Style	0.111	0.092	0.102	-
4	Rough lemon	Stigma	0.091	0.106	0.098	-
5	Variegated	Stigma	0.089	0.092	0.091	-
	Lemon	Style	0.083	0.084	0.084	-
+	Positive	-	1.096	0.880	0.988	+
-	Negative	-	0.108	0.081	0.095	-

+ Positive control (infected), - Negative control (healthy), R : replicate.

Results of RT-PCR analysis are shown in Fig. 2. They indicated detectable band resulting from RT-PCR products of 300 bp using the primers reported by

Rowhani et al. (1998), and all the tested samples were negative, no detectable expected size band (300 bp) as shown in (Fig. 2).

In fact, no CTV was detected in any of the regenerated plantlets using ELISA techniques or RT-PCR methods confirming that all plantlets resulted via somatic embryogenesis derived from stigma, style and ovary are CTV-free.

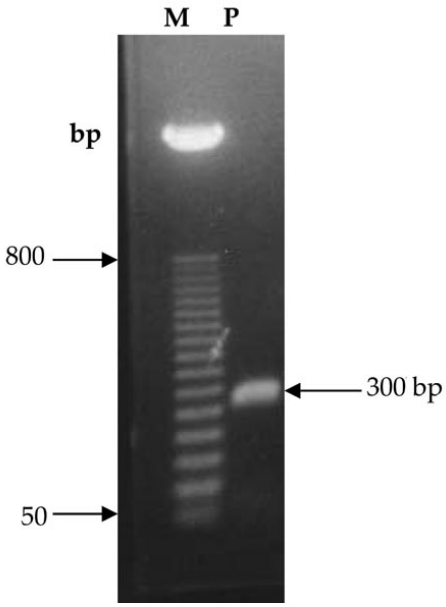

Fig. 2. Ectrophoresis analysis in 1% agarose gel of RT-PCR amplification products to detect CTV using primers reported by Rowhani et al. (1998). M: Marker 50 bp ladder; P: Positive control (CTV-infected).

Seven random oligonucleotide primers used and number of DNA fragments amplified in their presence are shown in (Table 7) and Fig. 3 out of seven (10-mers) primers, four were succeeded and turned out to give number of banding in the gel depend on the genotype; three did not produce amplification products. Otherwise, most of the succeeded primers, identify DNA polymorphism, under our PCR condition showed that, the percentage of polymorphism depended on the primer tested and the genotype. This may be due to short size of the primers tested as suggested by Caetano-Anolles and Gresshoff (1992) for DNA finger-printing.

As shown in (Table 7) and (Fig. 3) primers OPK01, and OPK02 succeeded with Washington navel orange, blood orange, lemon, grapefruit and sour orange and produced about 139 bands in the gel ranging from (100 to 1200 bp) in length , 22 out of these are polymorphic bands with 17.96% ranging from (300 to 1000 bp) depending on the genotype and the primer tested. But primers OPK05 and

OPK06 succeeded only with Shamouti orange and Local mandarin producing about 46 bands ranging from 250 to 1200 bp in length, 8 bands out of them were polymorphic with 17.3% ranging from 300 to 1150 bp .The results demonstrate here that the RAPD-PCR technique can be successfully applied to determine the genetic stability of citrus plantlets that are produced through somatic embryogenesis.

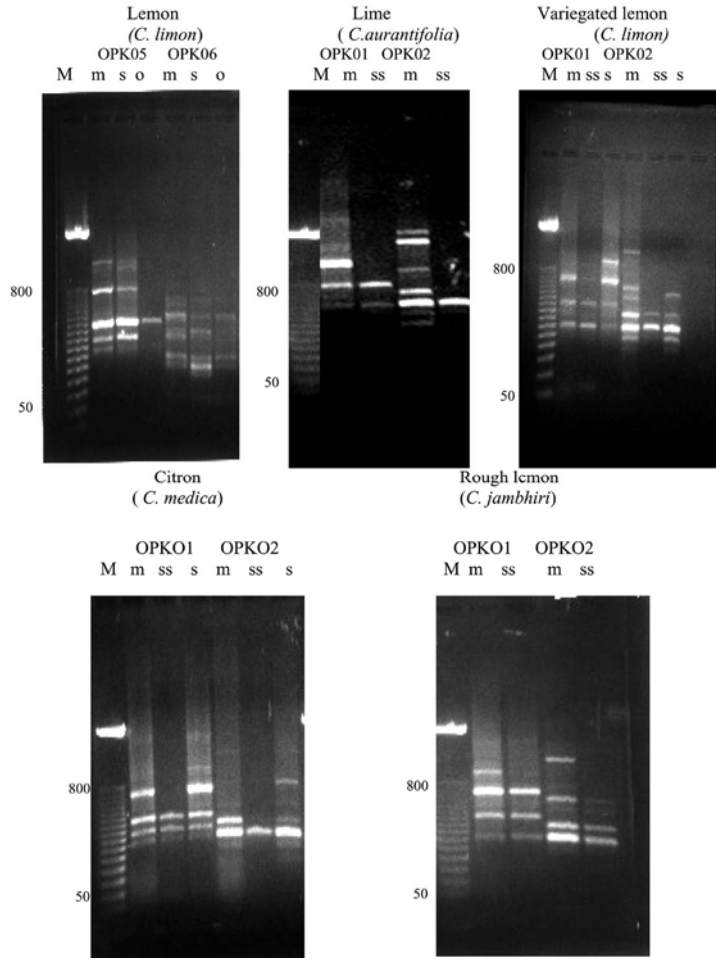

Fig. 3. Gel electrophoresis of RAPD fingerprints cbtained with primers OPK_1, OPK_2, OPK_5, OPK_6 of genomic DNA of 5 genotypes from style, stigma and ovary-derived plantlets of citrus. M : DNA marker 50 bp ladder; m: mother plant; s: style-derived plantlets; ss: stigma-derived plantlets; o: ovary-derived plantlets.

Table 7. Effect of arbitrary sequence primers tested in RAPD analysis of stigma, style, and ovary-derived-plantlets of citrus.

Genotype	Primer	No. of produced bands		Size range		No. of polymorphic band		% polymorphism		Size of polymorphic bands	
		Mother tree	*In vitro* derived plantlet								
Lemon			**S**	**O**	**S**	**O**	**S**	**O**	**S**	**O**	**S**	**O**
			6	1	325-1050		0	5	0	71.4		
	OPK_5	6				500					-	1050-1000-800-
			7	5	200-700		2	2	14.3	16.7		400-325
	OPK_6	7				200-700					650-200	650-400
Lime			**SS**		**SS**		**SS**		**SS**		**SS**	
	OPK_1	5	4		300-900		1		1.1		400	
	OPK_2	7	3		200-1000		4		40		1000-950-600-400	
Variegated			**SS**	**S**	**SS**	**S**	**SS**	**S**	**SS**	**S**	**SS**	**S**
lemon	OPK_1	4	4	5	300-650	300-900	0	1	0	22.2	-	900
	OPK_2	7	3	5	250-1000	200-1000	4	3	40	25	1000-600-500-200	1000-600-500
Citron			**SS**	**S**	**SS**	**S**	**SS**	**S**	**SS**	**S**	**SS**	**S**
	OPK_1	4	3	4	300-700	300-700	1	0	14.3	0	700	-
	OPK_2	4	2	5	250-500	250-700	2	1	33.3	11.1	500-250	700
Rough			**SS**		**SS**		**SS**		**SS**		**SS**	
lemon	OPK_1	4	3		300-850		1		14.3		850	
	OPK_2	4	3		300-950		1		14.3		950	

s: style-derived plantlets; ss: stigma-derived plantlets; o: ovary-derived plantlets.

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