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Efficient protoplast isolation from ovule-derived embryogenic callus in *Citrus volkameriana*

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Abstract: The present study reports on the isolation of viable protoplast from ovule-derived embryogenic calli of Volkameriana (*Citrus volkameriana* L.), which is a rootstock in high demand for lemon production.Ovules of *C. volkameriana* isolated at 3 different immature fruit stages, comprising4, 8, and 12 weeks after anthesis (WAA), were cultured on5 different media in order to produce embryogenic callus lines as a source material for protoplast isolation. EME medium (MT basal medium + 0.5 gL^{-1} malt extract), with the addition of phytohormones [kinetin (KIN), 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP)] at different concentrations, were tested for callogenesis. According to 2-way ANOVA, significant effects were determined as a result of the immature fruit stage and type of culture media ($P \leq 0.01$) on the callogenesis and embryogenic callus induction frequency. First, callus formation was recorded after 4 WAA on medium comprising EME + 2,4-D (1.0 mg L^{-1}) + BAP (0.5 mg L^{-1}). Callus induction frequency was the highest (90.00%) in the same culture medium when the ovules wereculturedat8 WAA. In addition, culturing the ovules isolated from 12 WAA immature fruits of *C. volkameriana* resulted in the highest indirect somatic embryogenesis (24%). Embryogenic callus initiation was the highest (25.56%) using EME + KIN (1.0 mg L^{-1}) and ovules cultured at 8 WAA (14%) resulted in the highest embryogenic callus formation. Effects of different enzyme concentrations on the efficiency of protoplast isolation were calculated using the hemocytometer cell counting method. The combination of 2% cellulase and 0.2% pectinase gave the highest numbers of protoplasts, at 12.33×10^5 protoplast/mL. Embryogenic callus lines obtained by culturing ovules of *C. volkameriana* yielded high-quality protoplasts after isolation and could be useful as a protoplast source for further somatic hybridization studies.

Key words: Citrus, rootstock, embryogenic callus, protoplast isolation

1. Introduction

Citrus-producing regions occupy an area of the world that extends approximately 40° north latitude to 40° south latitude from the Equator. The citrus-producing regions of Turkey are located in the northern half of the world's citrus-producing region. Turkey has exceptionally reasonable environmental conditions and citrus-producing potential, where 4,769,726 tons of citrus fruits were produced in 2017[1]. Citrus growing in Turkey is an important source of income, both in the domestic and export markets. Despite its productivity and planted area, citriculture suffers from many abiotic and biotic stress problems that cause economic losses, both before and after harvest.

Rootstocks play an extremely significant part in citriculture. Rootstock selection in citrus production has an important place in production success by helping to mitigate issues of climate, poor soil conditions, diseases, etc. In order to increase the quality and production of fruit crops, early fruit production, consistent crop production, short juvenility period, and controlling the tree size, planting density etc., growers have a wide variety of options whenchoosing citrus rootstocks. In spite the fact that a large number of citrus genotypes can be utilized as a rootstock, only a few are superior and best suited for particular conditions. There is, however, no ideal rootstock for all horticultural benefits, and abiotic and biotic stress conditions. For instance, sour orange (*Citrus aurantium* L.) is tolerant to root rot, calcareous soils, drought, and cold, and produces high yields and high fruit quality. However, it is susceptible to the citrus tristeza virus (CTV). In some countries, the use of this rootstock is declining due to the presence of CTV. However, in the presence of CTV, Volkameriana, rough lemon, and Rangpur lime (*Citrus limonia* L. Osbeck) have been shown to positively affect fruit yield (Cimen and Yesiloglu, 2016).

[1] Food and Agriculture Organization (FAO) (2019). FAOSTAT [online]. Website http://www.fao.org/faostat/en/#data/QC [accessed 08 April 2019].

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The demand for elite rootstock materials is continuously increasing to fulfill resistance/tolerance demands against various abiotic and biotic stress factors. Over the centuries, improved knowledge in genetics has allowed breedersto better manage crosses, to combine the most favorable characteristics. In the process of selection, certain characters have been emphasized over others, which have led to a decrease in other favorable traits. Nevertheless, genetic recombination is limited by reproductive biology and strong heterozygosity in most cultivars and rootstocks (Chetto et al., 2016). The lack of good results through conventional citrus breeding can be explained by various aspects of the biology of the genus *Citrus* and its relatives. The most important of these traits include high heterozygosity, pollen and ovule sterility, sexual incompatibility, nucellar polyembryony, and juvenility (Grosser and Gmitter, 1990).

Hence, plant tissue culture techniques can be applied as a helpful tool to reduce the time for improvement of citrus rootstocks. In comparison, somatic hybridization via protoplast fusion is an alternative to circumvent some of these difficulties, and may pave the way for transferring their resistance genes to citrus. Somatic hybridization through protoplast fusion allows the hybridization of sexually incompatible genera and species, which is often difficult or impossible by conventional breeding (Grosser and Gmitter, 1990; Grosser et al., 1996; Koç et al., 1999). Protoplasts can be isolated from different kinds of tissues, including leaf mesophyll, embryogenic calli, embryogenic suspension cultures, and nonembryogenic calli. Mesophyll protoplasts isolated from citrus leaves cannot divide and regenerate into plants under the currently used culture schemes (XiuXin Deng et al., 1992). However, protoplasts isolated from citrus embryogenic calli can be. Embryogenic calli have been induced, and their protoplast-derived plants have been regenerated from many species of the genus *Citrus* and its related genera (Guo and Deng, 2001; Guo et al., 2013). Somatic embryogenesis has been viewed as one of the most important techniques of plant tissue culture for mass propagation and synthetic seed production. As is well-known, plants can be regenerated from callus cultures via somatic embryogenesis or adventitious shoot organogenesis. Callus is an attractive target explant for studying germplasm and improvement of plant varieties (Cai et al., 2009, 2010). Grosser et al. (1988) reported that protoplasts can be isolated from friable embryogenic ovule-derived calli maintained on growth-regulator-free media. Chamandoosti (2017) reported that the production of embryogenic callus lines in citrus can be produced by the in vitro culture of excised nucelli, abortive ovules, unfertilized ovules, undeveloped ovules, juice vesicles, anthers, styles and stigmas, epicotyls, cotyledons, and root segments.Thus, for researches specifically focused on variety or rootstock improvements using somatic hybridization in citrus, a reliable callus induction protocol is essential. However, the efficiency of existing protocols may vary depending on the genotype used. Some species and varieties have shown to be recalcitrant to in vitro process in citrus, especially genotypes used as rootstocks (Tavano et al., 2009; Schinor et al., 2011). Although the induction of embryogenic callus lines, especially from ovule tissue, has been extensively investigated in citrus, there are only a few reports on embryogenic callus induction and protoplast isolation in recalcitrant rootstocks such as Volkameriana (*Citrus volkameriana* L.). For instance, Ricci et al. (2002) mentioned that the literature was lacking efficient protocols for the induction of embryogenic calli for many citrus genotypes, especially the recalcitrant ones. Researchers also remarked that the environmental conditions, genotype, the age of the explants, and the components of the culture medium, were factors that highly affected the induction of embryogenic calli (Gmitter and Moore, 1986; Grosser et al., 1994; Gloria et al., 2000; Machado et al., 2015; Hasan et al., 2019).

In view of this, the present study was undertaken with the objective of producing friable embryogenic callus lines in *C. volkameriana* in order to have source material for protoplast isolation. Experiments were conducted to investigate the effects of various combinations of cellulase and pectinase on protoplast isolation for further protoplast fusion experiments on somatic hybrid production.

2. Materials and methods

2.1. Tissue source and preparation of explants

Ovules extracted from the immature fruits of Volkameriana trees (*Citrus volkameriana* V. Ten. Pasq), grown in the Citrus Research Fields of the Department of Horticulture, Faculty of Agriculture, Çukurova University, were used as the explant source. *C. volkameriana*, also known as Volkamer lemon, is believed to originate from a cross between sour orange and citron. Similar to rough lemon, it has high adaptability to different soil conditions. *C. volkameriana* is well adapted to sandy and sandy loam soils and often performs poorly on heavy soils. It is moderately tolerant to salinity and tolerant to calcareous soils. *C. volkameriana* has no common incompatibilities with other species and varieties (Saunt, 2000; Roose, 2014). Immature fruits were harvested at 4, 8, and 12 weeks after anthesis (WAA) from open-pollinated mature trees. Fruits were surface-sterilized under running tap water for 10 min, followed by 70% (v/v) EtOH for 2 min. They were then exposed to a sodium hypochlorite solution (0.2% active chloride) for 15 min, followed by 3 rinses with sterile distilled water under aseptic conditions. After sterilization, the ovules were excised from the immature fruits using a stereoscopic microscope and used for embryogenic callus initiation.

2.2. Culture media and conditions for callus initiation

Ovules isolated from immature fruits of *C. volkameriana* after 4, 8, and 12 WAA were cultured on 5 different media formulated with the addition of various plant hormones, as shown in Table 1. The 5culture media basically contained EME: the MS basal salts (Murashige and Skoog, 1962), MT vitamins (Murashige and Tucker, 1969), 0.5 g $L^{-1}$of malt extract, and 50 g L^{-1} of sucrose (Grosser and Gmitter, 1990), and were supplemented with phytohormones 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), and kinetin (KIN). Gelrite was used as the solidifying agent in a 3.2-g L^{-1} concentration for the 5 culture media. The pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. After autoclaving, 20 mL of the culture media was poured into disposable Petri dishes (90 × 15 mm). Ovules were then inoculated on the medium for callus induction in alaminar air flow cabinet and the cultures were maintained at 26°C in the dark. After 30 days of culture, the first observations were recorded based on the callus induction rate. Counts of the callus induction and somatic embryos per explant were recorded after 90 days of incubation and expressed as below.

$$Callogenesis\ frequency = \frac{No.of\ ovules\ that\ produced\ calli}{Total\ No.of\ incubated\ ovules} x100$$

$$Embryogenesis\ frequency = \frac{No.of\ ovules\ that\ produced\ somatic\ embryos}{Total\ No.of\ incubated\ ovules} x100$$

Moreover, the best growing embryogenic calli were determined to establish long-term callus lines in order to be used as protoplast sources. Embryogenic calli were selected and subcultured in hormone-free EME medium for proliferation.

2.3. Protoplast isolation, purification, and counting

Selected embryogenic callus lines were then subcultured on modified MT medium with the addition of 0.5 g $L^{-1}$of malt extract (EME) for protoplast isolation. The general protoplast isolation protocol was adapted from that of Grosser and Gmitter (1990). At 3 weeks after transfer to fresh medium, about 1 g (fresh weight) of friable calli were placed into 60 × 15-mm Petri dishes containing 1.5 mL of enzyme solution (see Table 2) + 2.5 mL 0.7 M of BH3. Different enzyme solutions were formulated by combiningcellulase and pectinase concentrations in macerating solutions in order to achieve successful yielding protoplast isolation. The protoplast isolation experiment was designed as a 2-factorial experiment. Ovule-derived embryogenic calli of *C. volkameriana* were treated with 3 different cellulase concentrations (1.0%, 1.5%, 2.0%) and 3 different pectinase concentrations (0.1%, 0.2%, 0.4%). Enzyme solutions were sterilized by filtration through a 0.2-µm filter. Enzymatic digestions were performed in the dark at 26 °C on an orbital shaker (40 rpm) for 15 h. After digestion, the preparations were passed through a 40-µm nylon mesh filter to remove any undigested cell clumps and debris from broken cells. Following filtration and centrifugation, the callus-derived protoplasts were purified using CPW solutions (27.2 mg/L of KH_2PO_4, 100 mg/L of KNO_3, 150 mg/L of $CaCl_2$, 250 mg/L of $MgSO_4$, and 0.16 mg/L of KI, 0.00025 of $CuSO_4$, pH 5.8) containing 25% sucrose and 13% mannitol gradient centrifugation. The viable protoplasts at the interface between the 2 layers were then carefully collected and washed 2 times by centrifugation at 850 rpm for 6 min in protoplast washing solution (0.8 M of Mannitol + 0.5 mM of $CaCl_2$). After this step, yields of the protoplasts were determined using a double-chamber hemocytometer

Table 1. Culture media used for the embryogenic callus induction of the ovules of *C. volkameriana*.

Contents	Culture media				
	M1	M2	M3	M4	M5
MS basal salts	+	+	+	+	+
MT vitamins	+	+	+	+	+
Sucrose (50 g L^{-1})	+	+	+	+	+
Malt extract (0.5 g L^{-1})	+	+	+	+	+
2,4–D	–	1 mg L^{-1}	1 mg L^{-1}	–	–
BAP	–	0.5 mg L^{-1}	–	3 mg L^{-1}	–
Kin	–	–	0.5 mg L^{-1}	–	1 mg L^{-1}

2,4-D: 2,4-Dichlorophenoxyacetic acid, BAP: 6-benzylamino purine, Kin: 6-furfurylamino purine (kinetin).

Table 2. Enzyme solution for the protoplast isolation.

Components	Concentration
D-Mannitol	0.7 M
$CaCl_2$	18 mM
MES	6 mM
NaH_2PO_4	1.4 mM
Cellulase RS	1.0, 2.0, 4.0%
Macerozyme	1%
Pectolyase Y-23*	0.1, 0.2, 0.4%
pH	5.6

*Endo-polygalacturonase and endo-pectin lyase.

(Modified-Fuchs Rosenthal rulings, model B.S. 74B; Weber Scientific International Ltd., Teddington, UK). Protoplast counting was performed microscopically using an inverse microscope (Nikon Eclipse TS100, Tokyo, Japan) and repeated 3 times for each digestion condition.

2.4. Experimental design and data analysis

The ovule culture experiment was arranged in a 5× 3 × 5 design, comprising 5 culture media, for 3 different WAA, with 5 replicates (each replicate consisted of 5 Petri dishes containing 10 ovules each), respectively, in a completely randomized design. Data were subjected to a 2-way ANOVA. Significant differences between the means were evaluated using the Fisher LSD test at $P \leq 0.05$. Statistical analyses were performed using SAS v.9.00 statistics software (Carolina State University, CA, USA). Data for the induction (%) traits were arcsine transformed to increase normality (Fowler J, Cohen L, Jarvis P (1998). Practical Statistics for Field Biology. New York, NY, USA: John Wiley, pp. 272.).

3. Results

3.1. Callogenesis and indirect somatic embryogenesis

Callogenesis was initiated from the in vitro ovule culture of *C. volkameriana* rootstock. Observations were recorded 90 days after inoculation of the ovules onto the culture media; however, the first callus induction was observed after 30 days of culture from ovules of *C. volkameriana* incubated on M2 medium. Later, calli formed with varying characteristics of color and texture depending on the culture medium used. The2-way ANOVA indicated a significant main effect of the number of WAA ($P \leq 0.01$) and the type of culture media ($P \leq 0.01$), as well as their interaction ($P \leq 0.05$) on the callus induction frequency (Table 3). At 8 WAA, the highest callus formation was induced(78%), followed by 12 WAA (72%) and 4 WAA (55.33%). Among the culture media, the lowest callogenesis was recorded in ovules inoculated on M1 medium (36.67%), whereas callogenesis was the highest in those on M3 (82.22%), followed by M2 (81.11%) and M4 (77.78%).The frequency of callus induction varied from 26.67% to 93.33% depending on the number of WAA and type of culture media used. The highest callus formation occurred in ovules cultured for 8 WAA on M2 medium, whereas the lowest callus induction was observed at 4 WAA on M1 (Table 3).

Table 3. Effects of the number of WAA and type of culture media on the callus induction frequency (%).

Culture media	Number of WAA			
	4 WAA	8 WAA	12 WAA	Mean
M1	26.67 (31.01) i	43.33 (41.17) hi	40.00 (39.17) hi	36.67 (37.12) C
M2	63.33 (52.80) fg	93.33 (77.75) a	86.67 (68.89) abc	81.11 (66.48) A
M3	73.33 (59.04) c-f	90.00 (75.04) ab	83.33 (66.67) bcd	82.22 (66.92) A
M4	66.67 (54.81) ef	86.67 (68.89) abc	80.00 (63.96) cde	77.78 (62.55) A
M5	46.67 (43.10) gh	76.67 (61.25) c-f	70.00 (57.03) def	64.44 (53.79) B
Mean	55.33 (48.15) C	78.00 (64.82) A	72.00 (59.14) B	
P-value of the number of WAA			0.0091**	
P-value of the culture medium			0.0078**	
P-value of the number of WAA × culture medium interaction			0.0121*	

*,** Significance at $P < 0.05$ and $P < 0.01$, respectively; means with the same letter were not significantly different at $P < 0.05$ using the Fisher LSD test; lower case letters refer to differences based on separate ANOVA for each genotype; upper case letters refer to differences among the cultivars and plant growth regulator concentrations in the factorial analysis.

The structure of the calli depended on the plant hormones added to the EME medium, and varied from compact, slow-growing, yellowish calli to soft-, fast-growing, friable embryogenic calli (Figure 1). Calli formed after the inoculation of the ovules of *C. volkameriana* on M1 medium was hard and slow-growing without embryogenesis. Similarly, nonembryogenic fast-growing, spongy calli occurred on M2 medium. On the other hand, the formations of somatic embryos were observed on M3 and M4 media on soft-growing embryogenic calli. Ovules cultured on M5 medium formed fast-growing friable embryogenic calli, which was the most suitable callus formation for subcultures to maintain a totipotent isolation source for protoplasts, and the formation of somatic embryos was observed on M5 medium as well (Figure 1).

As previously mentioned, culturing ovules of *C. volkameriana* at different immature fruit stages and types of culture media resulted in embryogenesis on the induced calli. Significant main effects of the number of WAA ($P \leq 0.01$) and type of culture media ($P \leq 0.01$) on indirect somatic embryogenesis were determined using 2-way ANOVA (Table 4). The highest indirect somatic embryogenesis was observed in ovules cultured at 12 WAA (24.00%), followed by 8 WAA (19.33%) and 4 WAA (16.00%). In terms of culture media, embryogenesis frequencies were the highest in ovules cultured on M5 (40.00%) and M3 (30.00%) media, whereas the lowest indirect somatic embryogenesis induction frequency was 3.33% on M1. The 2-way ANOVA revealed a significant interaction effect ($P \leq 0.05$) of the number of WAA × type of culture media on indirect somatic embryogenesis. Ovules of *C. volkameriana* showed no response at 4 WAA cultured on M1 medium in terms of indirect shoot organogenesis. Embryogenesis was the highest, with a frequency of 43.33%, at 12 WAA cultured on M5medium.

Culture media was significant ($P \leq 0.01$) on embryogenic callus initiation (Table 5). Ovules of *C. volkameriana*

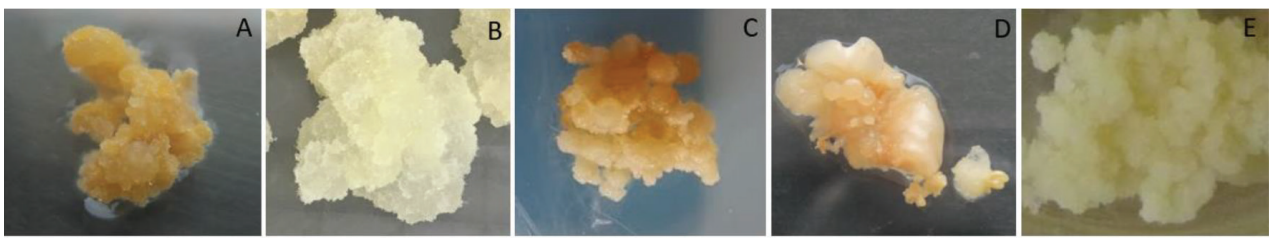

Figure 1. Callogenesis and indirect somatic embryogenesis derived by culturing ovules of *C. volkameriana*. A) Compact, slow-growing yellowish calli on M1; B) fast-growing, spongy calli without embryogenesis on M2; C, D) soft-growing embryogenic calli and indirect somatic embryogenesis on M3 and M4, respectively; and E) fast growing friable embryogenic calli.

Table 4. Effects of the number of WAA and type of culture media on the indirect somatic embryogenesis induction (%).

Culture media	Number of WAA			
	4 WAA	8 WAA	12 WAA	Mean
M1	0.00 (0.00) g	3.33 (6.15) fg	6.67 (12.29) ef	3.33 (6.15) D
M2	3.33 (6.15) fg	6.67 (12.29) ef	13.33 (21.15) de	7.78 (13.20) C
M3	26.67 (31.01) a-d	30.00 (33.23) abc	33.33 (35.24) ab	30.00 (33.16) A
M4	13.33 (21.15) de	16.67 (23.87) cd	23.33 (28.80) bcd	17.78 (24.61 B
M5	36.67 (37.24) ab	40.00 (39.17) ab	43.33 (41.17) a	40.00 (39.19) A
Mean	16.00 (19.11) B	19.33 (22.94) AB	24.00 (27.73) A	
P-value of the number of WAA			0.0037**	
P-value of the culture medium			0.0084**	
P-value of the number of WAA × culture media interaction			0.0413*	

*, ** Significance at $P < 0.05$ and $P < 0.01$, respectively; means with the same letter were not significantly different at $P < 0.05$ using the Fisher LSD test; lower case letters refer to differences based on separate analysis of variation for each genotype; upper case letters refer to differences among the cultivars and plant growth regulator concentrations in thefactorial analysis.

Table 5. Effects of the number of WAA and type of culture media on the embryogenic callus induction (%).

Culture media	Number of WAA			
	4 WAA	8 WAA	12 WAA	Mean
M1	0.00	0.00	0.00	0.00 (0.00) D
M2	0.00	0.00	0.00	0.00 (0.00) D
M3	13.33	16.67	16.67	15.56 (22.96) B
M4	6.67	16.67	10.00	11.11 (18.20) C
M5	16.67	36.67	23.33	25.56 (29.97) A
Mean	7.33 (11.46) B	14.00 (17.00) A	10.00 (14.22) AB	
P-value of the number of WAA			0.0035**	
P-value of the culture medium			0.0054**	
P-value of the number of WAA × culture medium interaction			0.0884	

** Significance at $P < 0.01$; means with the same letter were not significantly different at $P < 0.05$ using the Fisher LSD test; lower case letters refer to differences based on separate analysis of variation for each genotype; upper case letters refer to differences among the cultivars and plant growth regulator concentrations in the factorial analysis.

cultured for 4, 8, and 12 WAA showed no response onM1 and M2 media in terms of embryogenic callus formation. Calli initiated on M5 medium were fast-growing, highly embryogenic, and had lower somatic embryo formation in comparison to those on M3 and M4. In addition to the higher frequency of embryogenic calli on M5 medium, the average number of embryo-like structures per callus was lower in comparison to those on M3 and M4 (Figure 1). The highest embryogenic callus induction frequency was observed on M5 medium (25.56%), followed by M3 (15.56%). This clearly suggested that formulating EME together with KIN significantly increased the frequency of embryogenic callus initiation.

3.2. Protoplast isolation efficiency

Effects of the cellulase and pectinase concentrations on the protoplast yield were monitored through a 2-factorial experimental design. The 2-way ANOVA revealed significant differences ($P \leq 0.01$) among the different levels of cellulase, pectinase, and their interaction (Table 6). Using 2% cellulase resulted in the highest number of protoplast (7.8×10^5 protoplast mL^{-1}) after enzymatic digestion of the embryogenic callus followed by 4% cellulase (5.42×10^5 protoplast mL^{-1}) in the protoplast isolation solution. The lowest number of protoplasts was recorded in digestion solution supplemented with 1% cellulase (3.85×10^5 protoplast mL^{-1}). In terms of the pectinase concentrations in the enzymatic digestion solution, the highest number of protoplast (9.05×10^5 protoplast mL^{-1}) was determined using 0.2% pectinase followed by the addition of 0.1% pectinase in the digestion solution. The lowest number of protoplasts was counted as 2.96×10^5 protoplast $mL^{-1}$with 0.4% pectinase treatment. The results in Figure 2 show the significant interaction effects of cellulase × pectinase ($P \leq 0.01$) on the number of protoplasts isolated from the ovule-derived embryogenic calli of *C. volkameriana*. The yield of protoplast varied from 1.85×10^5 to 12.33×10^5 protoplast mL^{-1} under various protoplast isolation conditions. In Figure 3, isolated protoplast cell counts after digestion are presented. Thus, the optimal treatment for the isolation of *C. volkameriana* protoplasts was the addition of 2% cellulase and 0.2% pectinase, as digestion enzymes, to the protoplast isolation solution.

Table 6. ANOVAfor the number of isolated protoplasts (protoplast/mL) from the ovule-derived calli of *C. volkameriana*.

Source of variation	Df	MS	F	P-value
Cellulase	2	7.114×10^{11}	139.61	0.0001
Pectinase	2	1.725×10^{12}	338.55	0.0001
Cellulase*pectinase	4	1.497×10^{11}	14.69	0.0001
Error	18	4.586×10^{10}		

4. Discussion

The present study reported an efficient method for embryogenic callus induction. In order to have the necessary totipotent source material for protoplast isolation in *C. volkameriana* rootstock, as well as achieve high protoplast yields, different cellulase and pectinase concentrations must be combined in the protoplast digestion solution. Somatic hybridization in citrus is now

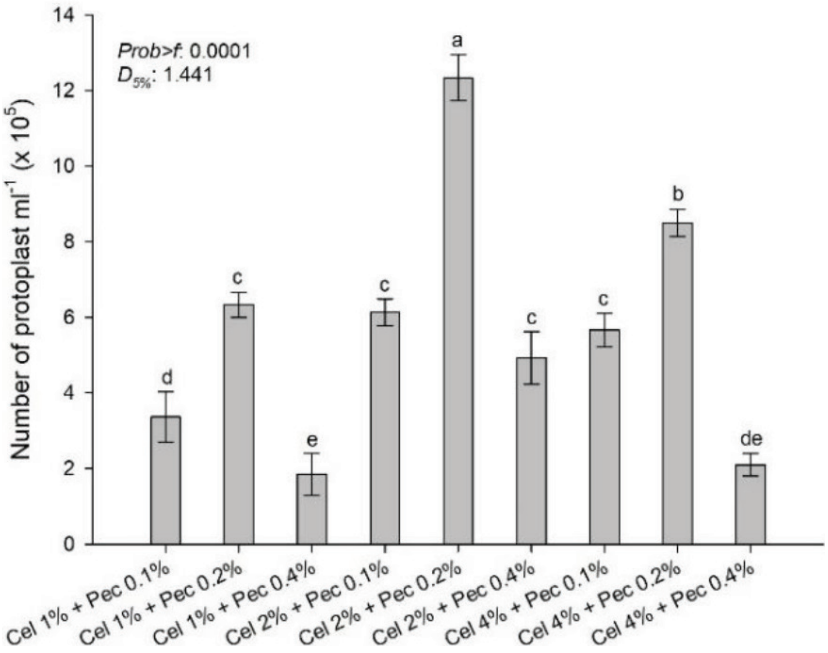

Figure 2. Effects of different cellulase and pectinase enzyme concentrations on the protoplast isolation efficiency of ovule-derived calli of *C. volkameriana*. Different letters indicate a significant difference at $P \leq 0.05$ according to the Tukey post hoc test.

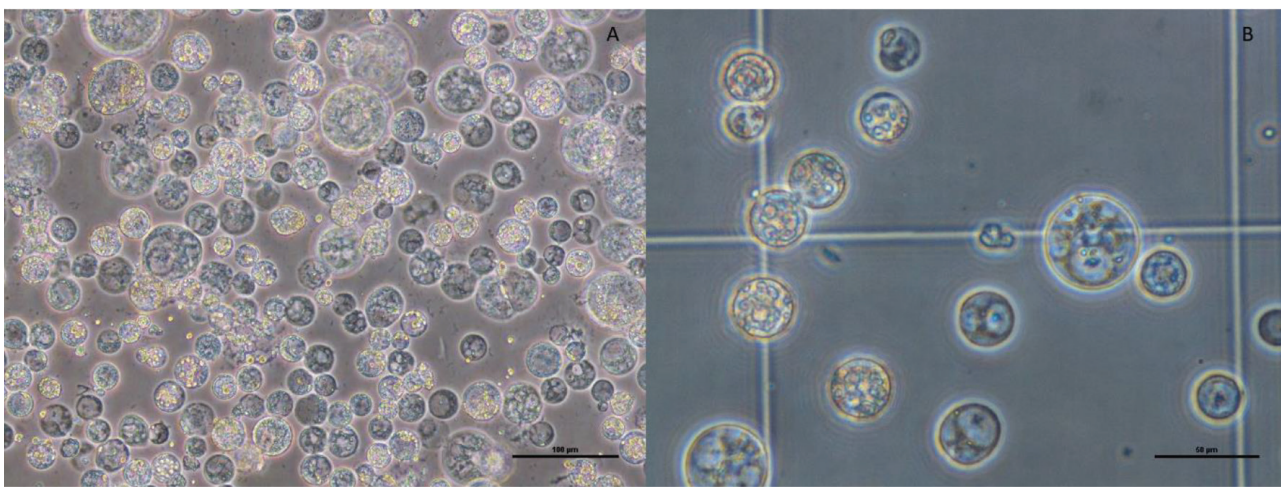

Figure 3. General views of the isolated protoplasts after digestion (A) and during the protoplast cell counting process by a double-chamber hemocytometer (B).

a useful tool for worldwide citrus breeding programs. This approach aimed to circumvent the biological obstacles encountered by conventional methods of breeding, such as sexual incompatibility, nucellar embryony, and male and/or female sterility (Grosser and Gmitter, 1990). The ability to produce distinct heterokaryon forms, by mixing nuclear and cytoplasmatic genomes of different species and genera, is thus given through the protoplast fusion (Abbate et al., 2019). Grosser et al. (2000) reported that citrus somatic hybrid plants were regenerated using more than 200 parental combinations. In terms of rootstock improvement, this technique is important in terms of fusing compatible rootstock genotypes in order to develop rootstocks with superior characteristics, such abiotic/biotic stress tolerance, broad soil adaptation, and high productivity. A precondition for an effective somatic hybridization is the introduction of embryogenic callus lines. In addition, leaf protoplasts have no potential for

plant regeneration, and protoplasts should be isolated from an embryogenic source for at least 1 parent (Grosser et al., 1994; Dambier et al., 2011). In the present study, ovules excised from *C. volkameriana* at 4, 8, and 12 WAA were cultured on five different culture media formulated with the addition of various plant hormones in order to obtain embryogenic callus lines. Success in the callogenesis efficiency was affected by a number of factors, such as genotypes and culture media, including phytohormones and culture conditions (Chen et al., 2019). The frequency of callus induction depended on the number of WAA and phytohormones used in the EME. Callus induction frequency varied from 26.67% to 93.33%. The highest initiation of calli was determined on medium with 2,4-D. Ramdan et al. (2014) reported that the highest callogenesis ratio of Cleopatra mandarin (*Citrus reticulata* Blanco), Rangpur lime, *C. volkameriana*, Trifoliate orange, and sour orange were determined on 2 culture media formulated with the edition of 1.0 mg $L^{-1}$of 2,4-D + 0.5 mg $L^{-1}$of BA. In addition, Savita et al. (2011) reported a callus induction rate of 96% on cotyledon culture for *Citrus jambhiri*, and mentioned that the existence of 2,4-D as an auxinwas necessary for callogenesis. Similar to the present study, it was reported that the best callogenesis was obtained with culture media formulated using 1mg $L^{-1}$of 2,4-D + 0.5 mg $L^{-1}$of KIN, in terms of producing calli in some citrus rootstocks (Chetto et al., 2016).

The first callus initiation was observed 30 days after inoculation of the ovules on M2 medium (EME + 1mg L^{-1} of 2,4-D + 0.5 mg $L^{-1}$of BAP). Later, in the experiment period, yellowish, white, compact, friable, embryogenic, nonembryogenic types of calli were produced from ovules on EME medium supplemented with the combination of 2,4-D, KIN, and BAP alone. Calli growing on EME and EME + 1mg $L^{-1}$of 2,4-D + 0.5 mg L^{-1} of BAP were compact, hard, and spongy, without embryogenesis, respectively. On the other hand, somatic embryos and embryo-like structures occurred in ovules inoculated on culture media formulated with EME + 1mg $L^{-1}$of 2,4-D + 0.5 mg $L^{-1}$of KIN and EME + 3.0 mg $L^{-1}$of BAP. Fast-growing friable embryogenic calli were observed in ovules cultured on EME + 1.0 mg $L^{-1}$of KIN. The results of the present study were in agreement with some of theearlier studies on different *Citrus* spp. which resulted in embryogenic calli under the influence of EME incombination with 2,4-D, BAP, or KIN (Grosser et al., 1988; Grosser and Gmitter, 1990; Singh et al., 1994; Ramdan et al., 2014).

Formation of somatic embryos and embryo-like structures were observed by inoculation of the ovules isolated from immature fruits of *C. volkameriana*. The number of WAA and the type culture media used significantly affected embryogenesis in the present study. Indirect somatic embryogenesis frequency varied from 3.33% to 43.33%, whereas the ovules isolated at 4 WAA showed no response on EME in terms of embryogenesis. On the other hand, the ovules inoculated at 12 WAA on EME + 1.0 mg $L^{-1}$of KIN resulted in the highest indirect somatic embryogenesis. Starrantino and Russo (1980) reported a higher ratio of 70% somatic embryogenesis of undeveloped ovules collected from fruits of *C. sinensis*, harvested at 8–15 WAA and cultured on MS medium supplemented with 0.5 g $L^{-1}$of malt extract. Similar results to those herein were reported by Carimi et al. (1998), who observed an average of embryogenic cultures (30.8%) from different *C. sinensis*. Those differences might have been due to the genetic differences between the cultured genotypes. In the present study, a high level of somatic embryogenesis was also observed in the ovules cultured on EME + 3 mg $L^{-1}$of BAP. Al-taha et al. (2012) documented that somatic embryogenesis was achieved in callus cultures from the undeveloped ovules of immature fruits of local orange. Similar to the results of the present study, the authors indicated that somatic embryos were developed on MS medium supplemented with BAP only (Al-Taha et al. 2012).

It has been proven that embryogenic friable calliwere essential for the development of cell suspension cultures that have played an important role in successful somatic hybridization (Davey et al., 2005; Chumakov and Moiseeva, 2012). In the present study, embryogenic calli were produced using the ovule culture of *C. volkameriana* at different immature fruit stages on EME medium supplemented with 2,4-D, BAP, and KIN. Effects of the number of WAA and type of culture media were significant on EC induction. Embryogenic callus induction without fully developed somatic embryos growing on theembryogenic calli varied from 6.67% to 36.67%. On the other hand, the ovules showed no response to culture media EME and EME + 1mg $L^{-1}$of 2,4-D + 0.5 mg $L^{-1}$of BAP at 4, 8, or 12WAA in terms of embryogenic callus initiation. On the other hand, embryogenic callus initiation was the highest in ovules cultured on M5 medium, which was formulated with 1mg L^{-1} of KIN, followed by M3(2,4-D + 0.5 mg $L^{-1}$of KIN). Similar to this, Oliveira et al. (1994) reported that using MT medium supplemented with KIN resulted in the highest embryogenic callus induction rate for Cleopatra mandarin and Rangpur lime. In addition, Carimi et al. (1998) documented that the culture of undeveloped ovules of the Navel orange group on EME + KIN resulted in high embryogenic callus initiation and cell proliferation. In the present study, embryogenic calli were also obtained on EME + 3.0 mg L^{-1} of BAP, which was supported by Al-Taha (2012), who reported a high frequency of embryogenic callus induction in the nucleus tissues of local orange using 1.0 mg $L^{-1}$of BAP as plant hormone. Chetto et al. (2016) also documented that the

production of friable embryogenic calli was induced in ovule culture Cleopatra when the culture media was formulated using 1mg $L^{-1}$of 2,4-D + 0.5 mg $L^{-1}$of KIN.

Ohgawara et al.(1985) developed the first intergeneric somatic hybrids between *Poncirus trifoliata* and *Citrus sinensis* in 1985, which led the new avenue for today's big development in citrus somatic hybridization. As a result of further studies, the somatic hybridization of plant protoplasts has become an increasingly important technique in citrus breeding programs. This technique can also be used in developing new rootstocks that are tolerant or resistant to abiotic and biotic stress factors (Grosser and Gmitter, 1990). Several abiotic factors, such as mixtures of enzymes, osmotic potential, and time and time of digestion, influence the isolation of protoplast (Sinha et al., 2003; Rizkalla et al., 2007). In the present study, different levels of cellulase and pectinase, and their interaction, significantly affected the number of protoplasts obtained from the embryogenic callus lines derived by the ovule culture of *C. volkameriana*. After transferring the selected embryogenic callus lines onto hormone-free medium and successful embryogenic callus growth was achieved, protoplast isolation studies were conducted on the produced embryogenic calli. The most favorable protoplast isolation efficiency was determined in the combination of 2% cellulase and 0.2% pectinase formulated protoplast digestion solution.The cellulase concentrations appeared to be crucial for callus protoplast isolation. Zhou et al. (2019) reported that different cellulase and pectinase concentrations in the protoplast digestion solution remarkably affected protoplast yield. However, using cellulase at a concentration of 4% reduced the number of protoplasts isolated from the ovule-derived embryogenic callus of *C. volkameriana* in the present study. This could have been related to the influence of cellulase on the integrity of the membrane and the reduction of the physical activities of the protoplasts (Raikar et al., 2008).

In conclusion, an efficient method for the embryogenic callus initiation and protoplast isolation of *C. volkameriana* was described in the present study. *Citrus* and its relatives have many elite resistance traits, which make them important germplasm reservoirs for breeding programs. Somatic hybridization by protoplast fusion has become an important tool for *Citrus* variety and rootstock breeding programs. Protoplast isolation from tissues is the first and most important step in somatic hybridization. In the present study, the responses of *C. volkameriana* ovules on 5 different culture media were variable. Generally, M3 and M5 media showed the best results in terms of embryogenic callus production from the ovules. Isolation procedures that yield highly purified and functional protoplasts have been described for many species, as well as *Citrus*. *C. volkameriana* has been used in many somatic hybridization manipulations as a leaf mesophyll protoplast source. However, this study reported the isolation of viable protoplast from ovule-derived embryogenic callus of *C. volkameriana*. The efficient protoplast isolation conditions described in this study should be practical and convenient for breeders. Furthermore, the selected calli of *C.volkameriana* can now be maintained and used for protoplast isolation and fusion for *Citrus* somatic hybridization.

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