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Original Research

Callogenesis and Plant Regeneration of Sweet Orange cv. Washington Navel from Floral Organ Cultures

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ABSTRACT

Washington Navel orange (*Citrus sinensis* L.) can be infected with virus and virus like diseases that affect not only the production but also fruit quality and the plant's longevity. For viral sanitation, Washington Navel regeneration was investigated *in vitro* via floral organ culture. Flowers were collected before opening from healthy Washington Navel trees kept under greenhouse. Floral organs (style/stigma and ovary) were cultured on Murashige and Skoog (MS) medium containing various plant growth regulators combinations of naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP). The highest rate of callogenesis (95%) was obtained from style/stigma explant cultures on MS medium enriched with 3 mgL^{-1} BAP, which also resulted in 100% rooted plantlets. Ovary cultures did not show any success on the culture medium with various plant growth regulators combinations. The acclimatization success of rooted plantlets by grafting on *Citrus volkameriana* rootstocks was about 83%. Thus, these results can be used for mass production of disease-free citrus plants and improve sanitation program of the local citrus genotypes in Tunisia.

Keywords: *Acclimatization, explant, in vitro, growth regulators, organogenesis, viral diseases.*

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INTRODUCTION

Tunisian citrus industry is of paramount importance in the national economy. The area under citrus in the country was estimated to be about 27,350 hectares with a total production of 440,000 tons during 2019. Washington Navel sweet orange in Tunisia is ranked at the second position with an average production of 110 thousand tons, preceded by the Tunisia's Maltese with 135 thousand tons (GIF, 2019). Besides other production issues, Washington Navel orange orchards suffer from numerous diseases especially of viral etiology (such as psorosis, Tristeza etc.) that continuously affect not only the production, but also the fruit quality and the tree longevity (Navarro, 1993). The use of healthy propagating materiel is of utmost importance in the control of these diseases. Therefore, a clean stock program has been established since 1994 in Tunisian using shoot tip grafting as described by Juárez et al. (2015). This technique has enabled the sanitation of the most important local cultivars and the production of thousands of healthy certified plants. Thermotherapy and shoot tip grafting used alone or in combination ensure the eradication of some citrus viruses such as citrus psorosis virus (CPsV) (Roistacher, 1993; Achachi et al., 2014). However, according to Roistacher (1993), the percentages of virus removal through these techniques vary from 70 to 80%. For organogenesis and somatic embryogenesis, floral organs provide the highest potential for the elimination of viruses and demonstrated its efficacy in the *in vitro* propagation of virus free citrus species (D'Onghia et al., 2000; Meziane, 2006).

The present investigation was undertaken to develop firstly an *in vitro* protocol for organogenesis induction and plant regeneration from style/stigma of Washington Navel orange (*Citrus sinensis* L. Osb.) using different combinations of plant growth regulators for mass production of disease-free plants and the improvement of existing elite cultivars.

MATERIALS AND METHODS

Unopened flowers were collected from the mother plants maintained in a greenhouse of the Technical Center of Citriculture of Tunisia (CTA/Unit of the Citrus Sanitation of Manouba).

Preparation of floral explants

As described by D'Onghia et al. (1997) and El-Sawy et al. (2013, 2014), unopened citrus flowers were surface-sterilized with

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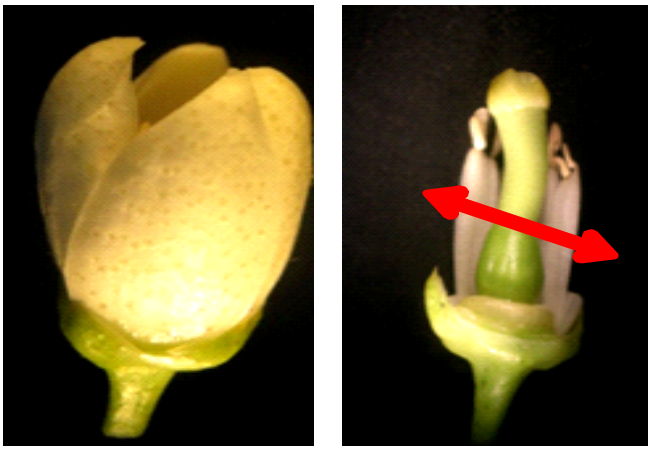

Figure 1: Citrus flower (left); style, stigma and ovary (right).

Figure 2: Different steps of rooted *in vitro* plantlets acclimatization: A. Acclimatization in pots; A1, transplantation of rooted plantlets in pots (plr); A2, covering pots by polyethylene bags (plr). B. Acclimatization uses a cleft graft; B1, downward sloping cut on both sides of the basal end of the scion to form it into a wedge; B2, wedge inserted in the made split on the rootstock. B3; covering of grafted plantlets (plr) with a plastic bag (sp.); C. Acclimatization by grafting on a rootstock *C. Volkameriana*; C1, grafting of rooted *in vitro* plantlets (plr); C2, Grafted plantlets (pg) and wrapped; C3, covering of grafted plantlets (gr) with a plastic bag (sp) ; C4, gradually removing bags when new leaves appear (4-5 leaves) (plgr).

Table 1: Concentrations of growth regulators added to the MS medium.

Reference Medium	NAA (mgL^{-1})	2,4-D (mgL^{-1})	BAP (mgL^{-1})
MS1	4	0	0
MS2	5	0	0
MS3	0	4	0
MS4	0	5	0
MS5	0	0	2
MS6	0	0	3
MS7	4	0	2
MS8	0	4	2
MS9	4	0	3
MS10	0	4	3

water added with Tween 20, then submerged in 70% (v/v) ethanol for 5 minutes, then immersed for 15 minutes in 30 per cent Clorox with Tween 20. The flowers were then opened under sterile conditions, and style/stigma and ovary were dissected with a scalpel.

Style/stigma and ovarian sections taken as explants were cultivated on the basic culture medium i.e. Murashige and Skoog (1962) (MS) supplemented with 10 different hormonal combinations of naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl-aminopurine (BAP) (Table 1). In the experiment, 6 organs (style/stigma or ovary) (Fig. 1) were placed in each Petri dish filled with 25 mL of medium and there were 12 replicates (petri dishes) for each treatment. The regenerated explants were sub-cultured on a fresh medium at 4 to 6 weeks and were maintained under the described culture conditions.

Emerged calli carrying shoots were transferred into plant growth regulator-free MS medium solidified with 6 g L^{-1} Agarose. Then, successfully rooted plantlets (3 to 5 months culture) were individually transferred into test tubes containing macronutrients and micronutrients diluted six times (1/6) MS medium free of plant growth regulators.

Culture conditions

Petri dishes were incubated at 25 ± 2 °C under 16 hours day length with illumination of 1000 lux (12 $\mu mol.m^{-2}. s^{-1}$) supplied by fluorescent lamps Mazda Fluor, daylight. These conditions were maintained throughout the entire experiment including the induction, the development and implantation phases .

Acclimatization

Well-developed rooted seedling (about 5 cm in length) were collected and transferred to the glass greenhouse for acclimatization following three techniques as given below.

1. **Direct acclimatization of seedlings in pots (Fig. 2A):** Rooted plantlets were washed thoroughly and transplanted in a greenhouse in 12 cm pots containing a mix of sand: peat moss: crushed oak-leaf (1:1:2). The pots were covered with polyethylene bags and then gradually removed at the appearance of new flushes.
2. **Acclimatization of cleft grafted plants (Fig. 2B):** The first

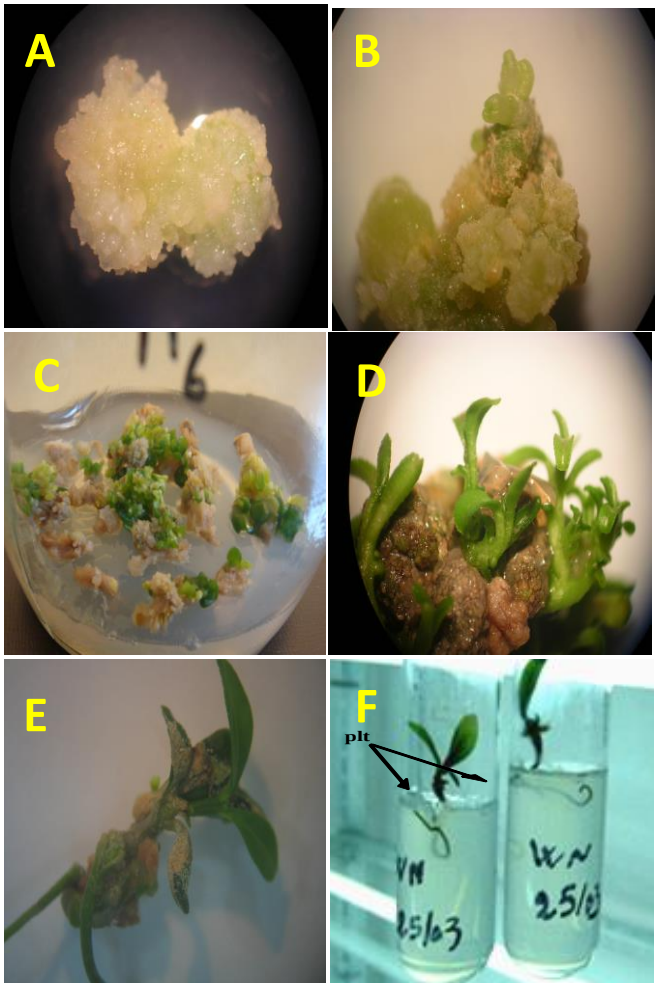

Figure 3: Regeneration of rooted plantlets from a Washington Navel orange (*Citrus sinensis* L.) callus. A. Callus induction from style/stigma explants on MS medium supplemented with 2-3 mgL^{-1} BAP; B. Initiation of shoot buds; C. Vigorous adventitious shoots development; D. Shoots elongation; E. Rooted plantlets 3 months after transferring on 1/6MS medium; F. Individual culture of rooted plantlets on solid medium.

step was to decapitate the *C. volkameriana* stock target stem. Then, a wedge or cleft was made on the cut end into which the scion was inserted. Using the sharp knife, sloping cuts on both sides of the basal end of the scion was made to insert into wedge/cleft on the rootstock. The grafted area was carefully wrapped with polyethylene sheet and bagged to ensure humidity. Bags were gradually removed when new leaves appeared.

3. **Acclimatization of T-grafted plants (Fig. 2C):** The rooted *in vitro* plantlets were grafted on *C. volkameriana* (8-10 months old) rootstock seedling under glass greenhouse to produce fast growth of grafted *in vitro* plantlets (Metoui et al., 2014). The rootstock shoots were bent at least one week before grafting to overcome apical dominance and prevent further growth of the graft. A T-shaped incision was made on the target rootstock shoot. A downward sloping cut on both sides of the basal end of the scion was made to insert in the T-shaped incision and then wrapped with parafilm. Grafted plantlets were also covered with plastic bags to ensure humidity. Bags were gradually removed when new leaves appeared.

Data analysis

Values of [number of calli/explant] were recorded monthly for eight months as reported by Carimi et al. (2004). The variance of analysis with two factors: (i) the nature of the explants (style/stigma and ovary) (ii) the hormonal composition of culture medium was adopted. Differences among means were tested by Student-Newman-Keuls range test ($p<5\%$). The percentage of rooted and successfully acclimatized plantlets were also evaluated periodically for three months.

RESULTS

The percentage of induced calluses was determined by the number of calli appearing for each cultured organ explant. Only "Organs/Medium" combinations showed results have been considered and discussed in this paragraph. The analysis of variance showed high dependence of produced calli on medium and organ.

Calli were induced at a higher rate (Fig. 3A) from floral organs with 3 specific plant growth regulator combinations with 2 and 3 mgL^{-1} of BAP and free of 2,4-D components. The maximum callogenesis (95%) was observed for style/stigma explants cultured on MS6 medium after 3 months. Percentages of calli were lower for MS5 (38%) and MS7 (35%) media (Table 2). A high level of shoot initiation (Fig. 3B) was achieved on the medium containing BAP with 71% on MS6, 29% on MS5 and 21% on MS7. All appeared shoots (Fig. 3C) after growth (Fig. 3D) and elongation (Fig. 3F), rooted 100% on MS/6 medium. Ovary cultures did not show success on the 10 described plant growth regulator treatments.

For the acclimatization, only the third technique by rootstock (8-10 months-old) graft gave good results as 83%, 78% and 48% with scion shoots regenerated on medium MS6, MS5 and MS7, respectively (Table 2).

DISCUSSION

Table 2: Response of explants (style/stigma) of Washington Navel orange to the different concentrations of growth regulators for organogenesis after 15 months.

Culture medium	Callus induction (%)	Shoots (%)	Rooted plantlets (%)	Acclimatized plantlets (%)
MS5 (2 mgL^{-1} BAP)	38^{b}	29^{b}	100^{a}	78^{a}
MS6 (3 mgL^{-1} BAP)	95^{a}	73^{a}	100^{a}	83^{a}
MS7 (4 mgL^{-1} NAA + 2 mgL^{-1} BAP)	35^{b}	21^{b}	100^{a}	48^{b}

*MS: Murashige and Skoog (1962) medium (MS5, MS6, MS7)

*Values followed by the similar letters were not significantly different by Student-Newman Keuls test at ($p<5\%$) level.

This study pointed out the ability of in *vitro* organogenesis to regenerate whole Washington Navel orange plantlets from style/stigma explants. It was based on a specific combination of the organ nature and medium composition. These results are consistent with previous reports on seedless sweet orange (*Citrus sinensis* L. Osbeck) (Cardoso et al., 2017), *Citrus sp.* (Schinor, 2006), Carrizo citrange rootstock (Moreira-Dias et al., 2000; Germanà et al., 2011), Navel orange and lemon (D'Onghia et al., 2000), Troyer citrange (Benyahia et al., 2011) and commercial citrus rootstocks (*Poncirus trifoliata*, *Citrus aurantium* and Swingle citrumelo) (Tzatzani et al., 2018). Thus, the success of citrus organogenesis is closely related to the hormonal composition of the culture medium (Khan et al., 2009), and explant types (Moreira-Dias et al., 2001; Costa et al., 2004; Khan et al., 2009).

In this research, only style/stigma explants showed good callogenic potential as previously reported by Miah et al. (2002) for *Citrus* varieties. It showed the highest rate of plantlets regeneration and acclimatization success (up to 83%). In ovary explants no callogenesis was observed. Further, according to this study, the supply of BAP in the medium appeared necessary for the shoot bud emergence on the calli surface as reported by Tavano et al. (2009). DNA markers and flow cytometry analyses of regenerated plantlets can investigate any somaclonal variability (Carimi et al., 2007). Only plants identical to the respective mother plant can be used and kept under greenhouse as healthy genotypes.

The optimization of the organogenesis protocol and the regeneration of citrus plantlets are considered important as it ensures a secure exchange of healthy germplasm with less risk of contamination and the conservation of local varieties (D'Onghia et al., 2000). It also allows for a high frequency of plant regeneration particularly for many of the economically important *Citrus* species (Chamandoosti, 2017). These results may also be used for the establishment of the regeneration process using somatic embryogenesis by the floral organ culture and sanitation of the local Tunisian citrus genotypes. Style/stigma somatic embryogenesis is considered one of the most effective methods for eradicating major citrus virus and virus-like diseases (Meziane et al., 2012).

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