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**Adventitious organogenesis induced in sweet orange (*Citrus sinensis* L.) var. “half-blood” maltese: morphogenetic and histological study**

**Kaouther Benmahmoud<sup>1\*</sup>, Emna Jedidi<sup>2</sup>, Asma Najar<sup>1</sup>, Rachida Ghezal<sup>2</sup>, Claire Kevers<sup>3</sup>, Ahmed Jemmali<sup>1</sup>, Nadhra Elloumi<sup>2</sup>**

*<sup>1</sup>Laboratory of plant protection, National Institute of Agronomic Research of Tunisia, Tunisia*

*<sup>2</sup>Laboratory of horticulture, National Institute of Agronomic Research of Tunisia, Tunisia*

*<sup>3</sup>Plant molecular Biology and Biotechnology, University of Liege, Belgium*

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**Abstract**

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Tunisian citrus crops are faced to several abiotic and biotic constraints among which virus and virus-like diseases are incurable. The production of virus-free plants systematically needs the use of in vitro techniques. In this context, somatic embryogenesis and further plantlet regeneration of the Tunisian “half-blood” Maltese orange were obtained using explants consisting in style/stigma collected from unopened flowers. Somatic embryos were induced on Murashige and Skoog medium containing 13.3  $\mu$ M 6-benzylaminopurine and 500 mg.l<sup>-1</sup> malt extract, but their germination was obtained on hormone free-medium. Somatic embryogenesis was induced indirectly from intermediate friable callus initiated at the basal part of the style. Somatic embryos exhibited central procambial cells and were surrounded by a protoderm isolating them from the callus. These embryos had bipolar structure confirmed by the presence of shoot and root apices at cotyledonary stage. The use of cotyledon excised from those embryos failed to regenerate somatic embryos, but gave rise to direct organogenesis in two forms, true buds and protuberances both evolved in shoots after transfer in hormone-free medium. According to histological observations, protuberances are induced from epidermal and subepidermal cells of the cotyledon explant and remain closely attached to their mother tissue even at the shoot stage.

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**\*Corresponding Author:** Kaouther Benmahmoud ✉ [benmahmoud\\_k21@yahoo.fr](mailto:benmahmoud_k21@yahoo.fr)

## Introduction

*Citrus* cultivation is an important agricultural sector in Tunisia that covers about 21 thousands hectares with 6,4 millions trees. Annual production is estimated to approximately 220 thousand tons. The main varieties actually cultivated in Tunisia are Maltese, Navels, Lemon and Clementine (Lebdi Grissa, 2010). Unfortunately, this sector is facing several constraints including viral and viral-like graft-transmissible diseases such as *Citrus Tristeza Virus* (CTV), *Citrus Psorosis Virus* (CPsV), *Citrus Exocortis Viroid* (CEVd), *Citrus Cachexia Viroids* (CCaV). These diseases may significantly cause considerable losses in crop yield and quality.

In order to stop or at least reduce spreading of these viral diseases, a national program has been held since 1990ies based on an integrated strategy including surveys in citrus orchards, installation of aphid traps and sampling for laboratory viral diagnosis (Lebdi Grissa, 2010). Concomitantly, our lab contributed with *in vitro* micrografting of shoot apices as a tool for virus elimination (Navarro *et al.*, 1980) for the main Tunisian citrus varieties. Although, this technique presents some disadvantages related to the difficulties to eliminate some viruses (Carvalho *et al.*, 2002). Looking for other alternative techniques, researches proposed somatic embryogenesis from different floral parts (Carimi *et al.*, 1994; Carimi *et al.*, 1998; Miah *et al.*, 2002; Carimi *et al.*, 2005), but style and stigma gave better results and became more and more useful for their specific advantages concerning sanitation and juvenility traits (D'Onghia *et al.*, 2000, Meziane *et al.*, 2012).

Genotype predisposition for SE remains an essential factor for the success of a such technique. This why, the present work was focused on the study of the ability of our target variety "half-blood Maltese" to respond to the SE process from style/stigma explant. This ability tested with the same SE protocol proposed by Carimi *et al.* (1994), has been explored at the morphological and the anatomical levels. Such approach will allow us to state on the usefulness of the adopted protocol for an optimal regeneration that

may efficiently contribute to the sanitation program cited above.

## Materials and methods

### *Plant material*

Unopened flowers were collected from the sweet orange (*Citrus sinensis* L.) var. "half-blood" Maltese trees cultivated in a citrus experimental field (located in the Northern-East region of Tunisia) belonging to the National Institute of Agronomic Research of Tunisia. They were surface-sterilized by immersion for 5min in 70% ethanol and 15 minutes in 2% sodium hypochlorite, followed by three 5-min rinses in sterile distilled water.

In a first step, styles and stigmas were used as explants. They were aseptically excised from flowers and placed vertically in Petri dishes (90x15mm) containing 25 ml of culture medium. Five explants were placed in each dish with the cut surface in contact with medium.

In a second step, aseptic cotyledons of previously regenerated somatic embryos, served as explant material for the same purpose.

### *Media and culture conditions*

#### *Culture of style/stigma explants*

Explants consisting in style/stigma were cultured on a semi-solid MS (Murashige and Skoog, 1962) medium supplemented with 146 mM sucrose, 500 mg.l<sup>-1</sup> malt extract and 13.3 µM BAP (6-benzylaminopurine), and solidified with 7 g l<sup>-1</sup> agar (Sigma). The pH was adjusted to 5.7 with 0.1 M KOH before autoclaving at 120°C for 30 min. Cultures were held in a growth chamber at 25 ± 2°C under 16h/8h photoperiod and photon flux of 4000 Lux provided by OSRAM Daylight lamps. Cultures were transferred on the same medium every 4 weeks.

Regenerated somatic embryos were either transferred into germination medium or used as a source of cotyledon explants.

### Germination of somatic embryos

Germination was realized in the same medium without growth regulators.

### Culture of excised cotyledon explants

Cotyledons of well developed somatic embryos regenerated from style and stigma explants, were excised and used for the induction of embryogenesis. Cotyledons were cut into segments and cultured in the same induction conditions applied to style/stigma explants.

### Morphological observations

Cultures were periodically observed under stereomicroscope (LEICA MZ6) in order to check the different morphological changes that occurred during induction phase and subsequent developmental stages. Numerical photographs were made to illustrate those changes with a digital camera (Canon S50).

### Histological observations

Samples used for the morphological observations served for the histological investigations to describe the

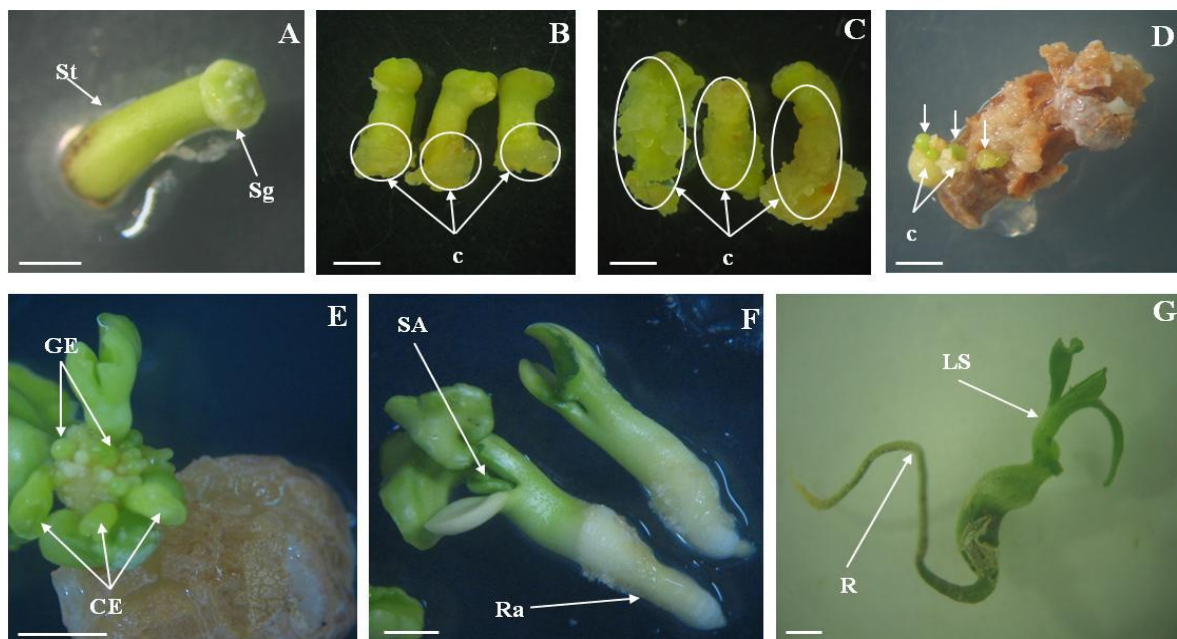
concomitant anatomical events subtending the morphological features. Samples were fixed overnight in FAA (formaldehyde: acetic acid: ethanol, 10:5:85) for 24 hours. The following steps of histological protocol consisted in dehydration through a graded ethanol-xylol series and embedding in paraffin wax. The samples were carefully oriented in the molds to facilitate subsequent longitudinal sectioning. Afterwards, 8  $\mu\text{M}$  sections were made with a rotary microtome (RM2125RT) and double-stained with hematoxylin (Regaud) for 15 min and safranin (Riedel-de Haën) for 24 hours. Slides were viewed with an optical microscope (Leica DMLB2) and photographed using the same digital camera (Canon S50).

## Results

### Morphological observations

#### Somatic embryogenesis from style/stigma explants

The described process of SE on style/stigma explants of "half-blood" Maltese sweet orange has been performed on MS semi-solid medium supplemented with 13.3  $\mu\text{M}$  BAP and 500  $\text{mg l}^{-1}$  malt extract.



**Fig. 1.** Sequential stages of indirect somatic embryogenesis from style/stigma explants of "half-blood" Maltese sweet orange cultured on BAP-enriched MS medium. (A) Swelling of style (St)/stigma (Sg) explants after a week of culture. (B) Friable callus (c) appeared at the basal part of the style after ten days. (C) Proliferation of callus along the style axis. (D) Green globular somatic embryos (SE) produced on callus after four months. (E) Coexistence of globular (GE) and cotyledonary (CE) embryos. (F) Germinated embryos showing cotyledons (Ct), shoot apex (SA) and radicle (Ra). (G) Plantlet showing leafy shoot (LS) and root (R). Scale bar=0,5cm.

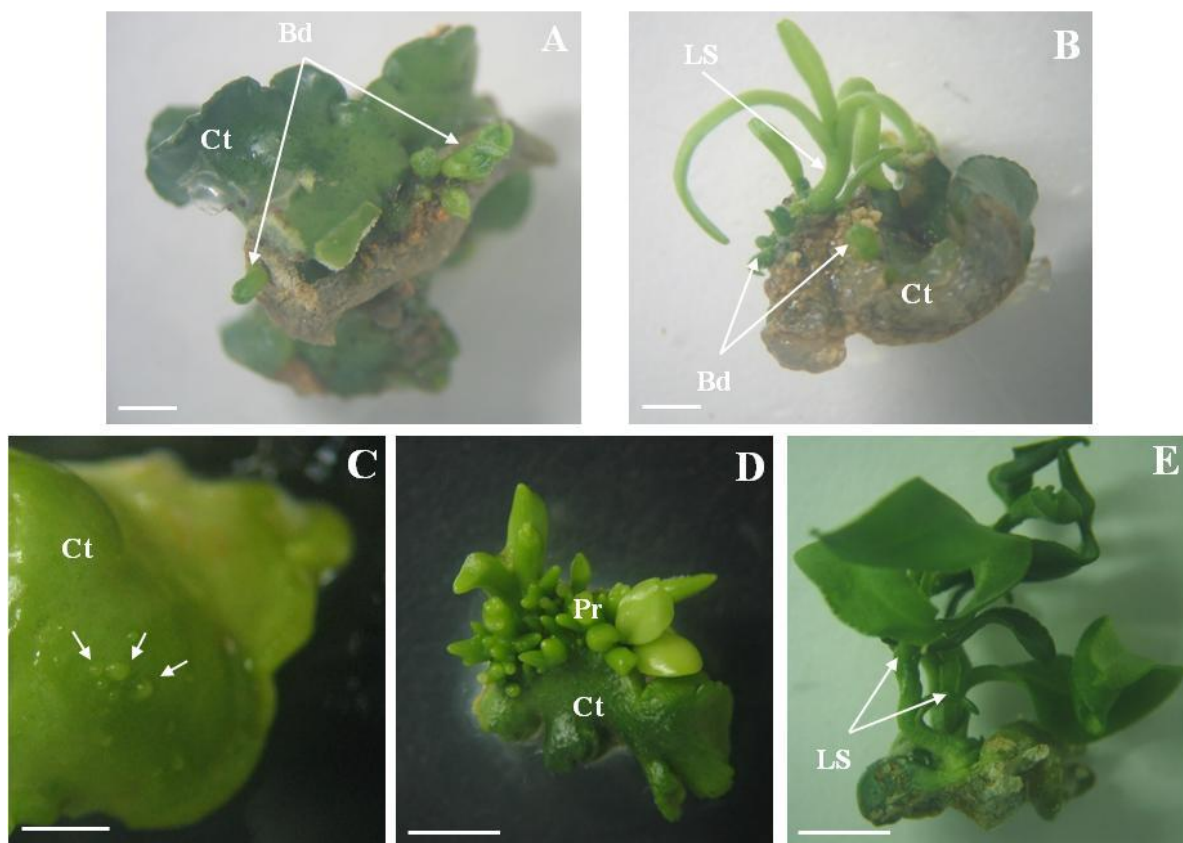
The main steps of this process were illustrated in Fig. 1. The first morphogenetic change consisting in a swelling of explants was observed after a week of culture (Fig 1A). After about 10 days, friable white callus appeared at the basal part of the style (Fig 1B). In some cases, callus proliferated along the style axis and might invade it (Fig 1C).

Somatic embryos became visible after approximately 4 months of culture. They consisted in small and green globular structures corresponding to the globular stage (Fig 1D). Later, clusters of somatic embryos ranging from globular to cotyledonary ones may be observed on callus (Fig 1E). Mature embryos, easily separated from the callus, showed well developed roots and shoot apices (Fig 1F). Those germinated embryos converted into plantlets when

transferred to hormone-free medium (Fig 1G). This embryogenic process took nearly 5-7 months to achieve the whole developmental steps.

#### *Organogenesis from cotyledon explants*

Excised cotyledons from the previous somatic embryos were cultivated adaxial surface in contact with medium. Unlike style/stigma, these explants showed a direct organogenesis (Fig. 2) consisting in true buds on the injured side (Fig 2A) or in globular protuberances on the abaxial surface (Fig 2C) that started emergence within the first four weeks of incubation without intervening callogenesis. Buds regularly grew into normal leafy shoots (Fig 2B) but globular protuberances became elongated (Fig 2D) before differentiating into normal shoots that remained closely attached to the mother tissue (Fig 2E).



**Fig. 2.** Direct organogenesis from the injured side (A, B) and from the upper epidermis (C, D, E) of somatic embryo-excised cotyledon (Ct) explants of “half-blood” Maltese sweet orange cultured on BAP-enriched MS medium. (A) Emerged young buds (Bd). (B) Coexistence of young buds (Bd) and leafy shoots (LS). (C) Small and green globular protuberances (arrows). (D) Evolution of globular protuberances (Pr) into elongated ones. (E) Normal leafy shoots (LS) issued from protuberances. Scale bar=0,5cm.

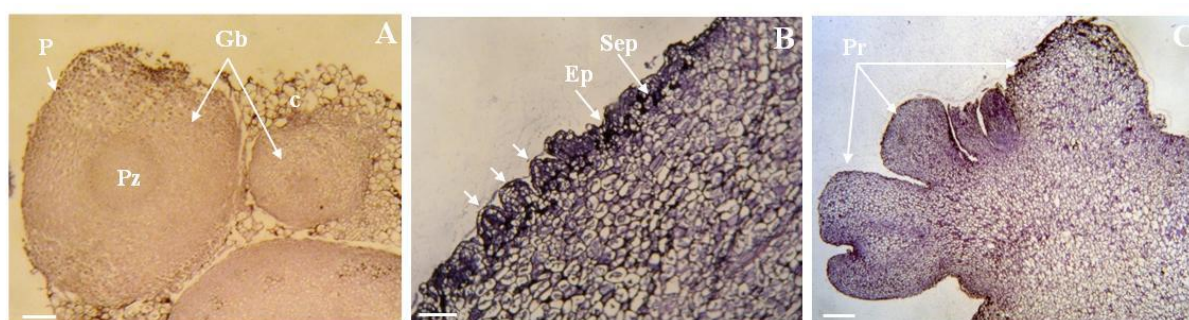


### Histological analysis

Histological observations were used to illustrate the main anatomical events that occurred during induction phase of embryogenesis and organogenesis respectively from style/stigma and excised cotyledons (Fig. 3).

In the case of style/stigma explants, an intermediate callus was developed before embryo induction, which then confirmed the indirect form of embryogenesis (Fig 3A). This last fig. concerning globular embryos, shows protoderm and procambial zone in the central core of the embryo.

When excised cotyledon explants were concerning, histological analysis was focused on protuberances regarding their intriguing developmental feature. Longitudinal sections showed that globular protuberances directly arose at the expense of epidermal and subepidermal cells (Fig 3B). The fact that these cells are densely stained is a synonym of their active division normally due to a dedifferentiation program that took place at this region of the cotyledon parenchyma.



**Fig. 3.** Histological sections on embryogenic callus induced from style/stigma explant (A) and on somatic embryo-excised cotyledon explant (B, C) of half-blood Maltese sweet orange cultured on BAP-enriched MS medium. (A) Indirect somatic embryos at globular stage (Gb) showing central procambial zone (Pz) and protoderm (P). (B) Protrusion of small protuberances (arrows) from epidermal (Ep) and sub-epidermal (Sep) cells of the cotyledon. (C) Elongated protuberances (Pr) with highly stained cells. Scale bar=300µm.

The evolution of those protuberances resulted in the edification of bigger structures among which, some resembled to embryos, but remained largely attached to their mother tissue (Fig 3C).

### Discussion

This paper described morphological and histological organogenic events that occurred during induction of organogenesis in "half-blood" Maltese sweet orange from different explants cultured on BAP-enriched medium. It is well reported that in vitro organogenesis in citrus is enhanced by cytokinin supplementation (Moreira-Dias *et al.*, 2000, Schinor *et al.*, 2011). The beneficial effect of cytokinin (BAP) reported in the induction of shoot bud primordia in different plant species may be due to an enhanced synthesis of nucleic acid and proteins required for organogenesis (Saini *et al.*, 2010).

In a first experiment, style/stigma excised from unopened flowers were used as explants to induce SE on MS culture medium containing 13.3 µM BAP, as it has previously been recommended for many citrus genotypes (De Pasquale *et al.*, 1994, Carimi *et al.*, 1998, 1999). The embryogenic process occurred indirectly on a friable callus initiated at the basal part of the style. The different sequential phases leading to the formation of somatic embryos took approximately 5-7 months. Normally germinating embryos (with caulinar and radicular apices) evolved into plantlets when transferred to hormonal-free medium.

The second experiment was based on the use of cotyledon excised from the previous embryos. Cotyledons of either zygotic (Rodriguez and Wetzstein, 1998, Tang *et al.*, 2000, Zhou and Brown, 2006) or somatic (Puigderrajols *et al.*, 2000, Zhou

and Brown, 2006) embryos, served as efficient source for in vitro plant regeneration of many species. Our explants, cultured on BAP-containing medium, reacted within one month by direct organogenesis (buds and protuberances) without intermediate callogenesis. This result corroborates those reported on the role of BAP in citrus regeneration (Saini *et al.*, 2010, Lombardo *et al.*, 2001, Schinor *et al.*, 2011).

Histological investigations were especially carried out on the protuberances because of their resemblance to the above-described embryos at the globular stage. The anatomy of these protuberances revealed their direct and superficial origin from epidermal and subepidermal cell layers of the cotyledon. Nevertheless, their tight insertion in the mother tissue and the absence of entirely developed protoderm allowed us to discard the hypothesis of their embryogenic nature. Comparable ambiguity between globular protuberances and embryos has been previously reported in the case of *Pelargonium x hortorum* (Haensch, 2004) and *Linum usitatissimum* (Salaj *et al.*, 2005).

In conclusion, our ultimate objective was to produce somatic embryos (SE) for citrus virus sanitation program. For this purpose, style/stigma explants have been successfully used in regenerating SE on BAP-enriched MS medium. Under the same conditions, the use of SE excised cotyledons failed to induce further somatic embryogenesis, but it led to direct generation of buds and protuberances. In all the cases, plantlets regeneration was possible.

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