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Production of Somatic Embryogenesis *via in vitro* Culture of Stigma and Style for Elimination of Citrus PsorosisVirus (CpsV) from Some Citrus Genotypes

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Abstract: The production of embryogenic callus lines was varied with different citrus genotypes and type of explants. The response of different genotype and explants to initiate callus has been observed. Initiation of callus has been observed from stigma and style explants taken from uninfected trees or trees infected by citrus psorosis virus (CPsV) of Washington navel orange or Valencia orange.While, those taken from local mandarin failed to initiate callus. Embryogenic callus and somatic embryogenesis has been observed only from callus developed from stigma explants taken from uninfected trees or CPsV infected trees of Washington navel orange. Callusing developed from stigma explants taken from uninfected or infected mother trees of Washington navel orange was the most successful for regeneration of indirect somatic embryogenesis. Citrus psorosis virus (CPsV) was tested using RT-PCR. The results indicated that the somatic embryogenesis *via* stigma is a tool for production of CPsV-free citrus plants. RAPD- PCR was used to examine the somaclonal variation in stigma-derived plantlets. The results indicated that most plantlets were belonging to the respective mother plants and no big variations were found.

Key words: Citrus - Stigma · Style - Somatic embryogenesis - RAPD - CPsV - RT-PCR

INTRODUCTION

The genus citrus has been recognized as one of the most economically important group of plants in the world. Plant regeneration by *in vitro* culture may offer a highly efficient approach to the improvement of citrus. In particular, tissue culture techniques have been exploited to determine genetic variability, to improve the state -of- health (absence of viral infection) of the plant material and to increase the available number of elite genotypes to plant breeders by *ex situ* conservation [1-4]. Somatic embryogenesis is a process through which plant cells differentiate into somatic embryos. The phenomenon first observed in *Daucus carota* and then has been widely described in several plants [5-8]. It represents an efficient micropropagation technique to obtain a sufficient number of plants on a yearly basis and can provide a powerful tool for crop improvement [9,10]. The development of efficient tissue culture protocols is necessary for conservation and genetic improvement of citrus. In citrus, the production of embryogenic callus lines was reported from excised nucelli, abortive ovules, unfertilized ovules, undeveloped ovules, juice vesicles, styles and stigmas as well as from leaves, epicotyls, cotyledons and root segments [11-17]. The embryogenic potential of citrus varied with genotype and type of explant. In citrus, somatic embryogenesis has been observed through the culture of entire fertilized ovules, or isolated nucellar embryos from polyembryonic citrus genotypes [18]. This technique involves the use of different explant types (thin cell layers of stigma and style or entire organs) excised from citrus flowers tissues, which are not ovular in origin and regenerate plantlets being genetically identical to the original source [16,19]. Psorosis virus, one of the most widespread and economically important viral disease of citrus, is caused by *Citrus psorosis virus* (CPsV), a novel type of virus with filamentous particles that was recently shown to belong to the genus *Ophiovirus* [20 - 22]. Psorosis is graft transmissible and is disseminated primarily by infected bud wood. Psorosis-free material can be obtained by heat therapy and shoot tip grafting alone or in combination

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with heat therapy [23, 24]. However, improvement in the efficiency of sanitation protocols is desirable because, as reviewed by Roistacher [20], virus elimination varies between isolates and rarely exceeds 70-80%. Somatic embryogenesis from *in vitro* culture of styles and stigmas is an excellent method for pathogen elimination in most infected *Citrus spp.* and has been successfully practiced in the regeneration of different citrus genotypes [25]. Tissue culture techniques such as somatic embryogenesis have been applied to a number of perennial crops to eliminate viruses. Somatic embryogenesis from stigma and style cultures of citrus was used to eliminate *Citrus psorosis virus* from common mandarin, sweet orange and Dweet tangor citrus species [1, 26]. Somatic embryogenesis was also effective in eliminating fan leaf viruses and leaf roll-associated viruses from grapevines [27, 28]. And eliminating the cocoa swollen shoot virus from infected cocoa tree [29]. In this study, the aim was to investigate *in vitro* response of some citrus genotypes to produce somatic embryogenesis from stigma and style explants for elimination of CPsV. In addition, RAPD analysis was used to evaluate genetic stability at the level of DNA of somatic embryogenesis-derived plants.

MATERIALS AND METHODS

This investigation was carried out at the Tissue Culture laboratory, Plant Biotechnology Department, National Research Center.

Source of Plant Material: Mature trees of three citrus genotypes, Washington navel orange (*C. sinensis L.)*, Valencia orange (*C. sinensis L.*) and Local mandarin (*C. deliciosa*) grown in the field of Horticulture Research station (Kanater), Agriculture Research Center, Ministry of Agriculture infected by CPsV or uninfected were used as a source of plant material Fig (1: a,b).

Preparation of Explants: Closed flower buds at balloon stage (just before opening) as shown in Fig. (2) were harvested just before opening from infected and uninfected mature trees of Washington navel orange (*C. sinensis L.)*, Valencia orange(*C. sinensis L.)* and Local mandarin (*C. deliciosa*) and washed carefully with running water and surface sterilized by immersion in 70% (v/v) ethanol for 1 min, then soaked in a 40% Clorox [commercial bleach (2% sodium. hypochlorite)] for 15 min, followed by three 5-min rinses in sterile distilled water as described by Carimi *et al.* [16,30,31] and D Onghia *et al*. [1].

Fig. 1: Infected and uninfected Washington navel orange tree.

Fig. 2: Citrus closed flower bud.

Stigma and style explants were excised from sterilized flower buds with a scalpel under aseptic conditions and placed vertically onto the culture medium.

Culture Media: Explants were cultured on Murashige and Skoog [32] MS basal medium solidified with 7 g/L Difco Bacto agar and supplemented with 50 g/L sucrose, 500 mg/L malt extract (ME) and 3 mg/L 6- benzylamino purine (BAP) as reported by Carimi *et al*. [16,30,31] and D Onghia *et al*. [1]. pH of the medium was adjusted to 5.8 with 0.5 M potassium hydroxide, then autoclaved at 121°C for 25 min.

Culture Conditions: Culture jars were incubated at 25 ± 2°C under a 16 hrs. day length with illumination of 100 ìmol/m^{-2}/s^{-1} Osram cool white 18 W fluorescent lamps. All culture explants were subcultured at four weeks intervals into fresh culture medium of the same composition.

Data and Record: Percentage of callusing was recorded weekly:

$$\frac{\text{Number of stigma or style explants - derived calli}}{\text{Total number of cultured explants}} \times 100$$

Percentage of embryogenesis was recorded monthly:

$$\frac{\text{Number of somatic embryogenesis - derived calli}}{\text{Total number of stigma explants - derived calli}} \times 100$$

Somatic embryos number/explant was recorded monthly for a period of eight months as reported by Carimi *et al*. [16, 30].

Germination of Somatic Embryos: Obtained somatic embryos (2-3 mm in length) were excised and cultured into culture tube (25 × 150 ml) containing 20 ml of MS basal medium solidified with7 g/L agar and supplemented with 50 g/L sucrose and 1mg/L of Gibberelic acid (GA_3) as described by Kobayashi *et al.* [33]; Hidaka and Omura [34] and Ling *et al*. [35]. The pH was adjusted to 5.8 ± 0.1 with 0.5 M potassium hydroxide before autoclaving at 121°C for 25 minutes. Each culture tube contains two somatic embryos. Culture tubes were sealed with alumium foil. The culture tubes were kept in the culture room at 25 ± 2°C and exposed to a 16 hrs day length, with illumination of 100 ìmol/m^{-2}/s^{-1} Osram cool - white 18 W fluorescent lamps. After the initiation of embryo germination, germinated embryos were subcultured to jars containing the same medium.Each treatment consists of three replicates and each replicate consists of three culture jars and each culture jar containing two-three somatic embryos.

Embryo germination was count weekly for 4 weeks per culture tube of each replicate and expressed as percentage of germination during 9 months.

$$\text{Percentage of germination} = \frac{\text{Number of germinated embryo}}{\text{Total no. of cultured embryos}} \times 100$$

Detection of Cpsv in Stigma-derived Plantlets: RT-PCR was performed on total RNA extracted from all regenerated plantlets and positive control using two primers were selected for RT-PCR detection of CPsV based on Published sequences of the cloned coat protein (CP) gene according to Martin *et al.* [36].

Molecular Analysis of Somatic Embryogenesis-derived Plantlets: Somatic embryogenesis-derived plantlets regenerated from stigma were tested for their genetic stability using RAPD-PCR according to Murray and Thompson [37].

Statistical Analysis: All the data were subjected to analysis of variances (ANOVA) and were analyzed using CoHort program [38] (Duncun's multiple range test at 5 % level) according to Snedecor and Corchan [39] to verify the differences between means of treatments. All experiments were designed in a completely randomized design; each treatment was the average of 3 replicates.

RESULTS AND DISCUSSION

Friable-yellow callus (Fig.3: a) was initiated from stigma and style explants taken from infected or uninfected mother trees of both Washington navel orange and Valencia orange,while, stigma and style explants taken from infected or uninfected mother trees of local mandarin could not able to initiate callus (Table 1).

Callus began to initiate after two weeks from stigma and style explants taken from infected or uninfected mother trees of Washington navel orange. While, from stigma and style explants taken from infected or uninfected mother trees of Valencia orange, callus began to initiate after six weeks.

For Washington navel orange, stigma and style explants taken from uninfected mother trees showed the highest significant callus percentage compared with callus percentage showed from stigma and style explants taken from infected mother trees. Maximum significant callus percentage for stigma and style explants taken from uninfected mother trees was observed after 2 months for stigma explants (38.4%) and after 4 months for style explants (35.4%).While, for stigma and style explants taken from infected mother trees, maximum callus percentage was observed after 4 months, (18.7%) for stigma explants and (20.3%) for style explants (Table 2).

For Valencia orange, stigma and style explants taken from uninfected mother trees showed the highest significant callus percentage compared with callus percentage showed from stigma and style explants taken from infected mother trees. Maximum significant callus percentage for stigma and style explants taken from uninfected mother trees was observed after 3 months (8.0%) for stigma explants and (8.1%) for style explants. While, maximum callus percentage was observed after 2 months (3.1%) for stigma explants and (6.2%) for style explants (Table 3).

Callus developed from stigma and style explants of Valencia orange taken from infected or uninfected mother trees failed completely to regenerate indirect somatic

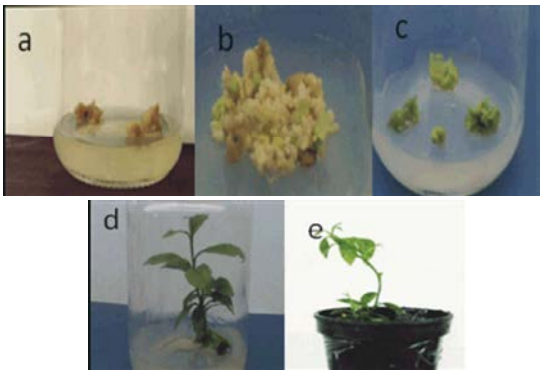

Fig. 3: Stages of initiation and development of somatic embryogenesis; a: Callusing stage from stigma explants after 1 month, b: Somatic embryogenesis after 3 months, c: Somatic embryos at cotyledonary stage on germination medium, d: Stigma- derived plantlet after 3 months on germination medium, e: Adapted plantlet of stigma derived-tissue culture (3 week-old) grown in soil mixture (1 peat: 1 sand v/v).

Table 2: Callusing percentage of stigma and style explants taken from infected and uninfected Washington navel orange mother trees (Through 7 subculture one-month for each).

	Stigma		Style	
Subculture (month)	Infected	Uninfected	Infected	Uninfected
1	4.2 d	17.4 b	6.7 cd	9.4 c
2	16.9 b	38.4 a	16.8 b	33.9 a
3	16.9 b	38.4 a	18.5 b	33.9 a
4	18.7 b	38.4 a	20.3 b	35.4 a
5	18.7 b	38.4 a	20.3 b	35.4 a
6	18.7 b	38.4 a	20.3 b	35.4 a
7	18.7 b	38.4 a	20.3 b	35.4 a
Mean	16.1 B	35.4 A	17.6 B	31.3 A

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

Table 3: Callusing percentage of stigma and style explants of infected and uninfected Valencia orange mother trees (Through 7 subculture one-month for each).

	Stigma		Style	
Subculture (month)	Infected	Uninfected	Infected	Uninfected
1	0.0 d	0.0 d	0.0 d	0.0 d
2	3.1 c	4.0 c	6.2 b	7.2 ab
3	3.1 c	8.0 ab	6.2 b	8.1 a
4	3.1 c	8.0 ab	6.2 b	8.1 a
5	3.1 c	8.0 ab	6.2 b	8.1 a
6	3.1 c	8.0 ab	6.2 b	8.1 a
7	3.1 c	8.0 ab	6.2 b	8.1 a
Mean	2.7 B	6.3 A	5.3 A	6.8 A

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

Table 4: Somatic embryogenesis percentage for stigma explants taken from infected and uninfected Washington navel orange trees (through 12 subcultures - one month for each)

Subculture (month)	Infected	Uninfected
1	0.0 c	0.0 c
2	0.0 c	0.0 c
3	0.0 c	27.8 b
4	0.0 c	33.3 a
5	0.0 c	33.3 a
6	0.0 c	33.3 a
7	28.6 b	33.3 a
8	28.6 b	33.3 a
9	28.6 b	33.3 a
10	28.6 b	33.3 a
11	28.6 b	33.3 a
12	28.6 b	33.3 a
Mean	14.3 B	27.3 A

Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

Table 5: Number of somatic embryos generated from stigma explants taken from infected and uninfected Washington navel orange trees (through12 subculture, one-month each).

	Total no. of embryos		No. of embryos/explant	
Subculture (month)	Infected	Uninfected	Infected	Uninfected
1	0.0 n	0.0 n	0.0 i	0.0 i
2	0.0 n	0.0 n	0.0 i	0.0 i
3	0.0 n	25.0 i	0.0 i	5.0 g
4	0.0 n	75.0 h	0.0 i	12.5 e
5	0.0 n	98.0 g	0.0 i	16.3 d
6	0.0 n	129.0 f	0.0 i	21.5 c
7	3.0 m	134.0 e	1.5 i	22.3 c
8	7.0 l	134.0 e	3.5 h	22.3 c
9	12.0 k	136.0 d	6.0 g	22.7 bc
10	13.0 k	143.0 c	6.5 g	23.8 ab
11	16.0 j	149.0 b	8.0 f	24.8 a
12	16.0 j	151.0 a	8.0 f	25.2 a
Mean	5.6 B	97.8 A	2.8 B	16.4 A

Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

Table 6: Germination percentage of somatic embryos regenerated from stigma explants taken from infected and uninfected Washington navel orange trees (through 9 months).

Month	Infected	Uninfected
1	10 i	8.2 j
2	60 f	21.3 h
3	60 f	48.4 g
4	76 d	73.8 e
5	96 b	92.8 c
6	96 b	92.8 c
7	100 a	92.8 c
8	100 a	92.8 c
9	100 a	92.8 c
Mean	77.6 A	68.4 B

Values followed by the same letters were not significantly different by Duncan's test at 0.05 level

embryogenesis. Otherwise, for Washington navel orange, callus developed from stigma explants taken from infected and uninfected mother trees was able to regenerate indirect somatic embryogenesis. On the other hand, callus developed from style explants taken from infected and uninfected mother trees failed completely to regenerate indirect somatic embryogenesis.

Callus developed from stigma explants taken from uninfected Washington navel orange trees started to regenerate indirect somatic embryogenesis after 3 months. While, callus developed from stigma explants taken from infected mother trees started to regenerate indirect somatic embryogenesis after 7 months. Maximum significant somatic embryogenesis percentage of stigma explants was observed after 3 months (33.3%) for uninfected mother trees and after 7 months (28.6%) for infected mother trees (Table 4).

Concerning the total number of embryos and number of embryo per explants regenerated from stigma explants of Washington navel orange data in Table 5 and Fig. (3-b) showed that total number of embryos and number of embryos/ explant was significantly affected by the infection of viruses, as the mean total number of embryos after 12 months was 97.8 embryos for the explants taken from uninfected trees and 5.6 embryos for the explants taken from infected trees. Also, total number of embryos for the explants taken from uninfected trees significantly increased gradually from 25 embryos after 3 months to 151 embryos after 12 months.While, for the explants taken from infected trees, total number of embryos increased gradually from 3 embryos after 7 months to 16 embryos after 12 months. Also, data in Table 5 showed a significant difference between number of embryos/ explant taken from uninfected trees and infected mother trees (16.4 and 2.8 embryos/ explants respectively). Number of embryos / explant taken from uninfected trees significantly increased gradually from 5 embryos/ explant after 3 months to 25.2 embryos / explant after 12 months. While, this number increased gradually from 1.5 embryos/ explant after 7 months to 8.0 embryo/ explant after 12 months when explants taken from infected trees.

Data in Table 6 recorded monthly (up to 9 months) the germination percentage of embryos regenerated from stigma explants taken from infected and uninfected Washington navel orange trees. Germination percentage of embryos regenerated from explants taken from infected trees was significantly higher compared with germination percentage of embryos regenerated from explants taken from uninfected trees (77.6% and 68.4% respectively). Germination percentage significantly increased gradually by increasing the months from 1 to 9 months and reached to the maximum significant germination percentage 92.8% after 5 months for explants taken from uninfected trees and to 100% after 7 months for explants taken from infected trees.

In our experiment, the genotype, type of explants and infection of the mother tree appeared to influence callus formation and somatic embryogenesis. In this study, Valencia orange and local mandarin failed to produce embryogenic callus. These results are similar to those obtained by Perez *et al*. [40] who mentioned the failure of lemon; grapefruit and Satsuma mandarin to produce embryogenic callus under a range of conditions assayed indicates that factors other than those related to *in vitro* tissue culture manipulations may be responsible for the lack of success. Stigma explants were significantly more responsive than style explants in both infected and healthy trees. On the other hand, Carimi *et al.* [16] reported that percentage of responsive styles ranged from 0% to 30%in different citrus genotypes. Greno *et al*. [41], reported that some virus and virus-like agents had a marked effect on the *in vitro* regeneration of citrus plantlets. Also, in our study it was observed that levels of response were generally higher in uninfected trees than those observed in infected trees.But, somatic embryos produced from infected trees successfully regenerated to plantlets with high frequency (100%). No virus was detected in any of the regenerated plantlets (obtained from stigma explants taken from infected mother trees). This indicates that, somatic embryogenesis from stigma explants is a successful protocol for producing virus -free citrus plantlets; these results are similar to those reported by D Onghia *et al.* [1, 26].

Detection of CpsV in Stigma-Derived Plantlets: Results of RT-PCR analyzed by electrophoresis shown in Fig. (4). They indicated that detectable band resulting from RT-PCR products with 218 bp when using the primers reported by Martin *et al.*[36]**.** But all the tested samples were negative because no detectable band (218 bp) was produced under our PCR conditions as shown in Fig. (4). In fact, no CPsV was detected in all regenerated plantlets using RT-PCR methods confirming that all plantlets resulted via somatic embryogenesis derived from stigma are CPsV-free.

The fact that no CPsV was detected in regenerated plants using sensitive techniques, such as RT-PCR. Confirms the results of previous studies involving other diseases and further show that somatic embryogenesis from stigma explants is a very promising technique for the production of virus-free citrus stocks [26].

Table 7: Effect of arbitrary sequence primers tested in RAPD analysis of stigma-derived-plantlets of Washington navel orange.

Primer	No. of produced bands: Mother tree (p)	No. of produced bands: *in vitro* derived plantlets	Size range Bp	No. of polymorphic bands	% Polymorphism	Size of polymorphic bands Bp
A1	5	7	200-800	3	25	900,700 400
A2	8	9	150-1000	6	35.5	1000,900,800,400,150,170

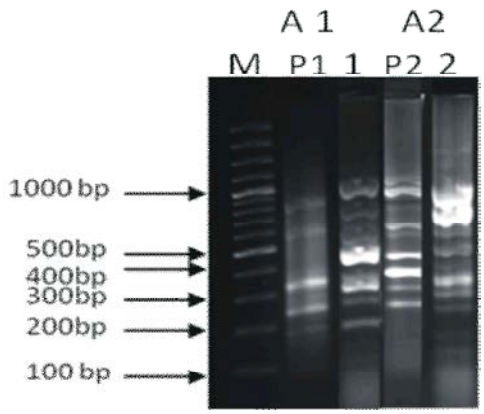

Fig. 4: Gel electrophoresis of RAPD fingerprints obtained with two Primers; M: DNA marker. P1, P2: mother plant. 1, 2: in vitro-derived plantlets; A1, A2: Random primers.

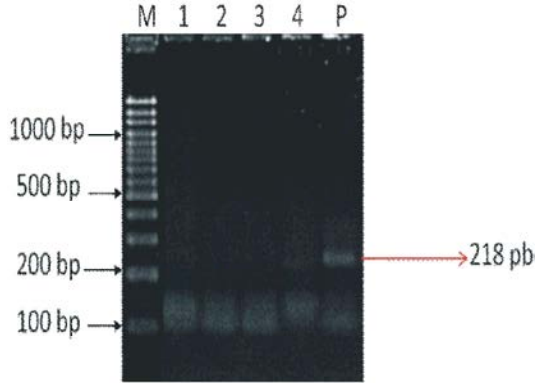

Fig. 5: Electrophoresis analysis in 1% agarose gel of RT-PCR amplification products to detect CPsV using primers reported by. M: Marker 100 bp ladder; P: Positive control (CPsV-infected.). 1, 2, 3, 4: *in vitro*-derived plantlets.

Molecular Analysis of Somatic Embryogenesis-Derived Plantlets: Five random oligonucleotide primers (A1, A2, M-07, Z-02, K7) were used and number of DNA fragments amplified in their presence are shown in Table 7 and Fig. 5, out of five (10 mers) primers, two were succeeded (A1,A2) and turned out to give number of banding in the gel depend on the used primer; three did not produce amplification products. Otherwise most of the succeeded primers identify DNA polymorphism under our PCR conditions 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 [42] for DNA fingerprinting.

As shown in Table 7 and Fig. 5, primers A1 and A2 succeeded with tested stigma-somatic embryogenesis-derived plantlets of Washington navel orange and produced about 29 bands in the gel anged from 150 to 900 bp in length, 9 out of these are polymorphic bands with 31% ranging from 150 to 900 bp depending on the primer tested. The results here indicated that the RAPD-PCR technique can be successfully applied to determine the genetic stability of citrus plants that are produced through somatic embryogenesis.

REFERENCES

1. D Onghia, A.M., F. Carimi, F. De Pasquale, K. Djelouah and G.P. Martelli, 2001. Elimination of citrus psorosis virus by somatic embryogenesis from stigma and style cultures. Plant Pathology, 50: 266-269.
2. Gambino, G., D. Di Matteo and I. Gribaudo, 2009. Elimination of Grapevine fan leaf virus from three *Vitis vinifera* cultivars by somatic embryogenesis. Eur. J. Plant Pathol., 123: 57-60.
3. Hawkes, J.G., N. Maxted and B.V. Ford-Lloyd, 2000. The *ex situ* conservation of plant genetic resources. Kluwer Academic Publishers, Dordrecht, The Netherlands Springer, 250: 32-42.
4. Sarasan, V., R. Cripps, M.M. Ramsay, C. Atherton, M. McMichen, G. Prendergast and J.K. Rowntree, 2006. Conservation *in vitro* of threatened plants-Progress in the past decade. *in vitro* Cell Dev. Biol. Plant. 42: 206-214.
5. Steward, F.C., M.O. Mapes and K. Hearks, 1958. Growth and organized development of cultured cells. II. Growth and division of freely suspended cells. Am J. Bot., 45: 705-708.

6. Ozias-Akins, P. and I.K. Vasil, 1982. Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): Evidence for somatic embryogenesis. Protoplasma, 110: 95-105
7. Fourre, J.L., P. Berger, L. Niquet and P. Andre, 1997. Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. Theor Appl. Genet., 94: 159-169.
8. Chen, K., X.B. Zhang, J.L. Jiang and X.Y. Wang, 2011. Plantlet regeneration from cotyledon, cotyledon petiole and hypocotyl explants via somatic embryogenesis pathway in roquette (*Eruca sativa* Mill). Plant Biosystems, 145: 68-76.
9. Redenbaugh, K., 1993. Synseed: Applications of Synthetic Seeds to Crop Improvement. CRC Press, Boca Raton, Florida, pp: 3-10.
10. Litz, R.E. and D.J. Gray, 1995. Somatic embryogenesis for agricultural improvement. World J Microbiol Biotech., 11: 416-425
11. Rangan, T.S., T. Murashige and W.P. Bitters, 1968. *in vitro* initiation of nucellar embryos in monoembryonic citrus. Hort Science, 3(4): 226-227.
12. Bitters, W.P., T. Murashige, T.S. Rangan and E. Nauer, 1970. Investigations on established virus-free plants thorough tissue culture. Calif. Citrus Nursery Soc., 9: 27-30.
13. Button, J. and C.H. Bornman, 1971. Development of nucellar plants from unpollinated and unfertilized ovules of the Washington navel orange *in vitro*. J. S. Afr. Bot., 37: 127-134.
14. Starrantino, A. and F. Russo, 1980. Seedlings from undeveloped ovules of ripe fruits of polyembryonic citrus cultivars. Hort Science, 15(3): 296-297.
15. Nito, N. and M. Iwamasa, 1990. *in vitro* plantlet formation from juice vesicle callus of Satsuma (*Citrus unshiu* Mare.). Plant Cell, Tissue and Organ Culture, 20(2): 137-140.
16. Carimi, F., F. De pasquale and F.G. Crescimanno, 1995. Somatic embryogenesis in citrus from styles culture. Plant Science, 105: 81-86.
17. Gill, M.I.S., Z. Singh, B.S. Dhillon and S.S. Gosal, 1995. Somatic embryogenesis and plant regeneration in mandarin *(Citrus reticulata* Blanco). Scientia Horticulturae, 63: 167-174.
18. Litz, R.E., G.A. Moore and C. Srinivasan, 1985. *in vitro* system for propagation and improvement of tropical fruits and palm. Horticultural Reviews, 7: 157-200.
19. De Pasquale, F., F. Carimi and F.G. Crescimanno, 1994. Somatic embryogenesis from styles of different cultivars of *Citrus limon* (L.) Burm. Aust. J. Botany, 42: 587-594.
20. Roistacher, C.N., 1993. Psorosis a review. In: Proceedings of the Twelfth Conference of the International Organization of Citrus Virologists, New Dehli, India. International Organization of Citrus Virologists, pp: 139-54.
21. Pringle, C.R., 1999. Virus taxonomy - 1999. Archives of Virology144, 421-9.DOI: 10.1007/s007050050515.
22. Milne, R.G., M.L. Garcia and O. Grau, 2000. Genus Ophiovirus. In: Van Regenmortel RHV, Fauquet CM, Bishop DHL, Carstens E, Estes MK, Lemon S, Maniloff J, Mayo M.A. McGeoch D. Pringle C.R. Wickner and R.B. eds. Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. New York, USA: Academic Press, pp: 627-31.
23. Calavan, E.C., C.N. Roistacher and E.M. Nauer, 1972. Thermotherapy of citrus for inactivation of certain viruses. Plant Disease Reporter, 56: 976-80.
24. Navarro, L., J. Juarez, J.F. Ballester and J.A. Pina, 1980. Elimination of some citrus pathogens producing psorosis-like leaf symptoms by shoot-tip grafting *in vitro*. In: Proceedings of the Eighth Conference of the International Organization of Citrus Virologists, Sydney, Australia. International Organization of Citrus Virologists, pp: 162-6.
25. D Onghia, A.M., F. Carimi, F. De Pasquale, K. Djelouah and G.P. Martelli, 2000. Somatic embryogenesis from style culture: A new technique for the sanitation, conservation and safe exchange of citrus germplasm. Proc. IX Int. Soc. Citric. pp: 147-149.
26. D Onghia, A.M., F. De Pasquale, F. Carimi, V. Savino and F.G. Crescimanno, 1997. Somatic embryogenesis from style culture as a possible means for virus elimination in citrus. J. Phytopathology, 145: 77-79.
27. Goussard, P.G., J. Wiid and G.G.F. Kasdorf, 1991. The effectiveness of *in vitro* somatic embryo genesis in eliminating fan leaf virus and leaf roll-associated viruses from grapevine. S. Afr. J. Enol. Vitic., 12: 77-81.
28. Goussard, P.G. and J. Wiid, 1993. The use of *in vitro* somatic embryo genesis to eliminate phloem limited virus and nepoviruses from grapevines. In: Extended Abstracts, vol. 11th Meet ICVG, Montreux, pp: 165-166.

29. Quainoo, A.K., A.C. Wetten and J. Allainguillaume, 2008. The effectiveness of somatic embryogenesis in eliminating the cocoa swollen shoot virus from infected cocoa trees. J. Virol. Methods, 149: 91-96.
30. Carimi, F., M.C. Tortorici, F. De pasquale and F.G. Crescimanno, 1998. Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explants of sweet orange navel group (*Citrus sinensis* (L.) Osb.). Plant Cell, Tissue and Organ Culture, 54: 183-189.
31. Carimi, F., F. De pasquale and F.G. Crescimanno, 1999. Somatic embryogenesis and plant regeneration from pistil thin cell layers of citrus. Plant Cell Reports, 18: 935-940.
32. Murashige, T. and F. Skoog, 1962. A reversed medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantanum, 15: 473-479.
33. Kobayashi, S., L. Ikeda and M. Nakatani, 1984. Induction of nucellar callus from orange (*Citrus sinensis* Osb.) ovules and uniformity of regenerated plants. Bulletin, Fruit tree Research station Japan E. Akitsu. 5: 43-54.
34. Hidaka, T. and M. Omura, 1989. Control of embryogenesis in citrus cell culture: Regeneration from protoplasts and attempts to callus bank. Bull. Fruit Tree Res. Stn. B. 16: 1-17.
35. Ling, J.T., N. Nito, M. Iwamas and H. Kunitake, 1990. Plant regeneration from protoplasts isolated from embryogenic callus of Satsuma. Hort Science, 25(8): 970-972.
36. Martin, S., D. Alioto, R.G. Milane, S.M. Garnsey, M.L. Garcia, O. Grau, J. Guerri and P. Moreno, 2004. Detection of citrus psorosis virus by ELISA, molecular hybridization, RT-PCR and immunosorbet electron microscopy and its association with citrus psorosis disease. Europ. J. Plant Pathol., 110: 747-757.
37. Murray, M.G. and W.F. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., 8: 4321-4325.
38. CoHort, 2004. CoHort Software Version 6.3. Monterey, CA, 93940, USA
39. Snedecor, G.W. and W.G. Cochran, 1982. Statistical Methods. 7th Ed. The Iowa State Univ. Press. Ames. Iowa, USA.
40. Perez, R.M., A.M. Galiana, L. Navarro and N. Duran-Vila, 1998. Embryogenesis *in vitro* of several citrus species and cultivars. J.of Hort. Scien. and Biotechnol., 73(6): 796-802.
41. Greno, V., L. Navarro and N. Duran-Vila, 1988. Influence of virus and virus-like agents on the development of citrus buds cultured *in vitro*. Plant Cell, Tissue and Organ Culture, 15: 113-124.
42. Caetano-Anolles, G. and P.M. Gresshoff, 1992. Staining with silver, promega Notes, 45: 13-18.