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*Full Length Research Paper*

# Regeneration of Algerian *Citrus* germplasm by stigma/style somatic embryogenesis

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**Stigma/style somatic embryogenesis is one of the efficient methods in plant regeneration of most *Citrus* ssp., without inducing somaclonal variations. Furthermore, somatic embryogenesis from style/stigma proved to be effective in the elimination of the main citrus virus and virus-like diseases. This technique was applied on Algerian citrus collection. Different *Citrus* species [*Citrus sinensis* (L.) Osbeck, *C. limon* (L.) Burm, *C. reticulata* Blanco, *C. paradisi* Macfad, *C. reshni* Hort. ex Tan., *C. jambhiri* Lush and *C. maxima* (Burm.) Merrill] were chosen and tested for the presence of the main virus and virus-like agents. Most of the genotypes showed to be infected, mainly by viroid agents. Closed flowers were collected and *in vitro* cultured on a Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine. All explants produced callus about 4 to 9 days after culture initiation, whereas embryogenesis occurred after 38 to 150 days in most of the cultured genotypes. Formed embryos were cultured in a single tube before *in vivo* acclimatization. After sanitary assays, regenerated plants were shown to be free from the agents detected in the mother trees.**

**Key words:** Algeria, citrus germplasm, plant regeneration, sanitation, somatic embryogenesis.

## INTRODUCTION

Somatic embryogenesis (SE) is the process by which somatic cells develop into plants through an orderly series of characteristic embryological stages without fusion of gametes (Jiménez, 2001). SE was described for the first time in *Daucus carota* in 1958 (Reinert, 1958), and since then it has been described in many and diverse plant species and it has been the subject of many studies. In *Citrus*, SE has been observed through the culture of entire fertilized ovules, or isolated nucellar embryos from polyembryonic citrus genotypes (Litz et al., 1985). 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 (De Pasquale et al., 1994; Carimi et al., 1995). SE 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 (D'Onghia et al., 2000).

Algeria has a citrus germplasm collection of 256 varieties/clones, which represents a reservoir of genetic resources of inestimable value (Larbi et al., 2009). Several virus and virus-like diseases are known to infect citrus groves in Algeria (Bovè, 1995). Infections induced

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**Abbreviations: BA,** 6-Benzylaminopurine; **SE,** somatic embryogenesis.

**Table 1.** Algerian citrus cultivars tested for somatic embryo regeneration by stigma/style culture.

| Species | Cultivar | Species | Cultivar |
|---|---|---|---|
| *C. jambhiri* | Rough lemon | *C. reshni* | Cleopatra mandarin |
| *C. limon* | Béni Abbes | *C. reticulata* | Mandarine de Blida |
| | Bornéo | | Mandarine Temple |
| | Citronnier Palerme | | Pam Américain |
| | Citronnier secile | *C. sinensis* | Alger navel |
| | Dellys | | Djirid |
| | Eureka | | Double fine améliorée |
| | Eureka Maroc | | Golden Bucky |
| | Femminello | | Maltaise de Tunisie |
| | Lisbon 16 | | Oranger de bey |
| | Lisbon 6 | | Oranger de Blida |
| | Lunario | | Oranger sidi Ali |
| | Poire de Commandeur | | Sanguine de Biskra |
| *C. maxima* | Pomélo Royal | | Shamouti Skikda |
| | Pomélo Ruby | | Shamouti station |
| | | | Thomson navel |
| *C. paradisi* | Pamplemoussier à chaire rose | | Washington navel |
| | Pamplemoussier commun | | |

by *Citrus tristeza* virus (CTV), the most severe viral agent of citrus were also reported by Larbi et al. (2009) in mandarin and sweet orange trees used as mother trees at the Beni Tamou farm of Institute Technique de l'Arboriculture Fruitière et de la Vigne (ITAF). Due to the threat posed by CTV, a program for the production of 'healthy' plants of the main *Citrus* species has been recently started.

The objective of the present study was the application of style and stigma somatic embryogenesis for the regeneration and sanitation of a number of citrus genotypes belonging to the citrus collection maintained at ITAF and the evaluation of the embryogenic potential of the local citrus genotypes.

## MATERIALS AND METHODS

Flowers were collected from 34 cultivars (Table 1) of seven different *Citrus* species belonging to the Algerian citrus collection of ITAF: *Citrus sinensis* (L.) Osbeck, *C. limon* (L.) Burm*, C. reticulata* Blanco*, C. paradisi* Macfad, *C. reshni* Hort. ex Tan., *C. jambhiri* Lush and *C. maxima* (Burm.) Merrill. Flowers were collected before opening from trees growing under field conditions during the blossoming period. The flowers were surface-sterilized by immersion for 5 min in 70% ethanol, 20 min in 2% (w/v) sodium hypochlorite, followed by three 5 min rinses in sterile distilled water. Stigmas and styles were excised with a scalpel and vertically plated as single explants in Petri dishes (100 × 15 mm) with the cut surface in contact with the medium. Five explants were placed in each Petri dish and 6 Petri dishes were used per treatment.

### Sanitary assays

Before applying somatic embryogenesis, the studied citrus genotypes were tested for the presence of the infectious agents. Flowers, leaves and phloematic tissues were sampled and processed by serological and molecular assays for the detection of the main viruses and viroid agents (Bar-Joseph et al., 1979; Djelouah et al., 2000; Palacio-Bielsa et al., 1999; Roistacher, 1991). *In vitro* plants regenerated from the infected genotypes were tested for assessing pathogen elimination.

### Culture medium

Styles and stigmas were *in vitro* cultured on MS (Murashige and Skoog, 1962) solidified medium (8 g/L Phyto agar) with 500 mg/L malt extract (M-0383, Sigma, St Louis, MO, USA) and 146 mM sucrose as carbon source. The pH of the media was adjusted to 5.7±0.1 with 0.5 M of KOH before autoclaving. Explants were cultured in the presence of 3 mg/L of 6-benzylaminopurine (BA, Sigma B-4308) (Carimi et al., 1994). BA was filter-sterilized through a 0.22 µM nylon filter and added to the medium after autoclaving. Plates were incubated in a growth chamber at 25±1ºC under a 16 h day length photoperiod and a photosynthetic photon flux of 50 µmol $m^{-2}$ $s^{-1}$ provided by Osram cool-white 18 W fluorescent lamps. Explants and calluses were subcultured into fresh medium at 4 to 6 weeks intervals and maintained under the same culture conditions.

### Germination and *in vivo* transfer of regenerated plants

Individual germinated somatic embryos (7 to 10 mm in length) were isolated from the culture and transferred into test tubes containing hormone-free solid MS medium (1 embryo per 55 × 23 mm glass

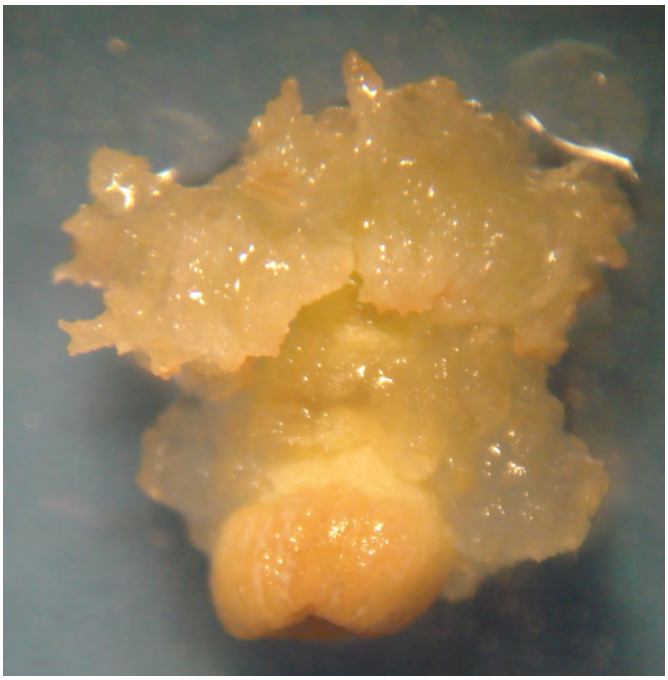

**Figure 1.** Callus formation from stigma/style explant of *C. sinensis* "Double fine améliorée" cultured on MS medium supplemented with BA. Picture was taken 9 days from the beginning of the experiment.

tube sealed with Parafilm M$^{TM}$) and incubated under the same culture conditions described above. Generated plantlets were individually grown in test tubes for 2 months before the *in vivo* transfer phase. When plantlets were 2 to 3 cm in length, they were grafted into 4 to 6 months old sour orange seedlings as described by De Pasquale et al. (1999).

#### Data and statistical analysis

Explants of 5 *Citrus* species were periodically observed to check when callus formation occurred and the first embryogenic event took place. Percentage of calluses and embryogenic explants were evaluated as percentages on a Petri dish basis. Statistical analysis was performed using percentages of callus and somatic embryo induction. The experimental design was completely randomized; for each genotype 6 Petri dishes were used (replicates), containing each five explants (30 explants for each genotype). Effects of treatment were tested by analysis of variance, while the differences among genotypes were tested by Tukey's test ($P=0.01$). Prior to analysis, percentage data were arcsine-square root transformed.

## RESULTS AND DISCUSSION

### Callus induction

About 4 to 9 days after the incubation, most of the explants of different genotypes produced a friable creamy-white callus at the cut end of the styles (Figure 1). The variable proliferation rates and size of callus production were observed during the callus induction period. Statistical analysis revealed that the frequency of callus induction was significantly affected by species and genotype. Indeed, the percentage of calluses production varied from 0 ('Rough lemon') to 100 % ('Shamouti station', 'Oranger de Blida', 'Femminello', 'Béni Abbes') according to genotypes and species (Figure 2). Among *Citrus* species, explants of *C. limon* and *C. sinensis* showed the highest callus induction rate which was also of large size (more than 1-2 cm in diameter, data not shown). On the contrary, a smaller size of calluses (less than 1 cm in diameter) was observed in explants of *C. reticulata*. Explants excised from *C. paradisi* showed high rate of browning 2 weeks after culture initiation, whereas 6 weeks later browning was also observed in explants of *C. reticulata*. Periodically, after 4 to 6 weeks from culture initiation, the produced callus was subcultured in the same culture conditions described above.

### Somatic embryos development

Indirect SE was observed and somatic embryos developed 38 to 150 days after culture initiation according to the genotype. Somatic embryos were green and easy to detach (Figure 3). Embryos at different stages of development were observed at the same time. The percentage of responsive styles varied from 2.35% ('Double fine améliorée') to 80% ('Femminello'), depending on species and genotype (Figure 4). The ANOVA showed that the embryogenic response of the explants was significantly influenced by the genotypes. Previous reports indicated that the addition of BA to the medium greatly increased the embryogenic response of styles (Carimi et al., 1994). Indeed, in our experiment, explants cultured on MS medium supplemented with BA showed a greater potential response in terms of the percentage of embryos produced.

*C. limon* showed a higher embryogenic potential (Figure 4A). After a similar experiment, Carimi et al. (1994) and De Pasquale et al. (1994) reported that lemon genotypes were successfully regenerated from stigma and style. Nevertheless, the highest somatic embryogenesis rate was obtained with 'Femminello' and 'Lisbon 16' with 80 and 34.78%, respectively. Very promising results were also obtained with the Algerian genotype 'Dellys' (17.85%). Somatic embryos were formed more rapidly in 'Eureka Maroc' explants (38 days after culture initiation) than in explants of other species. In accordance with previous reports (Carimi and De Pasquale, 2003; Carimi, 2005), the success of somatic embryos regeneration in lemon depends on the genotype, thus a great variability is shown within the same species.

D'Onghia et al. (2000) mentioned that not only lemon but also sweet oranges, particularly those belonging to the Navel group, showed a higher regeneration potential. Indeed, relatively to the cultured sweet orange genotypes (Figure 4B), embryos were obtained in 'Washington

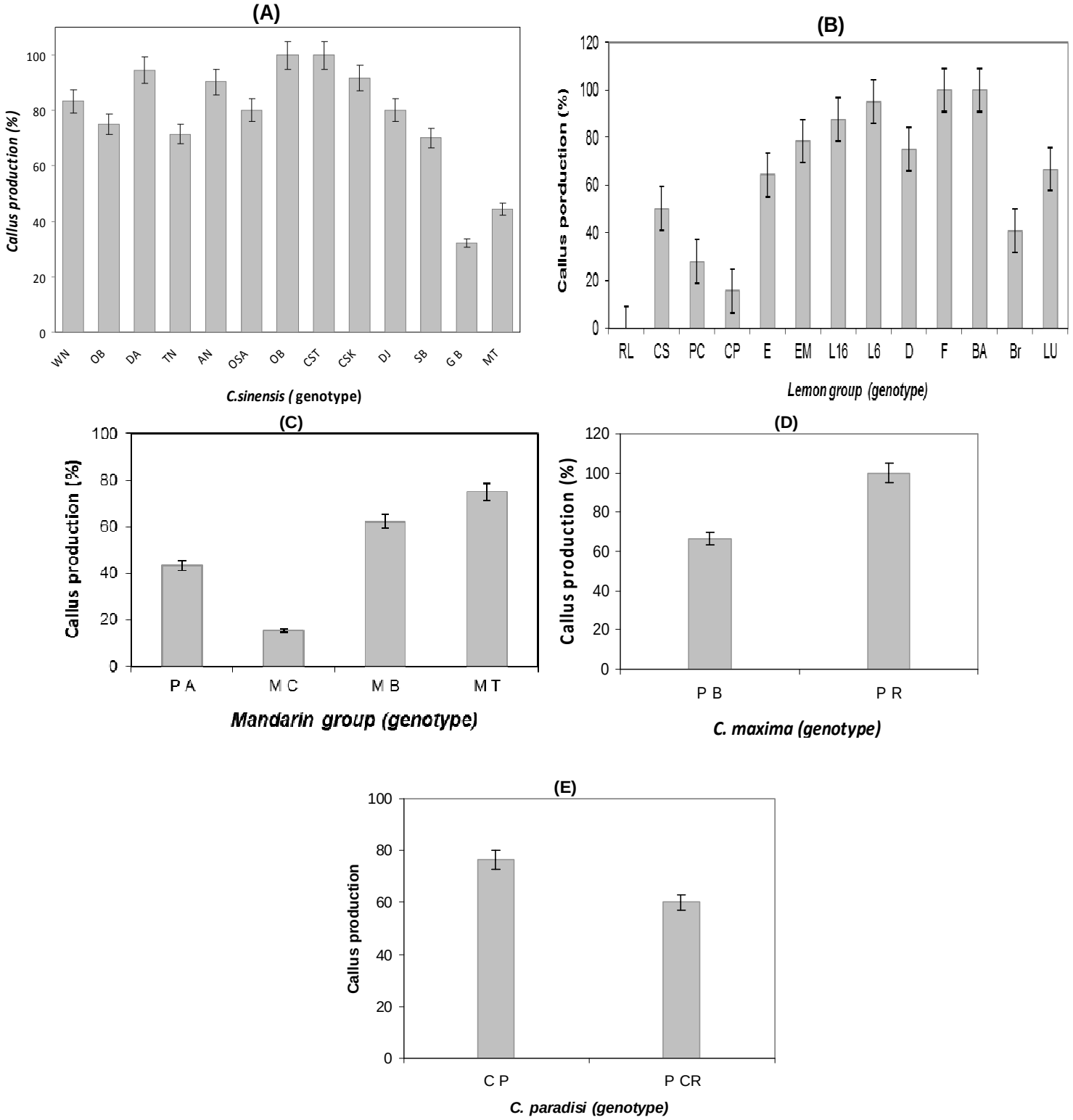


**Figure 2.** Percentage of callus induction from *in vitro* stigma/style culture of different *Citrus* species after 15 days from the beginning of the experiments. Vertical bars represent standard error of the means. **(A)** *C. sinensis*: WN, Washington navel; OB, Oranger de Bey; DA, Double fine Améliorée); TN, Thomson navel; AN, Alger navel; OSA, Oranger Sidi Ali; OB (Oranger de Blida); CST (Shamouti de station); CSK, Shamouti de Skikda; DJ, Djirid; SB, Sanguine de Biskra; GB Golden bucky; MT, Maltaise de Tunisie. **(B)** Lemon group; RL, Rough lemon; CS, C. Sécile; **PC,** Poire de commandeur; **CP,** Citronnier Palermo; E, Eureka; EM, Eureka Maroc; L16, Lisbon 16; L6, Lisbon 6; D, Dellys; F, Femminello; BA, Béni Abbes; Br, Bornéo); LU, Lunario. **(C)** Mandarin group*;* PA, Pam Américain; MC, Madarine Cléopatre; MB, Mandarine de Blida; MT, Mandarine Temple. **(D)** *C. maxima*; PB, Pomelo Ruby; PR, Pomelo Royal. **(E)** *C. paradisi;* CP, Pamplemoussier commun; P CR, Pamplemoussier à chair rose.

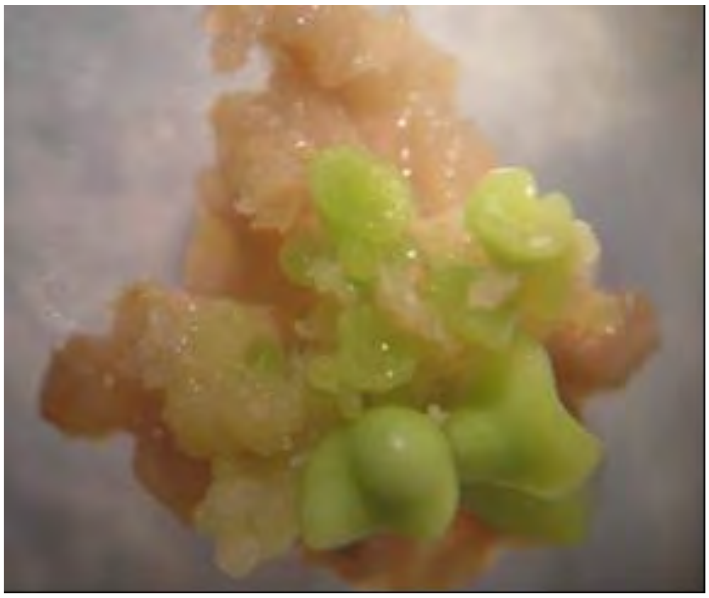

**Figure 3.** Embryos formed from *C. limon* (Dellys) stigma/style cultured on MS medium supplemented with BA. Picture was taken 60 days from the beginning of the experiment.

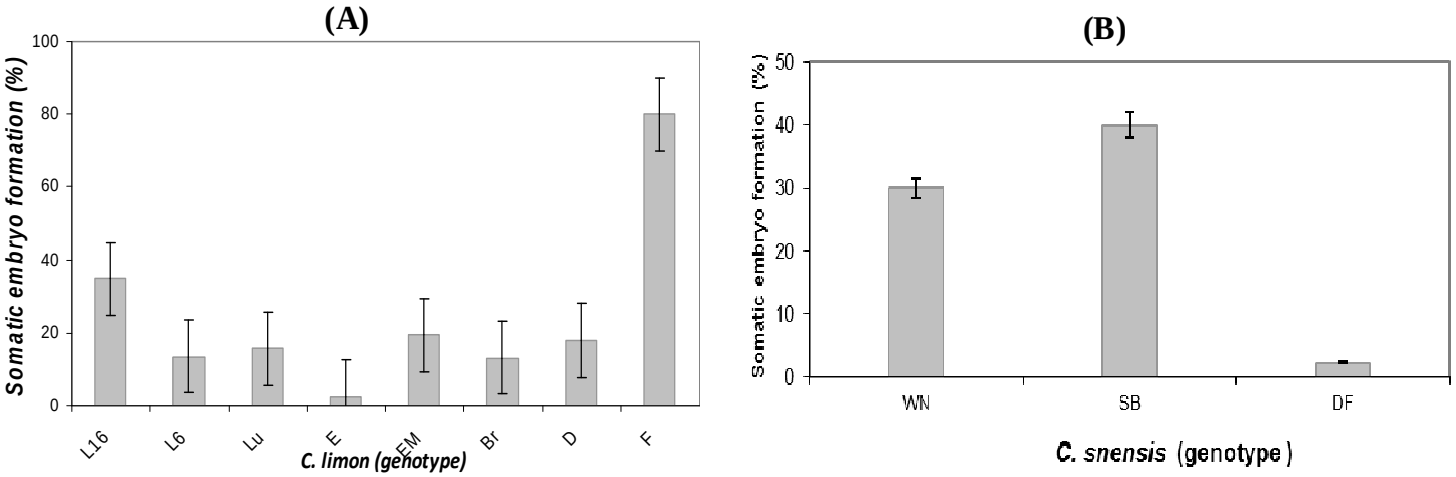


**Figure 4.** Percentage of somatic embryos formation from stigma/style explants after 5 months of culture of different *Citrus* species**.** Vertical bars represent standard error of the values. (**A)** *C. sinensis*: L16, Lisbon 16; L6, Lisbon 6; LU, Lunario; E, Eureka; EM, Eureka Maroc; Br, Bornéo; D, Dellys; F, Femminello; (**B)** *C. limon*: WN, Washington navel; SB, Sanguine de Biskra; DF, Double fine Améliorée.

navel' (30%) and in the local sweet orange 'Sanguine de Biskra' (40%). Different from the behaviour of other *C. sinensis* genotypes, embryogenesis in 'Double fine améliorée' occurred first at 47 days after culture initiation but with the lowest percentage of somatic embryos formation (2.4%). The potential embryogenic rate of other *Citrus* species such as *C. maxima* pomelo 'Ruby' (10%) was lower but consistent and very important; to the best of our knowledge this is the first report on somatic embryogenesis for this genotype. The attempts to induce somatic embryogenesis in all explants of *C. reticulata* and *C. paradisi* genotypes were unsuccessful. However, D'Onghia et al. (2000) reported that mandarin and grapefruit showed a lower percentage of embryo regeneration.

## Germination of somatic embryos and *in vivo* transfer of regenerated plants

After embryos differentiation occurred at the callus surface, they were transferred to germinate in Petri dishes containing hormone-free MS solid medium. Even the rate of germinated embryos varied according to

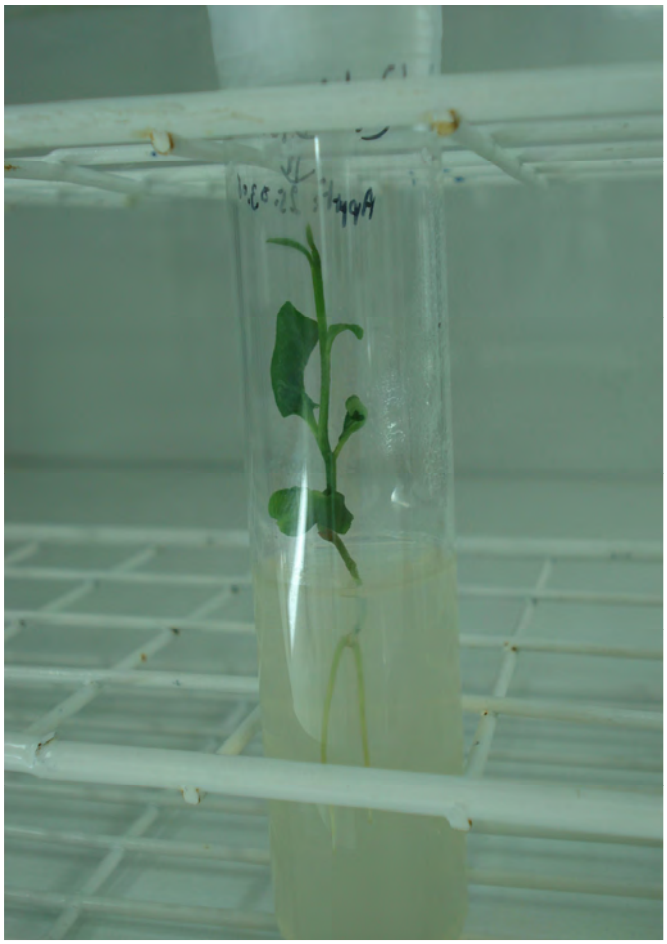

**Figure 5.** Plantlets regenerated *in vitro* from germinated somatic embryos of *C. limon* (Dellys). Picture was taken 30 days after transfer in test tube.

species/genotypes; in fact, the frequency of the germinated embryos into plantlets was higher in lemon than in sweet orange. Similar results (80 and 58%, respectively) have been. The regenerated plantlets were then transferred in the test tubes for 2 months before the *in vivo* transfer phase (Figure 5). All grafting into sour orange seedlings succeeded in the acclimatization phase. Regenerated plantlets will be investigated by DNA and flow cytometry analyses for somaclonal variation stability (Carimi et al., 2007).

## Sanitary assays

Most of the studied genotypes showed to be infected by one or more virus infections, primarily by *Citrus exocortis viroid* and hop stunt viroid (the agent of *Citrus cachexia*). Serological and molecular assays conducted on the regenerated plants from different embryogenic events showed no infection by the agents which were detected in the original source.

## Conclusion

Preliminary results of this investigation indicated that SE using stigma and style culture has been successfully applied to regenerate different genotypes of the main *Citrus* species grown in Algeria. Since the tested genotypes were local or international varieties grown in the country for a long time, the successful results obtained under Algerian conditions represent the very first report. Callus formation rates varied widely among 34 genotypes belonging to 7 *Citrus* species (*C. sinensis, C. limon, C. reticulata, C. paradisi*, *C. reshni*, *C. jambhiri* and *C. maxima*) but only 14 genotypes were regenerated by SE from stigma and style culture. A different regeneration potential was observed in different genotypes within the same species. In accordance with previous works, genotypes belonging to lemon and sweet orange species were successfully regenerated. Very promising results were obtained with the Algerian genotypes such as *C. sinensis* 'Sanguine de Biskra'and *C. limon* 'Dellys'. On the other hand, SE rate of pomelo 'Ruby' was lower but anyway consistent considering that this is the first report of regeneration of this genotype through this technique. All somatic embryos converted to plantlets were free of the infections assessed in the mother trees. Regenerated plantlets will be investigated by DNA and flow cytometry analyses for somaclonal variation.

Further studies need to be carried out in order to investigate the embryogenic potential of other citrus genotypes of economical importance for the Algerian citriculture. Moreover, the successful and easy application of this technique under Algerian conditions showed that SE could be largely applied not only for *Citrus in vitro* conservation but also for the production of healthy citrus plants to start up the citrus certification program in the country.

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