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Somatic embryogenesis and plantlet regeneration of pummelo 'Nambangan' (*Citrus maxima* (Burm.) Merr.)

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Abstract

Somatic embryogenesis is a method which needs to be mastered to support plant breeding in biotechnology. The induction of somatic embryos from various explants could be regenerated into plantlets. The aim of this study to establish somatic embryogenesis and plantlet regeneration of pummelo 'Nambangan', one of the local prime fruit varieties in Indonesia, from various explants. Juice vesicle and seeds from immature fruit, shoot tip and leaf segments from *in vitro* mature seed germination were used as explants for indirect somatic embryogenesis initiation whereas peeled seeds from immature fruit were used as explants for direct somatic embryogenesis. Explants for indirect somatic embryogenesis were cultured on MS medium supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) or Naphthalene acetic acid (NAA) at 0.5 and 1.0 mg L^{-1}. Explants for direct somatic embryogenesis were cultured on MS medium supplemented with combination of 3 mg L^{-1} of 6-benzyl amino purine (BAP), 1 mg L^{-1} of 2,4-D and 300 mg L^{-1} of Casein hydrolisate. The results showed that induction of indirect embryogenesis on media with the addition of 2.4-D produced non-embryogenic callus for all eksplan. Peeled immature seed with diameter 4-6 mm formed embryoid after 4 weeks cultured on direct embryogenesis medium and could regenerated into plantlet. Flow cytometry analyses indicated that regenerated plantlet were consistently diploid.

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Introduction

Pummelo 'Nambangan' is one of the local prime fruit varieties in Indonesia. Pummelo is a perennial plant belonging to family *Rutaceae*. Pummelo has the largest fruit size compared to other citrus fruits and has fresh sour sweet taste.

The fruit has long shelf life, until 4 months (Susanto, 2004). Pummelo fruit contains antioxidant, phenolics, ascorbic acid, gallic acid, hesperidin and naringin which has beneficial effects for human health (Caengprasath *et al.,* 2013, Buachan *et al.*, 2014). Buds and blossoms of pummelo flower possess a strong aroma containing β-myrcene, limonene, ocimene, linalool and caryophyllene as the major compounds potential for the fragrance industry (Zakaria *et al*., 2010b). Those potential characters are important for supporting citrus breeding program.

There are only a few reports on tissue culture technique for *C. maxima* compared to other citrus species, particularly in related to somatic embryogenesis process. Somatic embryogenesis produces embryos with higher propagation rate. Somatic embryos have bipolar axis, shoot meristem at one end and radicle at the other end. Plant regeneration via somatic embryogenesis indirectly occurs from callus culture or directly emerged embryo from explants (Ammirato, 1993). Meanwhile, development of adventives embryos from nucellar cells is a normal feature in several species of citrus and mangifera which are polyembryonic (Bhojwani and Razdan, 1983). Monoembryonic citrus gave low response when embryogenesis was initiated by culturing tissues around seed (Rangan *et al*., 1969).

It is known that citrus has repressive factor for asexual embryogenesis produced by nucellar tissue (Tisserat and Murashige, 1977). It is extremely difficult to obtain embryogenic callus from monoembryonic cultivars in citrus (Grosser and Gmitter, 2011). Therefore, it is important to explore suitable explant for pummelo to establish somatic embryogenesis.

Many different types of explants have been used to regenerate genus of citrus via direct and indirect somatic embryogenesis methods such as nucellar tissue of *C. grandis* (Rangan *et al*., 1969), juice vesicle of *C. unshiu* (Nito and Iwamasa, 1990), shoot segment of *C. limon* (Mukhtar *et al*., 2005), different parts of *C. grandis* flower (Zakaria *et al*., 2010a), immature seed of *C. limon* (Gholami *et al*., 2013) and embryo of five citrus rootstocks (Ramdan *et al*., 2014).

Growth regulators is an organic compound plays an important role in the growth and development of plant tissue culture. Induction of callus in citrus often used 2,4-D and NAA (Nito and Iwamasa, 1990; Mukhtar *et al*., 2005). Auxin 2,4-D is commonly used for embryogenic callus induction because of its resistance on enzymatic degradation reaction as well as for photooxidation. Cytokinin BAP and high concentration of sucrose could induce somatic embryo formation in *C. limon* (Gholami *et al*., 2013). Combination of 2,4-D with BAP were able to induce somatic embryos of *C. reticulata* (Merigo, 2011), also resulted in highest frequency of the callus induction rate and good quality of callus from embryos of five citrus rootstock (Ramdan *et al*., 2014).

Somatic embryogenesis involves a long process to regenerate into plantlets. This process often causes somaclonal variation. Somaclonal variation is defined as genetic variation observed among progeny of plants regenerated from somatic cells cultured *in vitro* (Morrison *et.al*. 1988).

Changes of ploidy level are one of genetic variation type (Grosser *et al*., 1997). Flow cytometry has been commonly employed to analyze genetic stability in regenerants of many fruit species including lemon (Orbovic *et al*., 2008), *Vitis vinifera* (Prado *et al*., 2010), and *Rubus fructicosus* L. (Vujovic *et al*., 2010). The objectives of this research were to establish somatic embryogenesis of pummelo 'Nambangan' and to regenerate their plantlets via indirect and direct somatic embryogenesis from several different explants and to analyze the ploidy level of its regenerants.

Materials and methods

Plant material

Explants consists of various part of mature and immature pummelo 'Nambangan' fruits. Immature fruits is a fruit with 8-10 cm in diameter. Pummelo fruits were collected from Cikabayan-field trials at Bogor Agricultural University, Indonesia.

Direct embryogenesis

Coat removed immature seeds were used as explants on direct embryogenesis induction. Direct embryogenesis experiment was conducted to compare three kinds of diameter size of explants for embryoid initiation on the same medium composition. Peeled immature seed were classified into three different group of diameters (1-3 mm; 4-6 mm and 7-10 mm), each group consisted of 25-55 explants. Medium for embryoid initiation on direct embryogenesis process was MS basal medium supplemented with 1 mg L^{-1} 2,4-D in combination with 3 mg L^{-1} BAP, containing 300 mg L^{-1} Casein hydrolisat (Merigo (2011) in modification).

Observation of cultured explants was done every week using stereo microscope (Leica EZ4HD) at 8-12.5 magnification. Number of explants were producing callus or embryoid on direct embryogenesis treatment was recorded after 4 weeks cultured.

Indirect embryogenesis

Induction of indirect embryogenesis were used 4 types of explants, that is juice vesicle, seed, shoot tip and leaf segments. Juice vesicle and seed with whole coats were extracted from immature fruit. Shoot tip and leaf segment were taken from germinated seed of mature fruit *in vitro*.

Seeds were dipped in 70% ethanol for 1 hour, then air dried in the laminar airflow cabinet. All seed coat was removed and seeds were germinated on MS0 medium until 6 weeks. All explants were planted on treatment medium in Petri dishes (100 × 15 mm) and cultured at 25-27^{o}C in dark condition.

Indirect embryogenesis experiment was designed to compare effect of 2,4-D and NAA for callus induction on each type of explants.

Three to ten explants were placed in each Petri dish per treatment with 3 replicates. Indirect embryogenesis experiments were set up as a completely randomized design.

Medium for callus initiation on indirect embryogenesis process were MS (Murashige and Skoog, 1962) containing 2,4-D at 0.5 and 1 mg L^{-1} also NAA at 0.5 and 1.0 mg L^{-1}. Observation of cultured explants was done every week using stereo microscope (Leica EZ4HD) at 8-12.5 magnification. Percentage of callus produced per explants on indirect embryogenesis treatment was recorded after 4 weeks.

Data from indirect embryogenesis treatments was subjected to ANOVA and significant differences among means were tested using Least Significant Differences (LSD) values at 5% level with DSAASTAT V1.1 software. Morphology of callus cell produced by explants was observed using inverted microscope (Leica DMIL LED) with a magnification of 400 times, after 8 weeks of incubation.

Plantlet regeneration

Globular stage of pummelo 'Nambangan' somatic embryos were proliferated and maturated to cotyledonary stage on MS basal medium containing 1 mg L^{-1} of BAP and 0.5 mg L^{-1} of 2,4-D at continuous light. Cotyledonary embryo germination was conducted on MS basal medium supplemented with 2.5 mg L^{-1} of Gibberellic acid (GA_3) grown at continuous light (Merigo, 2011; Karyanti, 2013). Regenerant plantlets were further cultured on MS containing 0.5 mg L^{-1} Kinetin.

Plantlet ploidy analysis

Regenerated plantlets from somatic embryogenesis formed ribbon-like (lingulatus) leaves. These leaves were analyzed for their ploidy level using flow cytometry (CyFlow ® Space. Partec, Germany) to verify somaclonal variation. They were compared to pummelo 'Nambangan' diploid leaves from *in vitro* seed germination and analysed according to the protocol developed for bananas (Doleel *et al.*, 2004). A small piece (approximately 0.4 cm^2) of one leaf blade of the second leaf of 4 weeks old planlets grown on propagation medium was chopped in the

extraction buffer, then they were passed through a 30 µm nylon mesh sreen and stained with Fluorescent dye Propidium Iodide (Partec, Germany). Diploid leaf plantlets were used to determine the position of diploid peak on the histogram presentation from the analyzer (standard).

Results and discussion

Direct embryogenesis

In this work, combination of BAP and 2,4-D on medium was effective plant growth regulator to induced embryoid directly from immature peeled seed explants with diameter 4-6 mm (Fig. 1).

The presence of auxin together with cytokinin could promoted adventitious shoot formation at low auxin: cytokinin ratio (George and Sherrington, 1984). Embryoids are adventive embryo. Peeled immature seed explant containing embryo and cotyledone with high starch contents.

Table 1. Growth response of pummelo 'Nambangan' explants on direct embryogenesis induction medium after 4 weeks.

Diameter of immature seed (mm)	Number of explant producing calli (%)	Number of explant producing embryoid (%)	Explant and callus growth response
1-3	0.00 (0/25)	0.00 (0/25)	Seeds persistent opaque
4-6	76.36 (42/55)	23.63 (13/55)	Emerged embryoid and white opaque, granular, compact callus
7-10	0.00 (0/30)	0.00 (0/30)	Seeds were green

Table 2. Responses of each type of pummelo 'Nambangan' explants on the indirect somatic embryo induction medium after 4 weeks.

Type of explant	Plant growth regulator	Callus per explant (%)±SE	Callus morphology
Juice vesicle from immature fruit	none	20.00±3.46b	Translucent callus, nodule-like, compact
	0.5 mg L^{-1} 2,4-D	70.83±3.82a	Translucent callus, nodule-like, compact
	1 mg L^{-1} 2,4-D	67.50±12.99a	Translucent callus, nodule-like, compact
	0.5 mg L^{-1} NAA	34.54±8.86b	Translucent callus, nodule-like, compact
	1 mg L^{-1} NAA	58.33±7.64a	Translucent callus, nodule-like, compact
Seed from immature fruit	none	25.00±4.33c	Translucent callus, nodule-like, compact
	0.5 mg L^{-1} 2,4-D	63.17±2.52a	Translucent callus, nodule-like, compact
	1 mg L^{-1} 2,4-D	47.83±8.40b	Translucent callus, nodule-like, compact
	0.5 mg L^{-1} NAA	28.50±12.17c	Translucent callus, cotton-like, compact
	1 mg L^{-1} NAA	38.50 ±6.26bc	Translucent callus, cotton-like, compact
Shoot tip from mature germinate seed	none	10.67±5.69c	No callus growth
	0.5 mg L^{-1} 2,4-D	58.33±10.41a	Emerged embryoid, white opaque callus, granular, friable
	1 mg L^{-1} 2,4-D	56.67±11.55a	White opaque callus, granular, friable
	0.5 mg L^{-1} NAA	10.67±5.51c	White callus, cotton-like, friable
	1 mg L^{-1} NAA	36.67±2.89b	White opaque callus, granular, friable
Leaf segment from mature germinate seed	none	0.00±0.00d	No callus growth
	0.5 mg L^{-1} 2,4-D	50.00±0.00b	White opaque callus, granular, friable
	1 mg L^{-1} 2,4-D	75.00±0.00a	White opaque callus, granular, friable
	0.5 mg L^{-1} NAA	30.56±9.62c	White opaque callus, granular, friable
	1 mg L^{-1} NAA	50.00±8.33b	White opaque callus, granular, friable

Mean ± s.d. followed by letter(s) are significantly different (P=0.05) according to LSD (for each type of explant).

These explants could promote adventitious embryo (embryoid) on that kind of medium.

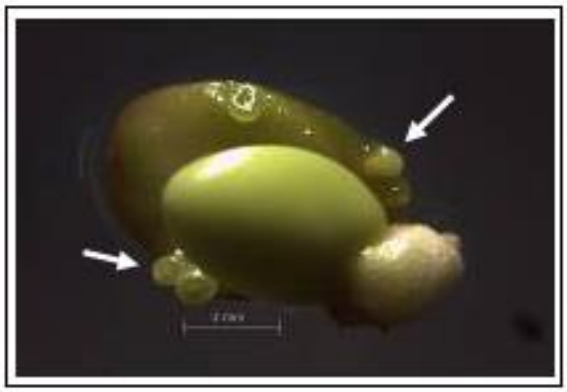

Fig. 1. Immature peeled seed of pummelo 'Nambangan' on MS containing 3 mg L^{-1} in combination with 1 mg L^{-1} of 2,4-D formed globular embryoid after 4 weeks cultured, observed with stereo microscope (bar=2mm; arrow = globular embryoid).

If treatment medium containing high concentration of auxin and low until middle concentration of cytokinin, explants would have produced callus. Mukhtar *et al.*, 2005 gived evidence that callus induction was highest when shoot segment of lemon were cultured on MS medium supplemented with 2 mg L^{-1} 2,4-D in combination with 20% of coconut milk.

Different seed sizes in this treatment showed different stages of seed development, which may cause different response to plant growth regulator added in the medium.

That is also shown that physiological age of explants could affect development of cells to morphogenesis (George and Sherrington, 1984).

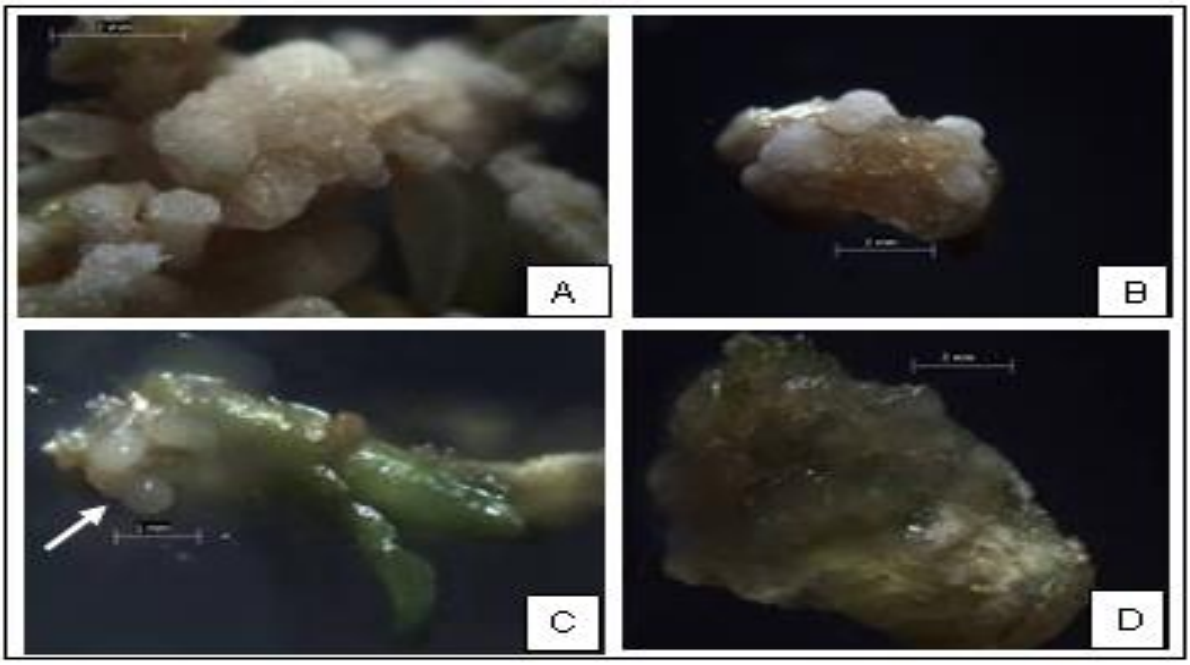

Fig. 2. Pummelo 'Nambangan' callus growth after 4 weeks culture from different explants and medium: A. Immature juice vesicle on MS containing 1 mg L^{-1} 2,4-D medium, B. Immature seed on MS containing 0.5 mg L^{-1} 2,4-D medium, C. Shoot tip on MS containing 0.5 mg L^{-1} 2,4-D medium, D. Leave segments on MS containing 1 mg L^{-1} 2.4-D medium (bar= 2 mm for A, B and C; bar = 1 mm for D; arrow=globular embryoid).

Response of different seed size on MS medium containing 3 mg L^{-1} in combination with 1 mg L^{-1} of 2,4-D after 4 weeks cultured was recorded on Table 1.

Indirect embryogenesis

Initiation of callus on indirect embryogenesis occurred on the first week of culture, whereas explants for direct embryogenesis formed embryoids after 3 weeks of culture and slightly callus initiation occurred on the fourth week (Fig. 2). Responses of forming callus were recorded after 4 weeks of culture (Table 2). Percentage of callusing explants was significant difference to treatment medium for each

kind of explants. 2,4-D clearly become an efficient plant growth regulator on pummelo callus inducing for all explants types.

2,4-D and NAA were members of plant growth regulator in auxin classes. Auxins are very widely used to promote the growth of callus (George and Sherrington, 1984).

Shoot tip explant besides of forming callus, they were capable of generating embryoid from meristematic point when cultured on medium supplemented with 0.5 mg L^{-1} 2,4-D.

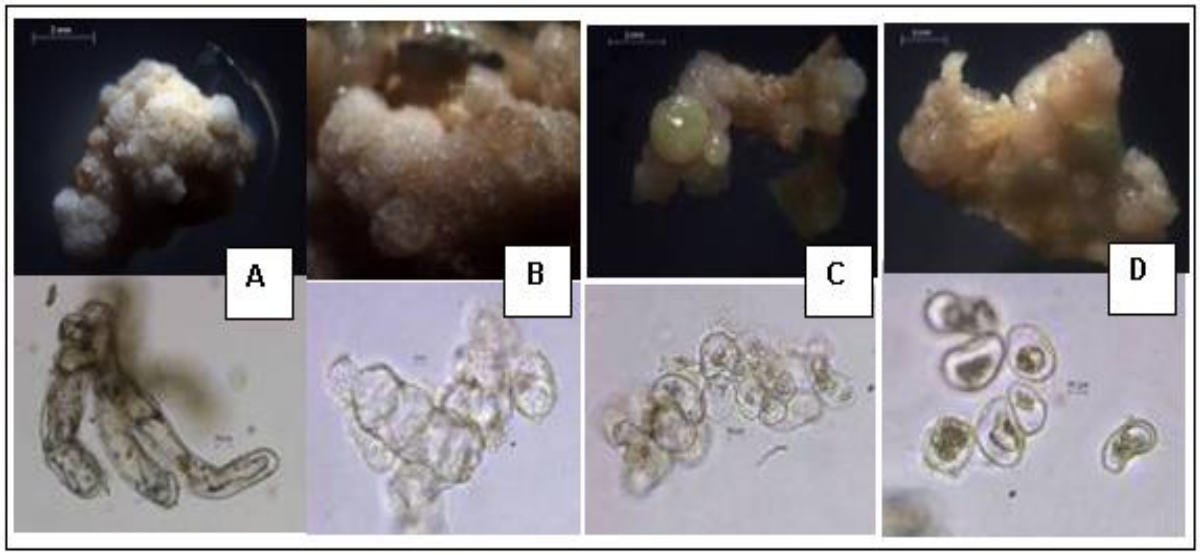

Fig. 3. Eight week-old callus of pummelo 'Nambangan' observed with stereo (top) and inverted microscope (bottom) from different explants and medium: A. Immature juice vesicle on MS containing 1 mg L^{-1} NAA medium, B. immature seed on MS containing 0.5 mg L^{-1} NAA medium, C. shoot tip on MS containing 0.5 mg L^{-1} 2,4-D medium, D. Leave segments on MS containing 1 mg L^{-1} 2,4-D medium (bar=2 mm for stereo microscope, bar= 10 µm for inverted microscope).

These results showed that auxin also involved with embryoid formation, although at low percentage with certain type of explant that is meristematic point of shoot tip culture. According to Nito and Iwamasa (1990), adventitious embryoids could arose from the callus tissue of *C. unshiu* juice vesicle when using medium containing 1 mg L^{-1} of NAA.

Callus which produced from explants, were growing until 8 weeks of culture. Observations of callus morphology with microscope were needed to detect callus embryogenic potential (Fig. 3).

The morphology of callus cells of pummelo 'Nambangan' were not uniform and depended upon the type of explants from which it was derived and upon the constituents of the medium and culture condition.

Microscopic observation showed that all types of callus were not embryogenic although it is friable (Fig. 3). Soft friable callus composed of undifferentiated and loosely attached cells, can be formed under conditions favoring rapid cell divisions (George and Sherrington, 1984).

Plantlet regeneration

Globular embryoids resulted from direct embryogenesis were proliferated and developed on the medium containing 1.0 mg L^{-1} of BAP in combination with 0.5 mg L^{-1} of 2,4-D.

It is shown that decreasing BAP concentration on this medium induced embryo maturation. Globular embryos developed onto heart, torpedo and cotyledon phase. Medium for embryo germination was MS containing 2.5 mg L^{-1} GA_3 (Merigo, 2011; Karyanti, 2013).

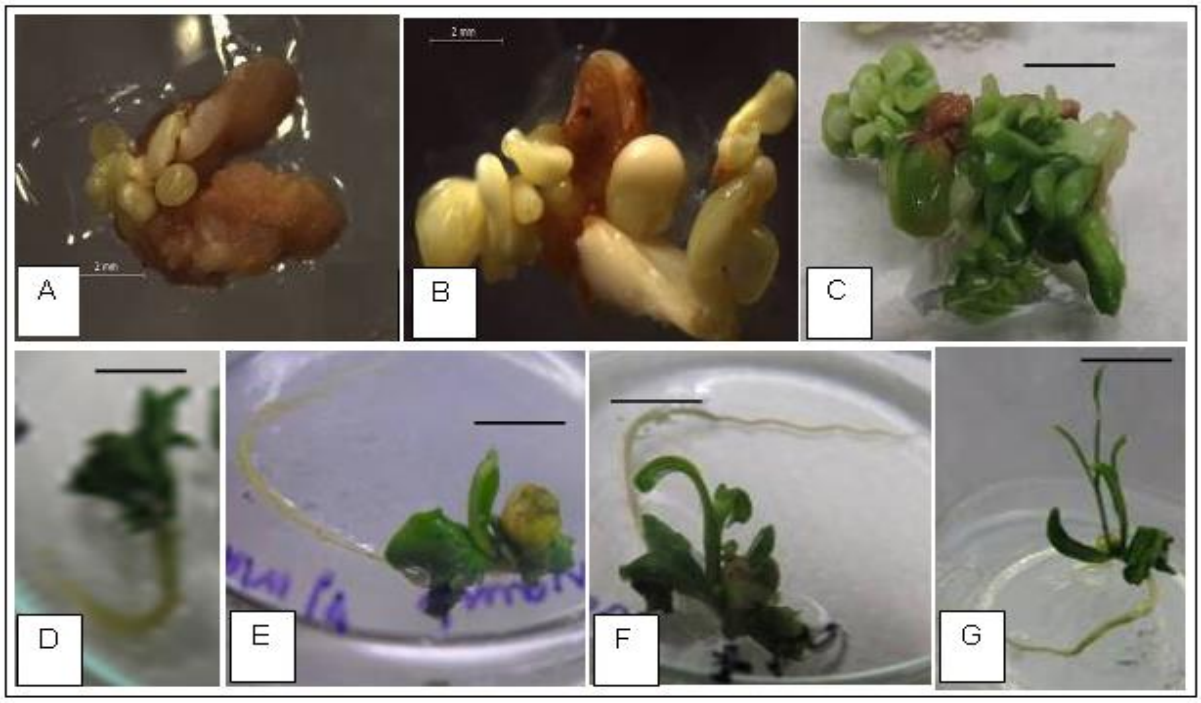

Fig. 4. Planlet regeneration process of pummelo 'Nambangan' via direct somatic embryogenesis initiated from peeled immature seeds. A and B. Embryoids produced from initiation stage on MS medium containing 3 mg L^{-1} BAP and 1 mg L^{-1} 2,4-D. C. Embyoid proliferation and maturation on MS medium containing 1 mg L^{-1} BAP and 0.5 mg L^{-1} 2,4-D. D. Embrio germination stage on MS medium containing 2.5 mg L^{-1}mg/l GA_3, root primordial formed. E. Embrio germination stage, shoot primordial formed. F. Embrio germination stage, shoot with full expanded leaves. G. Plantlet multiplication on MS containing 0.5 mg L^{-1} Kinetin. Bar = 2 mm (A and B), bar = 0.5 cm (C), bar = 1 cm (D, E and F), bar = 1.5 cm (G).

Regeneration of pummelo embryoids into plantlets is presented on Fig. 4. Pummelo somatic embryo produced root and shoot primordial within the first week of culture and developed further within 2 weeks and formed plantlets with fully expanded leaves within 6 weeks. Regeneration in pummelo 'Nambangan' was needed decreasing BAP concentrations for developing cotyledonary embryo, although forming many fused embryos (Fig. 3C). Rangan *et al.* (1969) also showed regenerated embryos of 3 monoembryonic citrus in all shapes and sizes and occasionally with fused callus formation at their radicular end. GA_3 applications is required for root and shoot development during embryo germination in citrus (Kochba and Spiegel-Roy, 1977). Nito and Iwamasa (1990) reported that 1 mg L^{-1} of GA_3 regenerated callus embryogenic of *C. unshiu* into plantlets. In pummelo 'Nambangan', 2.5 mg L^{-1} of GA_3 served as a dormancy breaking substance in meristems points of cotyledonary somatic embryos and induced plantlet regeneration (Fig. 4D-F). Plantlets could be propagated on MS medium containing 0.5 mg L^{-1} Kin (Fig. 4G).

Plantlet ploidy analysis

After undergoing a long process from embryoid induction, proliferation, maturation and germination with a number of plant growth regulators in varying concentrations, embryos regenerated into shoots. They formed ribbon-like leaves (lingualis) that emerged from plurycotyly embryos. Histogram of flow cytometry gave information that the regenerants were diploid consistent with standard diploid plants (Fig. 5).

This means that all regenerants were genetically normal plants. If genetic instability occurred, it could cause ploidy alteration and will affect growth of plantlets (Grosser *et al.*, 1997). Flow cytometric analysis is also powerful enough to analyze variability in various tissue culture-derived lemon plant

population combining with RAPD marker (Orbovic *et al.,* 2008) as well as to detect somaclonal variants in somatic embryogenesis-regenerated plants of *Vitis vinifera* combining with microsatellite marker (Prado *et al.,* 2010) and to assess genetic stability in regenerants of blackberry (Vujovic *et al.*, 2009).

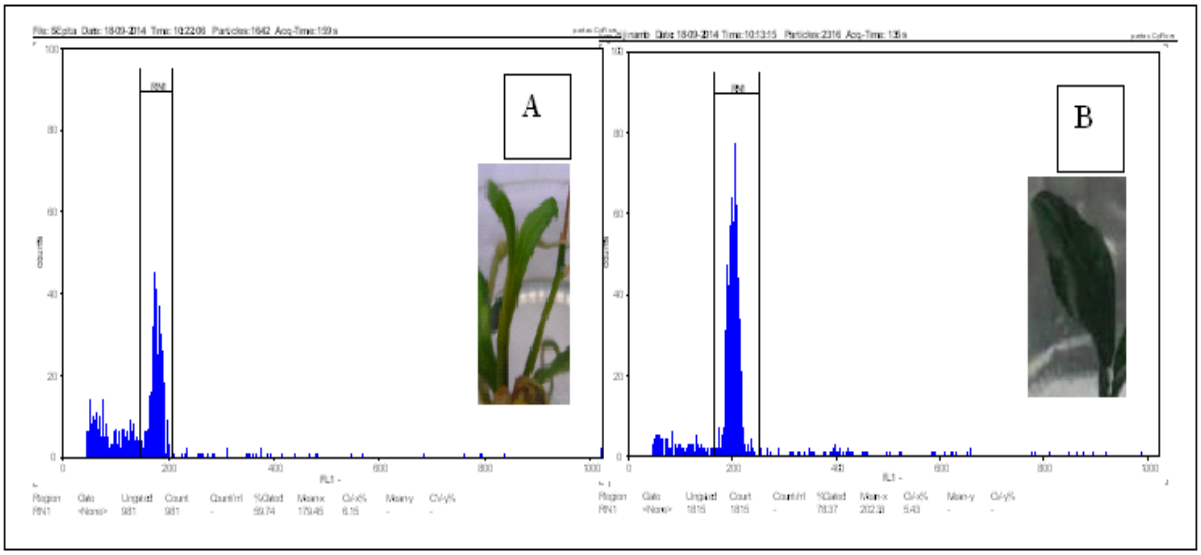

Fig. 5. Histogram of flow cytometric analysis of pummelo 'Nambangan' shows diploid level. A. ribbon-like leaf (abnormal) from direct somatic embryogenesis shoot, B. leaf from seed germination shoot (control). [X axis was value of Nuclear DNA content and Y axis was value of cell count].

Conclusion

Direct somatic embryogenesis of pummelo 'Nambangan' were successfully initiated from shoot tip and peeled immature seed explants. Meristematic point of shoot tip formed somatic embryos cultured on MS medium containing 0.5 mg L^{-1} 2,4-D, peeled immature seed with diameter of 4-6 mm formed somatic embryos on MS medium containing 3 mg L^{-1} of BAP and 1 mg L^{-1} of 2,4-D. Embryoids proliferated on medium MS containing 1 mg L^{-1} of BAP and 0.5 mg L^{-1} of 2.4-D reached cotyledonary stage and regenerated into plantlets on MS medium containing 2.5 mg L^{-1} of GA_3 after 2 weeks in culture. Diploid regenerants were obtained. All of the above protocol is promising to establish somatic embryogenesis on monoembryonic citrus species.

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