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An *In Vitro* Endosperm Culture of Anthurium

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ABSTRACT

A new route of in vitro endosperm culture of *Anthurium andreanum* Linden ex André aiming to explore feasibility production of triploid plants with more vigorous vegetative growth, superior quality of leaves, larger flower and more colorful was successfully established. Regenerative callus up to 100% with + callus score was determined on Vacin and Went (VW) medium (1949) fortified by 0.75 mg/l thidiazuron (TDZ) and 0.15 mg/l benzylaminopurine (BAP) after periodical subcultures on half-strength Murashige and Skoog (MS) medium (1962) supplemented with 0.12 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.75 mg/l TDZ and 0.5 mg/l BAP. Mature endosperm explants were sliced longitudinally into 2-4 parts, followed by removing embryo in the basal position, wounded their surfaces by tissue culture blade, cultured in the media, incubated in dark for one month, then transferred to light incubation in 12 h photoperiod under cool influorescent lamp with ~ 13 µmol/m^2/s, 24±1°C and 60.6 ± 3.8 relative humidity for next one month to increase callus regeneration potency of them. High regeneration of shoots was proved in New Winarto Teixeira da Silva (NWT) medium supplemented with 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l α-naphthalene acetic acid (NAA) and increased gradually from 3.6 - 13.5 shoots per callus incubated after periodical subcultures. The shoots were easily rooted on NWT medium containing 0.06 mg/l 2,4-D, 0.37 mg/l TDZ, 0.5 mg/l BAP and transferred ex vitro in a mixture of burned-rice husk, raw rice husk and organic manure (2:2:1, v/v/v) with acclimatization process resulting in 48-74% of survival plantlets. Based on chromosome and chloroplast counting, the acclimatized-plants derived from the in vitro endosperm culture successfully produced 5-12.5% or 2-4 triploid plants out of 22-37 survival plantlets. The research results can be applied and improved for producing triploid plants on different anthuriums.

Introduction

Anthurium (*Anthurium andreanum* Linden ex André) in one of important cut flowers and potted plants in Indonesia. The plant is widely cultivated commercially in several floriculture agribusiness area throughout the country. Total anthurium cut flower production per year reached 2.8 million stems in 2017 with 173,266 m^2 total cultivation area (Statistic Indonesia and Horticulture Directorate General, 2017), however there is no information about total number of potted plants produced per year. As the bigest anthurium grower in Indonesia, Ekakarya Graha Flora Ltd.

successfully produced 24,000 to 30,000 pots per year that were sold in high prices up to US $ 5.54. High economical value of the plant and increasing its market demand also lead to increasing customer need on new superior varieties (NSV) of the plants. To incresae and produce the high NSV as new alternative choices for Indonesian customers and markets, enhancing breeding activities shall be addressed.

Development of the NSV for anthurium can be resulted from conventional breeding through sexual breeding of two selected parental plants (Collette, 2004; Franz, 2007; Henny *et al.,* 2008) and cross breeding via mutation using gamma radiation (Puchooa and Sookun, 2003; Puchooa, 2005) and Agrobacterium-mediated transformation (Chen *et al.,* 1997; Kuehnle *et al.,* 2001; Zhao *et al.,* 2010). These methods were generally produced diploid regenerants characterized by higher results on percentage of seed germination, root length, seedling height with more vital and well-grown (Yildiz, 2013). While producing triploid plants that have more vigorous vegetative growth, faster growth rate, superior quality of leaves with higher photosynthetic capacity, larger flower and more colorful, larger organs and greater biomass, higher disease resistance, etc are a few on anthurium (Thomas and Chaturverdi, 2008; Wang *et al.,* 2016). Winarto et al. (2011ab) reported that triploid plants of anthurium were also successfuly produced via regenerated of callus derived from half-anther culture of *A. andreanum* 'Tropical'. Percentage of the triploid plants derived from the anther culture only reached 2.8%. However feasibility production of the triploid plants derived from in vitro endosperm culture in accordance to regenerate the NSV for the anthurium, so far, is not reported yet.

Triploid plants that have great economic value and have useful for developing new agronomic, horticultural and forestry plant varieties; are frequently studied via in vitro culture of endosperm (Wang *et al.,* 2016). The endosperm culture in vitro, especially, is a tissue culture method applied to regenerate triploid plants from triploid tissue. Successful producing of the triploid plants derived from the method is significantly affected by genotype, sampling times and culture media (Popielarska-Konieczna *et al.,* 2013). Recently, endosperm culture in vitro was successfully reported in Euonymus alatus (Thammina *et al.,* 2011), Santalum album (Sukamto 2011), Mallotus philippienensis (Sharma *et al.,* 2012), Actinidia deliciosa var Deliciosa (Popielarska-Konieczna and Kleszcz, 2015), Taxus chinensis var. Mairei (Li *et al.,* 2016). However, there is no report published on *A. andreanum*.

Feasibility production of triploid plants for anthurium as main objective was successfully explored in the study. Finding and overcoming critical factor on each step of establishing endopserm culture of anthurium were explained clearly. Successful regenerating and acclimatizing the triploid plants were evaluated and confirmed via chromosome and chloroplast counting methods.

Materials and Methods

Plant material and explant preparation

Anthurium andreanum Linden ex Andre´ with dark red spathe' was used as donor plants (Figure 1A). The donor plants were grown and maintained in a glass house as descibed in Winarto et al. (2011a). The *A. andreanum* seeds were prepared by sibling pollination between the donor plants both used as male dan famale plants. Viable pollen harvested from the donor plants then pollinated on receptive spadix. After pollination, the spadix were covered by transparen plastic bags (15 cm in height and 10 cm in diameter) to avoid unexpected further pollination due to insects. Subsequently, the mature inflorescent fruits containing seeds were harvested ± 6 months after pollination. The mature anthurium fruits were removed from spadix gently one by one (Figure 1B). The fruit was then suppressed softly to pull out seeds from it on tissue paper followed by gently pressure with hand to remove sticky and thin seed cover (Figure 1C). Furthermore the seeds were

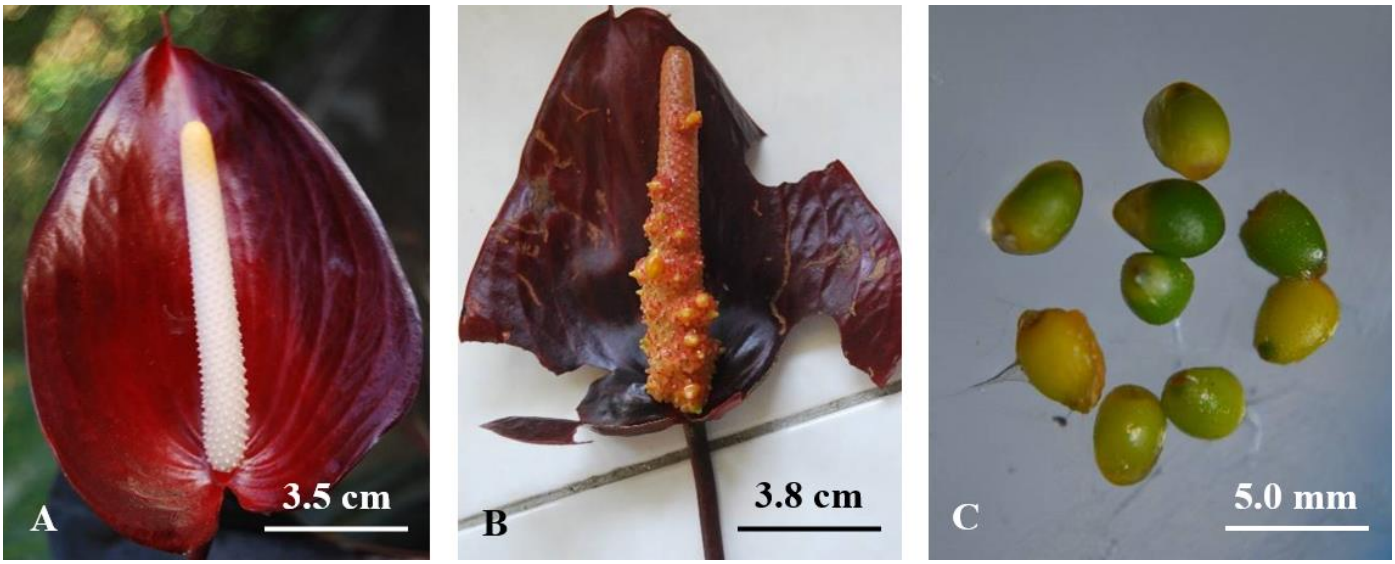

Figure 1. Morphological performance of flower donor plant, fruits and explant sources. (A). A. andreanum with dark red spathe used as donor plant, (B) inflorescence with mature fruits and (C) seeds harvested from mature fruits used as explant sources.

Table 1. Effect of different basal media and concentration of TDZ on callus formation derived from mature endosperm explants.

Initiation media	**Percentage of explant regeneration (%)**	**Percentage of explant browning (%)**	**Score of callus formation**	**Diameter of callus* (mm ± SD)**
WT medium + 1.5 mg/l TDZ and 0.15 mg/l BAP	100 a	0.0 c	+/++	**4.2 ± 0.25**
WT medium + 0.75 mg/l TDZ and 0.15 mg/l BAP	0.0 b	0.0 c	-	-
WT medium + 0.15 mg/l BAP	75.0 a	0.0 c	+	**2.3 ± 0.12**
VW medium + 1.5 mg/l TDZ and 0.15 mg/l BAP	81.3 a	0.0 c	+	**2.3 ± 0.15**
VW medium + 0.75 mg/l TDZ and 0.15 mg/l BAP	100 a	0.0 c	+	**2.0 ± 0.12**
VW medium + 0.15 mg/l BAP	100 a	0.0 c	+	**1.4 ± 0.09**
½ MS medium + 1.5 mg/l TDZ and 0.15 mg/l BAP	0.0 b	81.3 a	-	-
½ MS medium + 0.75 mg/l TDZ and 0.15 mg/l BAP	0.0 b	47.8 b	-	-
½ MS medium + 0.15 mg/l BAP	0.0 b	0.0 c	-	=
Coefficient variation (CV, %)	10.39	15.81		

* Diameter of callus was measured directly on callus placed on sterile medium using sterile Krisbow vernier caliper. Total regenerated-callus measured were 45-60 callus clumps.

washed under running tap water. After harvesting of seeds, the seeds were sterilized by immersing them in 1 % sodium hypochloride (NaOCl) for 10 min, 2 % NaOCl for 5 min, 80 % alcohol for 30 seconds, followed by 5–6 rinses in sterile distilled water (5 min each rinse).

Mature endosperm explants was prepared by slicing the sterile explant longitudinally into 2-4 parts depending the size of endosperm. Embryos attaching in the basal position of the sliced-mature endosperm explant were then removed carefully using tissue culture blade. Small basal and top parts of each piece of explant were cut using tissue culture blade to increase callus regeneration potency. The explants were then cultured on different initiation media.

Initiation of callus derived from endosperm under different culture media

Initiation of callus derived from endosperm was carried out by culturing of the endosperm explants on different combinations and concentrations of basal media and hormone and used as initiation media. The initiation media were Winarto and Teixeira (WT) medium (Winarto *et al.*, 2011a) supplemented with 1.5 mg/l TDZ and 0.15 mg/l BAP; WT medium containing 0.75 mg/l TDZ and 0.15 mg/l BAP; WT medium containing 0.15 mg/l BAP; Vacin and Went (VW) medium (Vacin and Went, 1949) fortified by 1.5 mg/l TDZ and 0.15 mg/l BAP; VW medium added by 0.75 mg/l TDZ and 0.15 mg/l BAP; VW medium containing 0.15 mg/l BAP; half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1.5 mg/l TDZ and 0.15 mg/l BAP; half-strength MS medium augmented with 0.75 mg/l TDZ and 0.15 mg/l BAP and half-strength MS medium fortified by 0.15 mg/l BAP. All the combination media were supplemented by 0.15 mg/l BAP, 20 g/l sucrose and 1.8 g/l gelrite. After preparation and adjustment pH of the media at 5.8, they were poured in the small bottles (5 cm height and 4.5 cm in diameter), 10 ml per bottles and then autoclaved in 121°C at 15 kPa for 20 minutes. The experimant was arranged in a randomized complete block design (RCBD) with four replications due to unsufficiency number of explants prepared for once proccess of experimen. Each treatment consisted of 3 bottles and each bottle contained 5 explants cultured. Total explants per treatment were 60 explants, while total explants used in the experiment were 540 explants. The cultured-explants were incubated in the dark for one months, then transferred to light incubation in 12 h photoperiod under cool influorescent lamp with ~ 13 µmol/m^2/s, 24±1°C and 60.6 ± 3.8 relative humidity for one month. Variebles observed in the experiment were (1) percentage of explant with callus (%), (2) percentage of browning explant (%), (3) score of callus formation, where -, there is no callus formation; +, 1-25% callus formation; ++, 26-50% callus formation; +++, 51-75% callus formation and ++++, more than 75% callus formation compared to total explant area and diameter of callus (mm). Periodical observation was carried out to evaluate explant changes during incubation. Observations were made after two month of culture.

Shoot regeneration of callus derived from endosperm explants

Optimation of hormone concentrations and combinations

From the preliminary studies, it was determined that callus initiated on VW medium containing 0.75 mg/l TDZ, 0.15 mg/l BAP, 20 g/l sucrose and 1.8 g/l gelrite was successfully regenerated and produced inital shoots on half-strength MS medium supplemented with 0.12 mg/l 2,4-D, 0.75 mg/l TDZ, 0.5 mg/l BAP, 10 mg/l maltose and 1.8 g/l gelrite after periodical subcultures. The medium was then optimized by reducing and eliminating concentration of 2,4-D and TDZ; adding Kinetin (Kin) and 2-isopentenyladenine (2-iP) as the regeneration media. In the experiment, the regeneration media in the first experiment were half-strength MS medium supplemented with 0.12 mg/l

Table 2. Effect of gradual reduction of TDZ and 2,4-D concentration and substitution them with Kin and 2.iP on shoot formation of callus derived from mature endosperm explants

Regeneration media	Percentage of shoot regeneration (%)	Number of initial shoots per explant	Number of shoots per explant	Height of shoots (cm)	Number of leaves per shoot
½ MS medium + 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP	13.3 b	11.3 b	1.5 bc	0.8 bc	2.3 cd
½ MS medium + 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP	18.2 ab	16.3 ab	2.8 ab	1.1 ab	4.9 ab
½ MS medium + 0.03 mg/l 2,4-D, 0.17 mg/l TDZ and 0.25 mg/l BAP	21.8 a	11.8 ab	2.5 ab	1.1 ab	3.5 bc
½ MS medium + 0.08 mg/l TDZ, 0.25 mg/l BAP, 0.5 mg/l Kin	13.6 b	9.3 b	1.3 bc	0.7 bc	2.1 cd
½ MS medium + 0.25 mg/l BAP and 1.0 mg/l Kin	0.0 c	6.2 b	0.0 c	0.0 c	0.0 d
½ MS medium + 0.25 mg/l BAP and 1.0 mg/l 2-iP	17.4 ab	22.0 a	4.0 a	1.4 a	6.1 a
½ MS medium + 0.25 mg/l BAP	16.4 ab	14.5 ab	2.5 ab	1.0 ab	3.6 bc
Coefficient variation (CV, %)	13.94	14.69	15.60	14.13	12.67

Table 3. Effect of basal media and gradual reduction of TDZ and 2,4-D concentration on shoot formation of callus derived from endosperm explants.

Regeneration media	Percentage of shoot regeneration (%)	Number of initial shoots per explant	Number of shoots per explant	Height of shoot (cm)	Number of leaves per shoot
NWT medium + 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA	27.9 a	58.0 a	3.6 a	1.6 a	6.5 a
NWT medium + 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP	24.1 b	24.3 b	2.1 b	1.2 b	3.7 b
NWT medium + 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP	16.9 c	21.5 b	1.6 c	0.8 c	2.4 bc
WT medium + 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP	5.0 d	17.5 b	0.3 d	0.3 d	1.3 c
WT medium + 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP	5.0 d	16.0 c	0.3 d	0.3 d	1.3 c
½ MS medium 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP	5.0 d	20.0 b	0.3 d	0.2 d	1.0 c
½ MS medium 0.06 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP	14.1 c	16.5 b	1.2 c	0.7 c	2.0 bc
Coefficient variation (CV, %)	17.79	18.01	13.02	14.57	13.45

2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP; 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP; 0.03 mg/l 2,4-D, 0.17 mg/l TDZ and 0.25 mg/l BAP; 0.08 mg/l TDZ, 0.25 mg/l BAP and 0.5 mg/l Kin; 0.25 mg/l BAP and 1.0 mg/l Kin; 0.25 mg/l BAP and 1.0 mg/l 2-iP and 0.25 mg/l BAP. All the combinations were fortified by 10 mg/l maltose and 1.8 g/l gelrite.

Optimation of media

In the second experiment, the regeneration media tested in the experiment were (1) New Winarto-Teixeira (Winarto *et al.*, 2011a; NWT) medium supplemented with 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA, (2) NWT medium augmented with 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP, (3) NWT medium fortified by 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP, (4) WT medium containing 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP, (5) WT medium added by 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP, (6) half-strength MS medium 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP and (7) half-strength MS medium 0.06 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP. All the media were supplemented with 10 g/l maltose and 1.8 g/l gelrite. The media were prepared and light incubation applied as described in the previous experiment. The two experiments were arranged in the RCBD with four replications due to limitaion number of callus clumps to carried out the experiment in once process. Each treatment consisted of 2 bottles and each bottle contained 5 callus-clumps cultured. Total callus clumps in each treatment were 40 clumps of callus, and total callus clumps in the first and second experiment were 560 callus cluamps (@ 280 each). Variables observed in the both experiments were (1) percentage of shoot regeneration (%), (2) number of initial shoots per explant and (3) number of shoots per explant. Periodical observation was carried out to evaluate explant changes during incubation. The observations were carried out 2 months after culture.

Plantlet preparation and its acclimatization

Plantlet for acclimatization in the study was prepared by culturing well-grow shoots on selected media as rooting media, i.e. NWT medium supplemented with (1) 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA and (2) 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP. The media was fortified by 10 g/l maltose, 2 g/l activated charcoal (AC) and 1.8 g/l gelrite. The experiment was arranged in RCBD with 10 replications. Each treatment consisted of 2 bottles (13 cm in height and 7 cm in diameter) and each bottle contained 8-10 shoots cultured. Total shoots cultured in each treatment were 160-200 shoots, while total shoots used in the stage were 320-400 shoots. Variables observed in the experiment were (1) number of roots per explant and (2) root length (cm). Periodical observation was carried out to observe explant alteration during incubation. Observations were carried out 1.5 month after culture. While acclimatization of plantlets was carried out as described on Winarto et al. (2011a). Each plastic tray was planted 50 plantlets and replicated 5 times. A total of 250 plantlets (5 trays) originating from the rooting experiment were acclimatized in the study with no experimental design applied due to there was no treatment tested.. Variables observed in the experiments were (1) percentage of survival plantlet (%) and (2) number of survival plantlets. Recording the variables was carried out 1.5 months after acclimatization.

Ploidy analysis

All plants derived from the mature endosperm culture successfully transferred to ex vitro condition were then analized their ploidy level. To confirm ploidy level of them, two direct analysis methods were applied i.e. chromosome and chloroplast counting methods as described on Winarto *et al.*, 2010. In each method, there were at least 10 cells observed and replicated 5 times. Well-chromosome and chloroplast cell performances were photographed under Nikon microscope type 104 on

Table 4. Effect of rooting media on root formation

Rooting media	Number of roots per shoot	Root length (cm)
NWT medium + 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA	1.7 b	0.6 a
NWT medium + 0.06 mg/l 2,4-D, 0.37 mg/l TDZ and 0.5 mg/l BAP	2.5 a	0.4 b
Coefficient variation (CV, %)	16.61	4.47

Table 5. Ploidy analysis of acclimatized-plants derived from mature endosperm explants

Explant source	Morphological Characteristics	Number of plants tested	Results	Number of chromosome	Number of chloroplast in stomatal guard cells
Donor plants	-	3	3 diploid	30	30
Hypothesized-diploid plants	Slow growth with low leaf length and width ratios	15	15 diploid	29-32	29-33
Hypothesized-triploid plants	Faster growth with high leaf length and width ratios	13	13 triploid	46-48	45-49

400 times maginification using Nikon D3100. Selected photos were used for the manuscript.

Data analysis

All data generated from the study were analyzed by two-way analysis of variance (ANOVA). Significant differences between means were assessed by Tukey's test at P = 0.05 (Mattjik and Sumertajaya, 2006).

Results and Discusion

Initiation of callus derived from endosperm under different culture media

Observation for callus initiation recorded twice a week was revealed that initial callus formation was obviously observed 19-25 days after culture (Figure 2B). The callus then grew and increased continually in size and diameter until the end of experiment (Figure 2C). Explant regeneration in the study was from 0-100% with 0-81.3% explant browning and – to ++ callus score. In the study, there was low regeneration capacity of callus derived from mature endosperm explants. Though percentage of explant regeneration reached 100% and 81.3% explant browning, while the high callus score was up to ++.

In the callus initiation step, from 9 media tested it was clearly revealed that the highest medium for callus initiation was established on WT medium supplemented with 1.5 mg/l TDZ and 0.15 mg/l BAP (Table 1). Other treatments stimulated lower results and no callus formation was recorded on WT medium + 0.75 mg/l TDZ and 0.15 mg/l BAP, half-strength MS medium + 1.5 mg/l TDZ and 0.15 mg/l BAP, half-strength MS medium + 0.75 mg/l TDZ and 0.15 mg/l BAP and half-strength MS medium + 0.15 mg/l BAP.. While high explant browning up to 81.3% was recorded on half-strength MS medium + 1.5 mg/l TDZ and 0.15 mg/l BAP.

Successful in callus initiation did not lead to easy step on regeneration of shoots. The regeneration of shoots under several experiments carried out were fail (Data not shown). In the latest experiment it was determined that callus derived from VW medium added by 0.75 mg/l TDZ and 0.15 mg/l BAP was successfully regenerated and produced initial shoots up to 7 initial shoots on half-strength MS medium supplemented with 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP (Figure 2D). The callus with initial shoots was multiplied for the next study.

Shoot regeneration of callus derived from endosperm explants

Gradual reduction of TDZ and 2,4-D concentration and substitution them with Kin and 2.iP

In the regeneration stage of shoot under periodical observation it was clearly known that initial shoots developed to produce new leaves 17-23 after subculture of callus (Figure 2D). The regenerated-shoots grew continually followed by increasing number and size of leaves and heigth of shoots. In the end of experiment, number of shoots, number of leaves, heigth of shoots were varied from 1-6 shoots per subcultured-callus, 1-3 leaves per shoot and 0.4-0.7 cm heigth of shoots. While percentage of shoot regeneration was from 0.0-30% derived from the first and second experiment.

Gradual reduction of TDZ and 2,4-D concentration and substitution them with Kin and 2.iP as regeneration media tested gave significant effect on inducing initial shoot formation and regenerating shoots. From the seven regeneration media, the highest percentage of shoot regeneration up to 21.8% was established on half-strength MS medium fortified by 0.03 mg/l 2,4-D, 0.17 mg/l TDZ and 0.25 mg/l BAP, however the highest number of shoots per subcultured-callus up to 4 shoots was found on half-strength MS medium containing 0.25 mg/l BAP and 1.0 mg/l 2-iP with 1.4 cm shoot height and 6.1 leaves per shoot (Table 2). Other media successfully produced 1.3-2.8 shoots per subcultured-callus with 0.7-1.1 cm height of shoots and 2.1-4.9 leaves per shoot (Figure 2E), while no regeneration of shoots was recorded on half-strength MS medium + 0.25 mg/l BAP and 1.0 mg/l Kin.

Basal media and gradual reduction of TDZ and 2,4-D concentration

Basal media and gradual reduction of TDZ and 2,4-D concentration as regeneration media in the experiment did not stimulate high effect on initial shoot induction and its regeneration. The highest percentage of shoot regeneration as high as 27.9% with 3.6 shoots per subcultured-callus with 1.6 cm shoot height and 6.5 leaves per shoot was established on NWT medium supplemented with 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA (Table 3). Though the medium had lower regeneration shoot capacity compared to half-strength MS medium supplemented with 0.25 mg/l BAP and 1.0 mg/l 2-iP; the medium successfully multiplied and increased number of regenerated shoots significantly from 3.6 till 13.6 shoots per callus subcultured periodically (Figure 2F and 2G). While other basal media and reduction of TDZ and 2,4-D concentrations caused reduction capacity on shoot regeneration with the lowest results noted on half-strength MS containing 0.06 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP.

Plantlet prepration and its acclimatization

Plantlet preparation was not a problem in in vitro culture of anthurium as well as noted in the study. High number roots produced per shoot up to 2.5 roots were successfully rooted on NWT medium containing 0.06 mg/l 2,4-D, 0.37 mg/l TDZ, 0.5 mg/l BAP with 0.4 cm root length (Table 4; Figure 2F). Successful plantlet acclimatization on a mixture of burned-rice husk, raw rice husk and organic manure (2:2:1, v/v/v) was also observed in the experiment. All treatments in the acclimatization stage resulted

in 48-74% of survival plantlet with 59.6% in average.

Ploidy analysis

Based on previous chromosome studies, it was revealed that somatic chromosome numbers of *A. andreanum* were 2n=2x=30 (Cotias-de-Oliveira et al 1999; Mohamed *et al.*, 2006) and 2n=2x=30 + 1-4B in special case of another anthurium. Winarto et al. (2010) determined that number of chromosome and chloroplast on acclimatized-plants derived from anther culture were varied with 17.13 ± 1.81 and 17.38 ± 1.56 for haploid plants, 30.1 ± 2.02 and 30.4 ± 1.63 for diploids, and 51.5 ± 3.03 and 50.2 ± 2.15 for triploid plants. Similar evidents, it was revealed that acclimatized plants derived from mature endosperm tissue of anthurium also resulted two varied-regenerants both hypothesized-diploid and triploid plants (Figure 2G). Percentage of hypothesized-triploid plants was varied from 5.4-12.5% with 9.6% in average. Thirteen plants indicated faster growth with high leaf length and width ratios hypothesized as triploid plants were analyzed their ploidy and revealed that the thirteen plants had chromosome number of 2n=3x=46-48 (Table 5; Figure 2K) with 45-49 number of chloroplast (Table 5; Figure 2L). While fifteen plants out of 136 hypothesized-diploid plants having slow growth with lower leaf length and width ratios was tested and resulted that the fifteen plants had 29-32 number of chromosome (Table 5; Figure 2I) with 29-33 number of chloroplast (Table 5; Figure 2J). Ploidy analysis of donor plant as control indicated that chromosom number of the plant was 2n=2x=30 (Table 5; Figure 2H).

From this research, an in vitro mature endosperm culture route for anthurium was successfully established. The route was started from callus initiation followed by callus regeneration, shoot rooting, plantlet acclimatization and ploidy analysis. The critical point in the route was determined on callus initiation and its regeneration. In the study, the point resulted in low rate of the callus initiation and regeneration. Lower result was also recorded on acclimatization step when was compared to the previous studies (Winarto *et al.*, 2011b and Winarto and Teixeira da Silva, 2012). The results of this study gave evidents that callus initiation and shoot regeneration were critical steps on establishing endosperm culture of anthurium. The results were also strengthened the previous studies carried out on Azadirachta indica (Chaturverdi *et al.*, 2003), Actinidia deliciosa cv Hayward (Góralski *et al.*, 2005), Carica papaya (Sun *et al.*, 2010), Santalum album (Sukamto, 2011) and Euonymus alatus (Thammina *et al.*, 2011)

Factor controlling callus proliferation and regeneration in establishing endosperm culture protocol closely related to development stage of seed and culture initiation media (Thomas and Chaturverdi, 2008). Mature endosperm of A. deliciosa cv. Hayward cultured on MS medium supplemented with 2 mg/l 2,4-D and 5 mg/l kinetin developed callus with 80% efficiency (Góralski *et al.*, 2005), 14% of mature endosperm explants formed compact, green calli after culture in the dark for 8 weeks and then under light for 4 weeks on MS medium augmented with 0.5 mg/l BA and 0.5 mg/l NAA (Thammina *et al.*, 2011). While in the study, mature endosperm explants of *A. andreanum* regenerated high callus initiation with 100% explant formation and +/++ callus score on WT medium supplemented with 1.5 mg/l TDZ and 0.15 mg/l BAP.

Shoot regeneration of callus derived from endosperm tissue is critical factor in establishing endosperm culture. In the study, though high callus initiation recorded on WT medium supplemented with 1.5 mg/l TDZ and 0.15 mg/l BAP, high regenerative callus was produced on VW medium added by 0.75 mg/l TDZ and 0.15 mg/l BAP that was subcultured on half-strength MS medium supplemented with 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP. The high shoot regeneration up to 4 shoots per explant was established on half-strength MS medium containing 0.25 mg/l BAP and

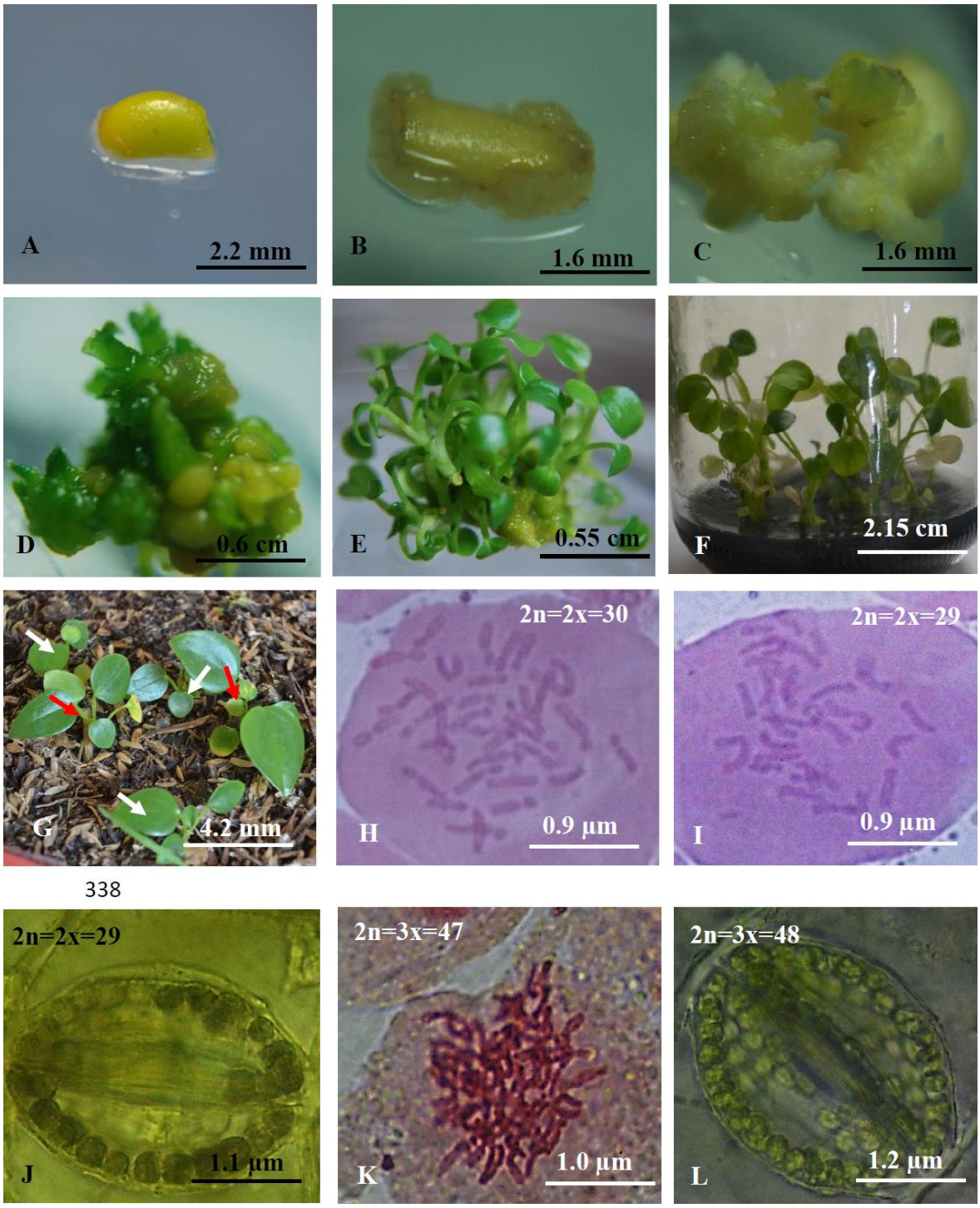

Figure 2. In vitro endosperm culture of anthurium. A. Endosperm explant condition in initial culture, B. Callus formation 20 days after culture on WT medium supplemented with 1.5 mg/l TDZ and 0.15 mg/l BAP, C. Regenerated-callus derived from multiplication stage 2.0 months after culture on half-strength MS medium supplemented with 0.12 mg/l 2,4-D, 0.75 mg/l TDZ and 0.5 mg/l BAP, D. Initial shoots developed to produce new leaves 17-23 after subculture of callus on NWT medium supplemented with 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA, E. Regenerated-shoots on the same medium 60 days after callus subcultured, F. Rooted-shoots on NWT medium containing 0.06 mg/l 2,4-D, 0.37 mg/l TDZ, 0.5 mg/l BAP 1.5 months after culture, G. hypothesized-triploid plants indicated by white arrows and hypothesized-diploid plants indicated by red arrows, H. Donor plant chromosome with 2n=2x=30, I. Chromosome of diploid plant with 2n=2x=29, J. Chloroplast number of diploid plant with 2n=2x=29, K. Chromosome number of triploid plant with 2n=2x=47, and L. Chloroplast number of triploid plant with 2n=3x=48.

1.0 mg/l 2-iP, but multiplication of shoots was established on NWT medium supplemented with 0.25 mg/l 2,4-D, 1.5 mg/l TDZ, 0.75 mg/l BAP and 0.02 mg/l NAA. Other studies reported that maximum shoot bud differentiation up to 6.2 shoots per subculture of A. deliciosa 'Hayward' were established on MS medium containing 0.5 mg/l TDZ (Góralski *et al.,* 2005) and 6.0 shoots of E. alatus were induced on MS medium with 3.0 mg/l BA (Thammina *et al.,* 2011). These studies clearly noted that successful regeneration of shoots derived from mature endosperm explant resulted in varied-results

Rooting of shoots and plantlet acclimatization were not a critical problem in the study as reported on Winarto et al. (2011a) and Winarto and Teixeira (2012). In the research, plantlets was easily prepared by culturing shoots on NWT medium containing 0.06 mg/l 2,4-D, 0.37 mg/l TDZ, 0.5 mg/l BAP. Winarto et al. (2011a) rooted shoots on NWT containing 0.2 mg/l NAA and 1.0 mg/l Kin or NWT medium hormone free (Winarto and Teixeira da Silva, 2012). While a mixture of burned-rice husk, raw rice husk and organic manure (2:2:1, v/v/v) and acclimatization process as described in Winarto et al. (2011a) and Winarto and Teixeira da Sila (2012) was successfully applied to acclimatize anthurium plantlets derived from endosperm explants. The percentage of survival plantlets were 48-74% with 59.6% in average. The results were lower than 83% on Winarto et al. (2011a) and 100% on Winarto and Teixeira da Silva (2012).

Developing in vitro endosperm culture of anthurium successfully produced triploid plants with low results between 5-12.5% or 2-4 plants out of 22-37 survival plantlets. In E. alatus 'Compactus', immature endosperm culture resulted in 0.45% of triploid plants (Thammina *et al.,* 2011). Other endosperm cultures did not clearly reported number of triploid plants produced (Chaturverdi *et al.,* 2003; Guzzo *et al.,* 2004; Góralski *et al.,* 2005; Sukamto, 2011; Sun *et al.,* 2010; Popielarska-Konieczna and Kleszcz, 2015).

Conclusion

The present study resulted in establishing endosperm culture protocol for anthurium using mature endosperm as explant sources. Critical factors noted in this study were obtaining (1) regenerative callus that was easily stimulated to produce shoots and (2) regenerated-shoots derived from the regenerative callus. Basal medium (MS, VW, WT and NWT) and application different combination and concentration of TDZ, BAP, 2,4-D and NAA had significant effect in different step of mature endosperm culture. The anthurium endosperm culture protocol was successfully applied to produce triploid plants. The protocol can be applied to induced triploid plants from different anthuriums that were important for their breeding programs. The protocol can also be used as a consideration to develop new reliable endosperm culture protocols for promising anthuriums with high economical values in commercial purposes

Conflicts of interest

We declare that there is no financial or other competing conflicts of interest dealing with authors and the Indonesian Ornamental Crops Research Institute and Indonesian Agriculture Research and Development Agency that facilitated and funded the research activity.

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