Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(2):154-159

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Detection of Two Viroids in the Tunisian Sweet Orange (*Citrus sinensis* L) Cv. Maltese and Sanitation via Somatic Embryogenesis

Kaouther Ben Mahmoud[1*], Asma Najar[1], Emna JedidI[2], Imen HamdI[3] and Ahmed Jemmali[1]

[1]*Laboratory of Plant Protection, National Institute of Agronomic Research of Tunisia, Rue Hédi Karray, 2080 Ariana, Tunisia*
[2]*Laboratory of horticulture, National Institute of Agronomic Research of Tunisia, Rue Hédi Karray, 2080 Ariana, Tunisia*
[3]*Regional Center of Agricultural Research of Sidi Bouzid, Tunisia*

ABSTRACT

In vitro somatic embryogenesis from citrus style and stigma culture is reported to be highly effective in the elimination of the main virus and virus-like diseases. This method was applied for the regeneration and sanitation of the Tunisian sweet orange (Citrus sinenesis L.), var "Half-blood" Maltese, which were infected with Citrus dwarfing viroid (CDVd) and Citrus bark cracking viroid (CBCVd). For this purpose, style/stigma explants were excised from unopened flowers and cultured in vitro on MS medium containing 0, 6.65 or 13.3 µM BAP to induce somatic embryogenesis. No reaction was observed in explants cultured on BAP-free medium. Embryogenic frequency was relatively high in presence of 13.3 µM BAP. Unfortunately, many cases of teratological forms affecting embryos ontogeny were scored during their maturation and hampered their conversion into plantlets. Even though the high frequency of those abnormalities, we succeeded to obtain well developed shoots after transfer of normal embryos on growth regulator-free MS medium. These shoots were then grafted on Troyer citrange rootstock and acclimatized in greenhouse. One year later, RT-PCR carried out on growing grafted plants revealed that they were free of viroids CBCVd and CDVd identified in the source material.

Keywords: Citrus; Somatic embryogenesis; Viroids, sanitation; RT-PCR

INTRODUCTION

Viroids, the smallest disease-causing agents, cause serious diseases in citrus trees and are highly contagious, as they are not only graft-transmissible, but are also transmitted by mechanical means such as infested tools (Roistacher, 1991). There is no chemical way to manage virus and viroid diseases.The production of clean material is therefore essential for the conservation and use of citrus genetic resources. For this purpose, the production of healthy material has been obtained by in vitro shoot tip grafting alone or in combination with heat therapy (Navarro *et al*., 1980). More recently, somatic embryogenesis developed from stigma and style (D'Onghia *et al*., 1997; 2001), proved to be highly effective in the elimination of viruses and viroids in some plant species (D'Onghia *et al*., 2001; Gambino *et al*., 2010).

In Tunisia, the citrus orchards are faced to several constraints such as virus and virus-like diseases that may significantly cause considerable losses in crop yield and quality (Najar and Duran-vila, 2004). In this context, the present study was conducted on the Tunisian sweet orange (*C. sinensis*) cv 'Maltese' in which *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) were identified. The main aim of this study was then to try elimination of these viroïds via somatic embryogenesis according to the protocol described by Carimi *et al*. (1994).

MATERIALS AND METHODS

Pre-checking of viral state

In the course of our regular checking work of the viral state of the experimental citrus collection of INRAT, we realized a complete investigation including two virus Citrus tristeza virus (CTV) and Citrus psorosis virus (CPsV) and Five groups of viroids: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CVd-IV or CBCVd). Viruses were checked by serological ELISA-DAS test (Clark and Adams, 1977) using monoclonal antibodies from plant print (Spain) for CTV and Agritest (Italy) for CPsV. Viroids were checked by RT-PCR.

For RT-PCR analysis, total RNA was extracted following the method reported by Foissac *et al*. (2001). cDNAs were synthesized with random primers using the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the supplier. The resulting cDNA was subjected to PCR amplification with Taq DNA polymerase (Invitrogen) using specific primers for each viroid (table 1) to amplify sequences of 371, 315, 300, 300 and 168 pb. The cycling profile consisted of 30 cycles of 1 min at 94 °C, 1 min at 60°C, and 2 min at 72°C; with an initial denaturation at 94°C for 4 min and a final extension at 72 °C for 10 min. Electrophoresis of the RT-PCR products has been realized in a 2 % (w/v) agarose gel and followed by ethidium bromide staining.

Table 1: Specific primers used for PCR amplification of cDNA of five citrus viroïds

CEVd	R:5'-GCCCCGGGGCTCCTTTCTCAGGTAAG-3'and F: 5'CGCCCGGGGCAACTCTTCTCAGAATCC 3'	Semmancick *et al*, 1993
CBLVd	CV-I-cp: 5'TTCGTCGACGACGACCAGTC3' and CV-I-hm: 5'GGCTCGTCAGCTGCGGAGGT3'	Gandia and Duran-vila 2004
HSVd	R: 5'CGCCCGGGGCAACTCTTCTCAGAATCC 3' and F: 5'GCCCCGGGGCTCCTTTCTCAGGTAAG3'	Kofalvi *et al*, 1997
CDVd	R:5'GGGGGAAACACCAATCGTGTG3' and F:5'GGAGGAAACTCCGTGTGGTC3'	Rakowski *et al*.,1994
CVd-IV	R:5'CCGGGGATCCCTCTTCAGGT3' and F: 5'GGTGGATACAACTCTTGGGTTGT3'	Puchta et al., 1991

Somatic embryogenesis for sanitation

Induction of somatic embryos:

Explants used for induction of somatic embryogenesis consisted in style/stigma. For this reason, unopened flowers were collected from two infested trees (one viroïd/tree) and were surface sterilized by immersion for 5 min in 70% ethanol and 15 min in 2% sodium hypochloride, followed by three 5-min rinses in sterile distilled water. Styles and stigmas were excised with a sterilized scalpel and placed vertically in (90 x 15 mm) Petri dishes with the cut surface into contact with the growth medium. Five explants were placed in each Petri dish.

Explants were cultured on MS semi-solid medium (Murashige and Skoog, 1962) containing 146 mM sucrose, 500 mg L^{-1} malt extract and supplemented with 6.65 or 13.3 µM BAP (6-benzylaminopurine). The pH was adjusted to 5.7 with 0.1M KOH before autoclaving at 120°C for 30 min. Petri dishes (90 x 15 mm) were filled with 25 ml of medium and sealed with Parafilm M. Explants and calli were maintained in a growth chamber at 25°C ± 2°C with a 16h/8h photoperiod and photon flux of 4000 Lux provided by OSRAM Daylight lamps. Subculturing occured at 4 weeks intervals. Cultures were periodically observed to check when callus formation and the first embryogenic events took place. The experimental design was completely randomized; 10 Petri dishes were used (replicates), containing each five explants (50 explants for each tree). The frequency (%) of callogenesis and embryogenesis was estimated as the percentage of style/stigma explants forming callus and embryo respectively.

Germination of somatic embryos and transfer of plantlets to soil:

Individual somatic embryos reaching 3-5 mm in length were collected and allowed to germinate in test tubes (15 x 55 mm), each containing 25 ml of MS basal semi-solid medium supplemented with 146 mM sucrose and 500 mg L^{-1} Malt Extract. No growth regulators were added. When plantlets were 1-2 cm in length, they were grafted onto *Troyer citrange* (*C. sinensis* x *Poncirus trifoliata*) using parafilm M for wrapping. To protect young scions against dehydratation, they were covered by a plastic bag providing high humidity through soaked cotton placed under the graft union.

Grafted plants were planted into plastic pots containing a 1:1 (v/v) mixture of peat:sand and transferred to greenhouse for acclimation and further growth.

Scanning Electronic Microscopy (SEM)

SEM was also used to illustrate the superficial morphology structure of the embryogenic calli and their derived somatic embryos at different developmental stages. Fresh samples were fixed with super glue on the plane surface of a metallic support and inserted inside the chamber of a Scanning Electron Microscope (EDAX Quanta200). Observations were made at different magnifications and photos were taken for the targeted features.

RESULTS

Checked virus and viroids

All samples collected from "half-blood" Maltese sweet orange cultivar were negative for CTV and CPsV. However, RT-PCR assays for the five viroïds cited above revealed the presence of two viroïds CVBVd in the tree 1 and the CVDd in the tree 2 as shown in figure (1). This result is illustrated by the two amplicons of the expected sizes 168pb and 300pb using respectively CVBd and CDVd specific primers.

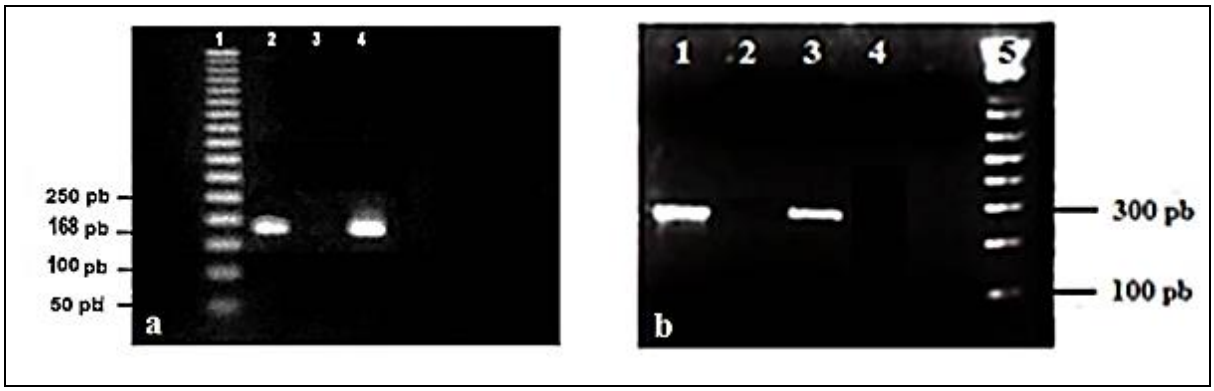

Figure 1: Electrophoresis analysis of RT-PCR products to detect CBCVd (a) and CDVd (b) on the "half-blood" Maltese sweet orange; (a) Lane 1: DNA size marker (50 bp DNA Ladder (Invitrogen), Lane 2: CBCVd infected tree1, Lane 3: CBCVd uninfected tree 2, Lane 4: Positive control, Lane 5: negative control; (b) Lane 1: positive control, Lane 2: CDVd uninfected tree 1, Lane 3: CDVd infected tree 2, Lane 4: negative control, Lane 5: DNA size marker (100 bp DNA Ladder (Invitrogen)

Callogenesis and Somatic embryogenesis

In order to regenerate viroid-free plants from infected trees (tree 1 and tree 2) via somatic embryogenesis, unopened flowers were collected from each tree and style/stigma explants were excised and cultured on the MS basal medium supplemented with BAP growth regulator. Callogenesis was the first morphogenetic change and it was initiated from the style base; although, only in few cases stigma was reactive. Callogenesis occured after 4 weeks of incubation in explants cultured on BAP-containing medium; but no reaction was observed in explants cultured on BAP-free medium (table 2).

Table 2: Callogenesis and somatic embryogenesis frequency (%) of Maltese "half-blood" style/stigma explants cultured on BAP-containing medium

BAP concentrations (µM)	Callogenic explants (%)	Embryogenic calli (%)
0	0±0	0±0
6,65	60±10	8±8
13,3	89±7	19±8

Data are reported as mean values ± SDM, *n=10*

SEM observations (Figure 2) allowed to see embryos at their first globular stage on the callus surface after approximately 4 months of culture (Figure 2a). Higher magnification (Figure 2b) proved that these structures were still amorphous at this stage, but they evolve into elongated bodies resembling heart-shape stage of the habitual somatic embryos (Figure 2c). Nearly one month after, cotyledonary embryos were observed (Figure 2d). This figure also indicates the asynchronous ontogeny of the somatic embryos even on the same callus. The embryogenic response of the explants was influenced by the BAP concentration. In fact, BAP at 13.3 µM was more efficient than 6.65 µM in induction of embryogenesis (table 2).

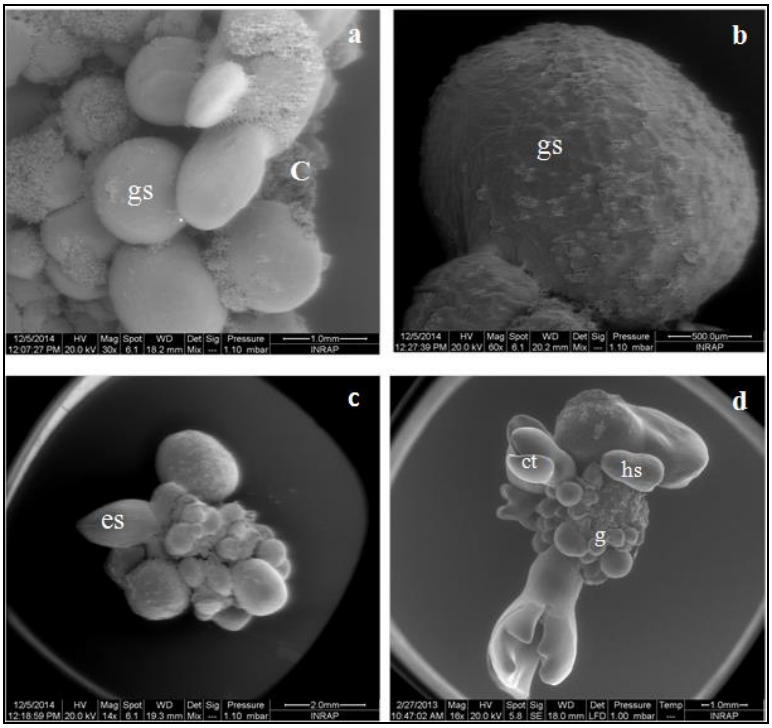

Figure 2: SEM micrographs showing main stages of somatic embryogenesis from style/stigma explants of 'half-blood' Maltese sweet orange cultured on BAP-enriched MS medium. (a) Aggregate of some globular structures (gs) on the surface of 4 months aged embryogenic callus (C). (b) High magnification of the central globular embryos (gs). (c) 5 month-aged callus showing elongated structures (es) similar to embryo at heart-shape stage. (d) Co-existence of all developmental stages of somatic embryos illustrating the asynchronous process of somatic embryogenesis. g: globular stage; hs: heart-shape stage; ct: cotyledonary stage

Abnormalities affecting embryogenesis process

As it was reported above, the number of converted embryos into normal shoots was relatively low. In this context, it should be precised that, in the context of our experiment, frequent teratological forms took place concomitantly with normal structures. Those malformations appeared either early after transfer in a fresh medium or later in the case of a prolonged period in the induction medium. Whatever their morphotypes, all of these abnormal structures usually failed to develop into regular shoots and sometimes turn in more fasciated form (lettuce aspect) even after transfer in a germination medium. Multiple forms of abnormalities were observed, but we only selected those occurred more frequently. They varied from mono (Fig. 3a) to pluri-cotyledonary forms (Figure 3b), fused embryos (Figure 3c), and development of secondary embryos on the rudimentary primary ones (Figure 3d). In addition, well developed embryos showed another form of malformation when they stayed on the induction medium for prolonged duration. In this case, embryo cotyledons undergone disturbance of their development (Figure 3e) that led to fasciated forms (Figure 3f) incompetent to develop into plants. Its outline has ribs and furrows with wavy and undulated margins.

Another aberrant shapes also observed in our culture model were trumpet-shaped embryos with collar-like cotyledons. Cotyledons formed circularly around a centrally located pore containing sometimes developed shoot meristem and radicle (Figure 3g).

Germination, grafting and acclimation

Despite of the occurrence of many cases of morphological abnormalities described above, we succeeded to obtain well developed plantlets. Normal somatic embryos were isolated from callus and cultured in test tubes containing MS basal medium supplemented with 500 mg/l malt extract. They developed into plantlets constituted by leafy elongated shoots (Fig. 4a). In some cases, the roots grew, but the shoot collapsed. When germinated plantlets were 1-2 cm in length, they were grafted onto *Troyer citrange* (*C. sinensis* X *Poncirus trifoliata*) (Fig. 4b). Grafted plantlets succeeding to the acclimation step, grew in a greenhouse with cooling system at 25°C ± 2°C until reaching 1 to 2 meters in high (Fig. 4c). At this stage, they are ready to be tested against viroid presence.

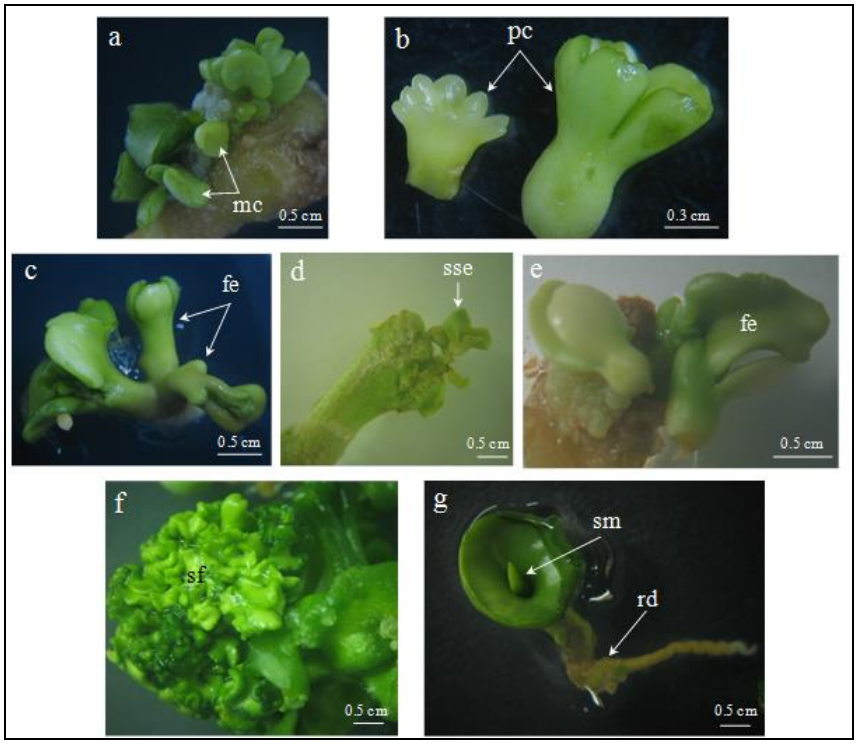

Figure 3: Some teratological forms observed during maturation phase of somatic embryogenesis regenerated in "half-blood" sweet orange. (a) Monocotylated embryos (mc). (b) Polycotylated embryos (pc). (c) Fused embryos (fe). (d) Secondary embryos (sse) developed on a rudimentary one. (e) Embryo showing disturbance in the development of their cotyledon (fc). (f) Fasciated form (ff). (g) Trumpet-shaped embryo showing shoot meristem (sm) and radicle (rd)

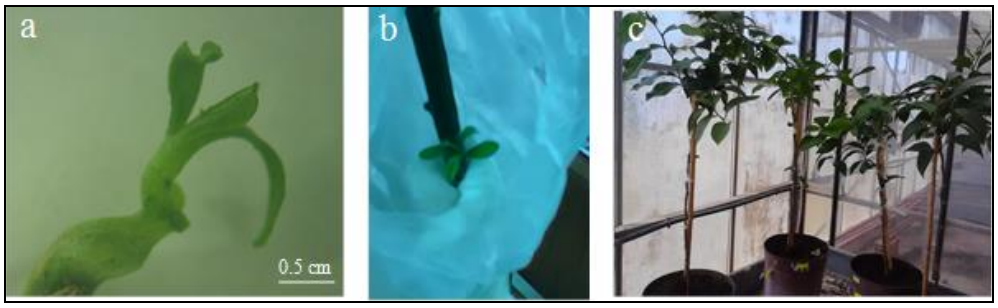

Figure 4: Growth steps of the somatic embryo-derived plants of the "half-blood" Maltese sweet orange. (a) Germination of somatic embryo into plantlet showing an elongated shoot (sh). (b) Grafted somatic plantlet and acclimatization. (c) 1-2 m high plants in conditioned greenhouse

CDVd and CVBd detection

The sanitary control by RT- PCR conducted on regenerated plants from somatic embryogenesis two years after culture initiation, revealed that they were free of viroids CVBd and CDVd identified in the source plant materials (Figure 1).

DISCUSSION

Somatic embryogenesis was carried out from style/stigma explants of "half-blood" Maltese sweet orange cultured on MS medium containing BAP. The achievement of somatic embryogenesis in presence of BAP confirmed that this is an effective cytokinin for citrus somatic embryogenesis as previously demonstrated (Carimi *et al*., 1994; D'Onghia *et al*., 2001; Lambardo *et al*., 2011). The concentration of growth-regulators used in this study is within the range used in the above mentioned works. The use of 13.3 µM BAP stimulated embryogenic response in styles (De Pasquale *et al*., 1994).

Unfortunately, the developmental process is accompanied by the occurrence of abnormal embryos that hamper the achievement of whole embryogenic process. Abnormal somatic embryos, including malformed cotyledons, mono ou pluri-cotyledonary forms, trumpet-shaped embryos and fusion of embryos in "half-blood" maltese genotype, seem to be a common phenomenon as in other species (Tang *et al*., 2000; Ricci *et al*., 2002; Santana-

Buzzy *et al*., 2009, Benelli, 2010). This phenomenon could be attribuated to genetic, epigenetic, or physiological factors (Santana-Buzzy *et al*., 2009). In the case of our genotype, the permanent exposition of the cultures to BAP during induction and expression of somatic embryos seemed to be one of the reasons of abnormalities previously cited. These morphological abnormalities were generally associated to the failure and/or low frequency of conversion in plantlets (Chengalrayan *et al*., 1997). According to this interpretation of the events, the improvement of the somatic embryogenesis technique should involve a deeper investigation regarding both the physical and chemical conditions in the vessels and of the timing of explant transfer.

In spite of the occurrence of many cases of teratological forms of somatic embryos, we obtained well developed plantlets regenerated from normal somatic embryos. These embryo-derived plants subjected to RT-PCR analysis for checking their viral state were no viroid was free of the viroïds found in the mother trees. This result confirms those obtained in previous studies (D'Onghia *et al*., 1997; Gambino *et al*., 2010) proved the fiability of our somatic embryogenesis work from style/stigma explants which is a very promising technique for the production of healthy citrus stocks.

Regarding genetic stability of the progeny plants that show many thorns attributed to a juvenile behavior (D'Onghia *et al*., 2001), we plan to submit them to molecular and flow cytometry analyses.

CONCLUSION

This efficiency of the technique of somatic embryogenesis from style/stigma explants to eliminate the targeted viroids from infected citrus plants allow this technique to be adopted in sanitary selection scheme against virus and virus-like diseases.

ACKNOWLEDGMENT

The authors would like to thank ministry of high education and scientific research for financial support.

References

[1] C Benelli; MA Germanà, T Ganino; D Beghe; A Fabbri. *Biol. Plantarum*, **2010**, 54, 224-230.
[2] F Carimi; F De Pasquale; FG Crescimanno. *Plant. Cell. Tiss. Org*., **1994**, 37, 209-211.
[3] K Chengalrayan; VB Mhaske; S Hazra. *Plant. Cell. Rep*., **1997**, 16, 783-786.
[4] MF Clark; AN Adams. *J. Gen. Virol*., **1977**, 4, 475-483.
[5] F De Pasquale; F Carimi; FG Crescimanno. *Aust. J. Bot*. **1994**, 42, 587-594.
[6] AM D'Onghia; F De Pasquale; F Carimi; V Savino; FG Crescimanno. *J. Phytopathol*. **1997**, 45, 77-79.
[7] AM D'Onghia; F Carimi; F De Pasquale; K Djelouaha; GP Martelli. *Plant Pathol*. **2001**, 50, 266-269.
[8] X Foissac; L Svanella-Dumas; P Gentit; MJ Dulucq; T Candresse. *Acta Hortic*. **2001**, 550, 37-43.
[9] G Gambino; R Vallania; I Gribaudo. *Eur. J. Plant Pathol*. **2010**, 127, 557-570.
[10] M Gandia; N Duran-Vila. *Arch. Virol*. **2004**, 149, 407-416.
[11] SA Kofalvi; JF Marcos; MC Canaizares; V Pallàs; T Candresse. *J Gen Virol.* **199**7, 78, 3177-3186.
[12] T Murashige; F Skoog. *Physiol. Plant*. **1962**, 15, 473-497.
[13] A Najar; N Duran-Vila. *Plant Dis*. **2004**, 88, 1286.
[14] L Navarro; J Juárez; JF Ballester; JA Pina. Elimination of some citrus pathogens producing psorosis-like leaf symptoms by shoot-tip grafting *in vitro*. *Proceedings. 8th Conf. Int. Org. Citrus Virol* (Sydney, Australia), **1980**, 162-166.
[15] H Puchta; K Ramm; R Luckinger; R Hadas; M Bar-Joseph; HL Sanger. *Nucl. Acids Res.* **1991**, 19, 6640.
[16] AG Rakowski; JA Szychowski; ZS Avena; JS Semancik. *J Gen Virol*. **1994**, 75, 3581-3584.
[17] AP Ricci; FAAM Filho; BMJ Mendes; SMS Piedade. *Sci Agr*. **2002**, 59, 41-46.
[18] CN Roistacher. Psorosis. In Graft-Transmissible Diseases of Citrus. Handbook for Detection and Diagnosis (Italy: FAO Publication Division), **1991**, 115-126.
[19] M Santana-Buzzy; G Lopez-Puc; A Canto-Flick; F Barredo-Pool; E Balam-Uc; S Aviles-Viñas; D Solís-Marroquín; C Lecona-Guzman; J Bello-Bello; E Gomez-Uc; JO Mijangos-Cortes. *HortScience*, **2009**, 44, 113-118.
[20] JS Semancik; SJ Szychowski; AG Rakowski; RH Symons. *J. Gen. Virol*. **1993**, 74, 2427-2436.