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Influence of isolation technique of half-anthers and of initiation culture medium on callus induction and regeneration in *Anthurium andreanum*

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Abstract Optimization of the isolation technique and initiation culture medium are two critical aspects that can determine the success of anthurium half-anther culture. Both aspects in half-anther culture of *Anthurium andreanum* Linden ex André cv. 'Tropical' were studied and successfully improved. Untreated half-anthers, when cultured abaxial side down on medium, was the most suitable means of inducing callus. Callus formation was further improved by culturing half-anthers adaxial side down on Winarto-Teixeira (WT) medium (Winarto et al. in Plant Growth Regul 65:513–529, 2011b) supplemented with 0.01 mg/l α-naphthaleneacetic acid (NAA), 1.0 mg/l 6-benzyladenine (BA) and 0.5 mg/l thidiazuron (TDZ). Gelrite enhanced callus formation (compared to agar) when the concentration was reduced from 2.0 to 1.5 g/l on WT medium. Application of 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) in WT medium increased callus formation. Most callus formed when half-anthers were cultured adaxial-side down on WT medium supplemented with 0.5 mg/l 2,4-D in combination with 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ using 1.5 g/l Gelrite. This ideal medium induced the growth of half-anthers, with as much as 38 % of half-anthers producing callus, or, on average, 3.4 half-anthers/treatment. Callus derived from this optimized protocol regenerated easily and could be multiplied on New Winarto-Teixeira medium (NWT) (Winarto et al. in Plant Growth Regul 65:513–529, 2011b) containing 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA, 1.5 mg/l TDZ and 2.0 g/l Gelrite. Shoots rooted well on hormone-free NWT medium with 2.0 g/l Gelrite. The plantlets could be easily acclimatized in a substrate of raw rice husk, burned-rice husk and organic manure (1:1:1, v/v/v) with a high survival (100 %) ex vitro in a greenhouse. The results of this study would benefit half-anther culture of other *Anthurium* cultivars, particularly at the initial stage of callus induction.

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Introduction

Anther culture has recently been emphasized as being an important tool to produce double haploid (DH) plants (Germaná 2011). The technique is widely applied to cereals, vegetables and fruits (Atanassov et al. 1996; Wędzony et al. 2009). DH plants derived from anthers can be used to produce pure lines and uniform F_1 hybrid seed of some commercial varieties (Seguí-Simarro and Nuez 2007; Germaná 2011; Salas et al. 2011; Seguí-Simarro et al. 2011), to reverse ploidy level to the diploid status in tetraploid interspecific hybrids (Rotino et al. 2005), for quantitative trait loci (QTL) analysis (Chen et al. 2007; Muñoz-Amatriaín et al. 2008), to determine the reciprocal effects in plant breeding (Bruins and Snijders 1995; Yildirim et al. 2008), and to study genetic manipulation in plants (Atanassov et al. 1996).

The application of anther and/or microspore culture to ornamental crops remains limited. The method was utilized

to produce homozygous lines in several ornamentals such as *Lilium longiflorum* (Arzate-Fernández et al. 1997; Han et al. 1997, 2000, 2006; Niimi et al. 2001), *Helianthus anuus* (Thengane et al. 1994; Saji and Sujatha 1998), interspecific hybrids of *Cyclamen persicum* and *Cyclamen purpurascens* (Ishizaka 1998), *Anemone coronaria* (Laura et al. 2006; Laura and Allavena 2007) and *Dianthus chinensis* (Fu et al. 2008). In the Araceae, especially in *Anthurium*, the use of the technology (except for the studies conducted by our group) has not been reported yet, although DH plant production was tested in *Spathiphyllum* via ovule culture, but with poor results (Eeckhaut et al. 2001).

In most cases, anthers were isolated by removing whole anthers from their filaments and then culturing them on various initiation media without slicing or treating them. In *Lilium longiflorum*, successful callus initiation was possible when whole anthers were cultured on N6-medium (Chu et al. 1975) (Arzate-Fernandez et al. 1997), on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 2 mg/l picloram and zeatin (Han et al. 1997; Niimi et al. 2001), or on MS medium supplemented with half-strength MS macronutrients, full micronutrients and MS vitamins, and 0.01 mg/l α-naphthaleneacetic acid (NAA) (Han et al. 2006). In *Helianthus annuus*, callus was prolific when anthers were cultured on MS basal medium supplemented with 0.2–2.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 and 1.0 mg/l 6-benzyladenine (BA) and 40 g/l sucrose (Thengane et al. 1994) and MS supplemented with 2.0 mg/l NAA and 1.0 mg/l BA (Saji and Sujatha 1998), *Cyclamen persicum* and *C. purpurascens* on B5 medium (Gamborg et al. 1968) containing 0.1 and 1.0 mg/l NAA, 0.1 mg/l 2,4-D and 90 g/l sucrose (Ishizaka 1998), and *Dianthus chinensis* on MS medium supplemented with 9 μM 2,4-D and 4.44 μM BA (Fu et al. 2008). However, in *Anthurium andreanum*, a tropical plant, culturing whole anthers on initiation culture medium resulted in the browning of anthers and no callus formation (Winarto and Rachmawati 2007; Winarto and Mattjik 2009a, b; Winarto et al. 2009; Winarto et al. 2010a, b; Winarto et al. 2011a, b).

Our group has studied anther culture of *A. andreanum* since 2003. No other group in the world has done such intensive studies on this ornamental. To date, the primary findings of these studies were: (1) *A. andreanum* Linden ex Andre cv 'Tropical' is an appropriate plant model to develop and establish an anther or half-anther culture system for *Anthurium*; (2) half-anthers are the most responsive and suitable explant to achieve this; (3) responsive anthers are isolated from the transition area of the spadix with 50 % of the stigma being receptive, indicated by a highly sticky solution (glue-like) at the tip of the stigma; (4) Winarto-Teixeira medium (WT) supplemented with 0.5 mg/l thidiazuron (TDZ), 1.0 mg/l BA and 0.01 mg/l NAA and New Winarto-Teixeira medium (NWT) containing 1.5 mg/l TDZ, 0.75 mg/l BA and 0.02 mg/l NAA are the best initiation culture media for callus induction and formation, respectively (Winarto and Rachmawati 2007; Winarto and Mattjik 2009a, b; Winarto et al. 2009; Winarto et al. 2010a, b; Winarto et al. 2011a, b). However, further improvement of the technique and culture initiation is required, and these are the foci of this study, particularly at the initiation stage, which is where the biggest risk of losing the potential to initiate and form callus is lost, primarily because of the ease with which explants can brown (i.e., oxidize) and necrose.

Several modifications of previously established half-anther culture protocols such as the application of slight pressure on half-anthers (thus releasing a portion of all microspores), testing the effect of different agars and their concentrations on callus induction, and adding 2,4-D are all novel factors of *Anthurium* tissue culture covered by this study that have not yet been studied or published.

Materials and methods

Plant material and explant preparation

Anthurium andreanum Linden ex André cv 'Tropical', a very popular cultivar in international markets, was the target plant material for our study. Moreover, several previous studies indicated this to be a model plant due to the ability of establishing an effective anther culture protocol that would serve as the basis for other *Anthurium* cultivars. Mother plants were purchased from Eka Graha Flora Ltd., Kanoman, Cianjur, West Java, Indonesia when in their first year of growth and flowering and used in experiments after the second to third flowers formed. The plants were grown in plastic bags (35 cm in diameter, 40 cm in height), with 38.5 cm³ of potting substrate, which consisted of burned rice-husk, rice husk and bamboo peat (1:1:1, v/v/v). Plants were placed in a glasshouse at 35–40 °C during the day and at 15–20 °C at night (temperature assessed by a thermo-hygrometer, Haar-Synth-Hygro, Germany), 50–90 % relative humidity during the day and 25–60 % at night, assessed with a Haar-Shynth-Hygro, and a 12-h photoperiod with 185–370 μmol/m²/s light intensity during the dry season (April to October) and 37–111 μmol/m²/s in the rainy season (November to March). Light intensity was measured using a Digital Lux Meter, Lutron LX 101 (Lutron Electronic Enterprise Co., Ltd., Taiwan). Measurement of data using the Lutron LX 101 was originally in lux but was then converted to μmol/m²/s by multiplying each data point with a conversion factor for sunlight i.e. 0.0185 (Thimijan et al. 1982). The plants were watered

with liquid fertilizer (2 g/l of N:P:K, 20:15:15; Nusa Tani, Ltd., Jakarta, Indonesia) at 3-day intervals. No pesticides, either as spray or as soil drench, were applied during the maintenance period of donor *Anthurium* plants to minimize the reduction of microspore vitality. Spadices with 50 % of their stigmas in a receptive condition—indicated by the profuse secretion of a sticky substance at the tip of the stigma—were harvested from 20.1 ± 2.03-days-old flowers (counted from when the spathe started to open) between the second and fourth flower.

Spadices were then sterilized by placing them under tap water for 30–60 min then immersed in a 1 % pesticide solution of 50 % benomyl (Benlox® 50WP, Dharma Guna Wibawa Ltd., Jakarta, Indonesia) and 20 % streptomycin sulphate (Agrept® 20WP, Mastalin Mandiri Ltd., Jakarta, Indonesia) for 30 min and rinsed with sterile distilled water 5 times (5 min each rinse). After pretreatment, spadices were sterilized by immersing them in 1 % sodium hypochloride (NaOCl, Bayclin-Johnson Home Hygiene Products Ltd., Jakarta, Indonesia) for 10 min, 2 % NaOCl for 5 min, 80 % alcohol for 30 s, followed by 5–6 rinses in sterile distilled water (5 min each rinse). Material preparation was consistent with the protocol used in our previous studies (Winarto et al. 2009, 2010a, b, 2011a, b).

After the sterilization step, one spadix was placed in a sterile Petri dish. The transition area was cut and used for anther isolation. Petals were then carefully removed and anthers were isolated using a tissue culture blade. The top part of the anther (half-anther) without the adjoining filament was isolated and cultured in initiation culture medium. All activities related to anther isolation were conducted under a Stemi SV8 stereo microscope (Zeiss, West-Germany).

Two pre-selected (based on previous success rates) initiation culture media used and tested in the experiment were WT basal medium containing 0.5 mg/l TDZ, 1.0 mg/l BA and 0.01 mg/l NAA and NWT basal medium supplemented with 1.5 mg/l TDZ, 0.75 mg/l BA and 0.02 mg/l NAA (Winarto et al. 2011b). To each medium 30 g/l sucrose and 2 g/l Gelrite (except when agar was used as a treatment) were added and the pH was adjusted to 5.8. Thereafter, 10 ml were poured into a culture vessel (5 cm in diameter; 7 cm in height; 80 ml in volume) and sterilized at 121 °C, 15 kPa for 20 min using a Pressure Steam Sterilizer Vertical Cylindrical LS. 001 (SMIC, Shanghai, China).

In a series of three experiments (detailed next), half-anthers were incubated in the dark for ± 2 months to induce callus; thereafter, callus cultures were placed under fluorescent lamps (TL-Philips, The Netherlands) under ∼13.5 μmol/m^2/s (light intensity measured with a Digital Lux Meter, Lutron LX 101) with a 12-h photoperiod, 23.5 ± 1.1 °C, and 60.6 ± 3.8 % relative humidity

(temperature and relative humidity measured by a Haar-Synth-Hygro thermo-hygrometer) to allow callus to develop and grow. Lux was converted to μmol/m^2/s using the conversion factor for cool white fluorescent lamps i.e. × 0.0135 (Thimijan et al. 1982). Callus cultures were maintained in these conditions until plantlets with fully developed shoots and roots formed.

Effect of isolation technique and type of gelling agent on callus induction

Two different treatments tested in the first sub-experiment were: (a) type of isolation method (abbreviated as IT and described in Fig. 1) and (b) type of gelling agent, abbreviated as TA. Three different isolation techniques used in both sub-experiments were: (1) half-anthers without any treatment (i.e., to which no pressure was applied) served as the control (IT-1), (2) half-anthers to which light pressure was applied with a blunt pair of forceps on the half-anther wall to release a part of the microspores (IT-2) and (3) half-anthers to which stronger pressure using the same pair of forceps was applied to the half-anther wall in order to release all the microspores, following which the half-anther wall was removed and all released microspores were subsequently cultured onto the medium (IT-3). In IT-3, 5.000–7.000 microspores could be isolated from each half-anther, based on a tenfold calculation of microspores harvested from half-anthers. The types of agar studied were: (1) Gelrite (TA-1, Duchefa, 2.0 g/l), (2) Phytagel (TA-2, Sigma, 3.0 g/l) and (3) Swallow agar (TA-3, PT Bola Dunia Walet, Jakarta-Indonesia, 7.0 g/l). The basal medium for all treatments was WT containing 0.5 mg/l TDZ, 1.0 mg/l BA and 0.01 mg/l NAA with 30 g/l sucrose.

Effects of Gelrite concentration on callus formation

IT-1 and IT-2 were tested with different Gelrite concentrations (GC): (1) 2.0 g/l (GC-1), (2) 1.5 g/l (GC-2), (3) 1.0 g/l (GC-3) and (4) 0.5 g/l (GC-4). WT containing 0.5 mg/l TDZ, 1.0 mg/l BA and 0.01 mg/l NAA with 30 g/l sucrose was used as the basal medium for all treatments. IT-1 and IT-2 were re-tested or repeated twice to confirm or verify the effect of both isolation techniques in improving callus formation in combination with different Gelrite concentrations.

Effect of anther culture position and inclusion of 2,4-D in culture induction media on callus formation

Two different treatments applied in this experiment were anther culture position (ACP, Fig. 1) and the addition of 2,4-D to induction culture media. ACPs tested in this experiment were: (1) abaxial side down on medium (ACP-
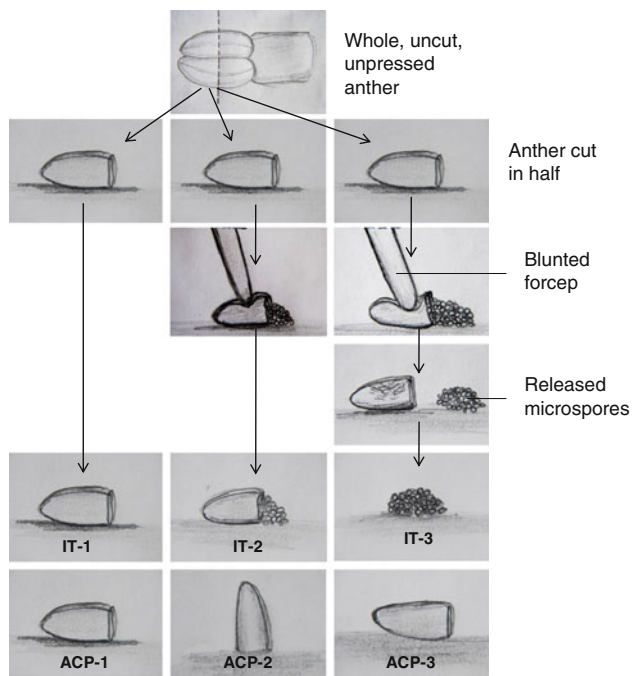
Fig. 1 Scheme of explant preparation in anther culture of *A. andreanum* Linden ex André cv. 'Tropical'. IT-1, half-anthers without any treatment, which served as the control; IT-2, half-anthers to which light pressure on the half-anther wall was applied with a blunt pair of forceps in order to release some microspores; IT-3, half-anthers to which stronger pressure using the same blunt pair of forceps was applied to the half-anther wall in order to release all of the microspores, after which the half-anther wall was removed and all the released microspores were subsequently cultured onto the medium. ACP-1, abaxial side down on medium; ACP-2, upright in medium; and ACP-3, adaxial side down on medium

1), (2) upright on medium (ACP-2), and (3) adaxial side down on medium (ACP-3). The inclusion of 0.5 mg/l 2,4-D or the lack of 2,4-D were tested in two culture initiation media, namely WT medium containing 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ, and NWT medium supplemented with 0.02 mg/l NAA, 0.75 mg/l BA and 1.5 mg/l TDZ (Winarto et al. 2011b). Both media were solidified with 1.5 g/l Gelrite. In summary, the four treatments were: (1) WT medium containing 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ (WT-D), (2) NWT medium supplemented with 0.02 mg/l NAA, 0.75 mg/l BA, 1.5 mg/l TDZ (NWT-D), (3) WT medium containing 0.5 mg/l 2,4-D, 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ (WT + D), and (4) NWT medium supplemented with 0.5 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA, 1.5 mg/l TDZ (NWT + D).

Regeneration of callus derived from half-anthers, plantlet preparation and acclimatization

Callus derived from half-anthers from any experiment was regularly subcultured every 1.5–2.0 months on NWT

medium supplemented with 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ, 0.75 mg/l BA and solidified with 2 g/l Gelrite (Winarto et al. 2011b) and incubated under light. In the first subculture, callus clumps had an average size of $2 \times 1.5 \times 2$ mm (l × w × h), $4 \times 3.5 \times 3.5$ mm in the second subculture, and $5 \times 5 \times 5$ mm from the third until the eighth subculture. Eight subcultures were needed for shoots to regenerate from callus. Well-developed shoots (1.5–2.0 cm in height with 2–3 leaves) were harvested from regenerated shoots produced from the eighth subculture and cultured on hormone-free NWT medium to prepare only the best plantlets suitable for acclimatization. The cultures were incubated under ∼ 13.5 µmol/m²/s light intensity in a 12-h photoperiod, 23.5 ± 1.1 °C, and 60.6 ± 3.8 % relative humidity for 2 months. Well-rooted shoots were acclimatized as described in Winarto et al. (2011b).

Experimental design, parameters assessed and data analysis

A multifactorial experiment was arranged using a randomized complete block design with four replications. Each treatment consisted of 3 bottles, each of which contained 6 half-anthers (or clusters of microspores in the first experiment). All cultures were incubated in the dark for 2 months; thereafter, the cultures were placed in the light.

Parameters observed in all experiments were: (1) percentage of half-anthers producing initial callus (PHAPC, %) and (2) average number of half-anthers producing callus per treatment (NHAPC). PHAPC was assessed after 2 months while NHAPC was assessed after 3 months. PHAPC was the total number of half-anthers that produced initial callus compared to total number of half-anthers cultured in any treatment. The PHAPC was important due to the fact that there were half anthers produced initial callus died due to explant browning. NHAPC was the number of half-anthers that produced callus and continued to grow without browning symptoms that caused them died due to necrosis.

Quantitative data in all experiments 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

Effect of different isolation methods and types of agar on callus induction

Following regular periodic observations, callus initiation was obvious 20–35 days after culture initiation. It was rare

to find all anthers (6 half-anthers/bottle) alive. In general, some of them survived, continued to grow and produced callus while the remainder turned brown and finally necrosed (Fig. 2a). Three months after culture initiation, approximately one-third of the callus clump was able to proliferate.

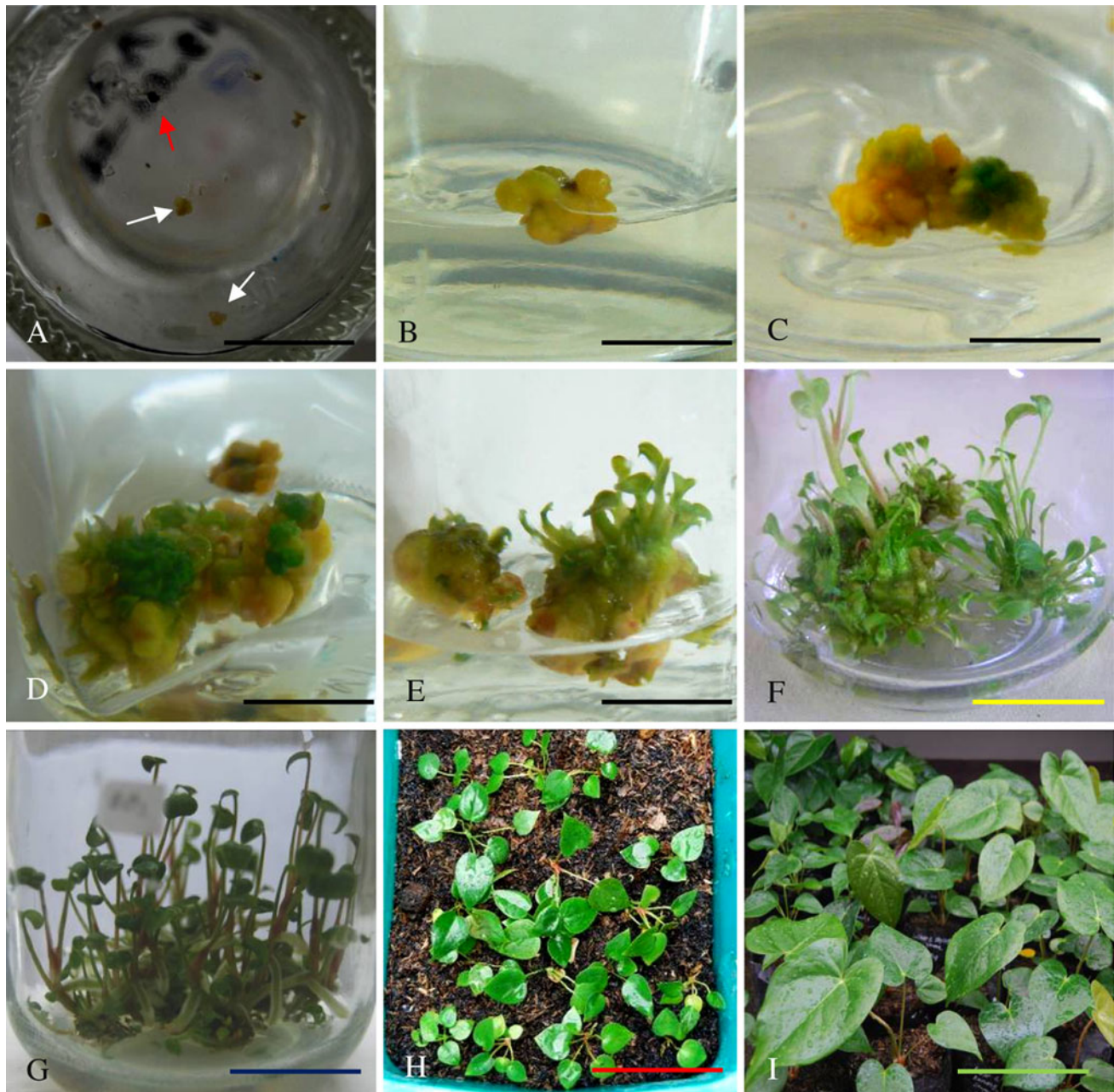The isolation technique (IT-1, IT-2 and IT-3) and type of agar (GA-1, GA-2 and GA-3) significantly affected

Fig. 2 Regeneration of callus derived from *Anthurium andreanum* Linden ex André cv. 'Tropical' half-anthers until acclimatization. **a** Callus derived from half-anther two months after culture initiation after incubation in the *dark*. Part of the callus continued to grow and produced more callus (*white arrows*) while another part of turned *brown* and finally necrosed (*red arrow*). **b** Callus growth 2.5 months after incubation in light. **c** Callus growth 4 months after incubation in light. **d** Initial shoot regeneration 5.5 months after incubation in light. **e** Shoots regenerating from half-anther-derived callus 1.5 months after first subculture. **f** High number of regenerated-shoots 2 months after the second subculture. **g** Well-rooted shoots derived from the fifth subculture 2 months after culture. **h** Acclimatized-plantlets in a plastic *box* containing a mixed medium of raw rice husk, burned-rice husk, and organic manure (1:1:1, v/v/v). I. Variation in individual plants derived from half-anther culture in the same potting medium 4 months after acclimatization. *Black bars* = 0.55 cm, *yellow bar* = 1.75 cm, *blue bar* = 2.0 cm, *red bar* = 4.5 cm, *green bar* = 11.5 cm. (Color figure online)

callus formation, although there was no interactive effect between both treatments. Development of a new isolation technique by lightly pressing the half-anther wall to release a part of the microspores (IT-2; observe Fig. 1) did not, in fact, have a positive effect on increasing the number of half-anthers that produced callus. Rather, this treatment increased contamination by between 17 and 50 %, relative to IT-1. Simultaneously, the anther became brown and died in response to the damage to the anther wall. In contrast, when clusters of microspores (IT-3) were placed on semi-solid medium after having been released by a greater force, little or no contamination was observed. Moreover, growth of microspores was observed 5–10 days after culture initiation and no callus formed from the microspore clusters until the end of the observation period i.e. 2 months of culture. Half-anthers without treatment (IT-1, i.e., the control) was the most appropriate isolation technique for anthurium half-anther culture (Table 1) since callus formation was significantly higher than in other isolation techniques. In IT-1, the growth of anther wall cells of half-anthers was stimulated as much as 7 % PHAPC and 1.1 NHAPC (Table 1). Callus formation was significantly reduced in IT-2 (0.6 NHAPC) and IT-3 (0 NHAPC).

The choice of agar significantly affected callus formation. Gelrite (TA-1) and Phytagel (TA-2) almost equally stimulated both parameters significantly more than Swallow agar (TA-3) although TA-1 tended to give higher values than TA-2 with 7 % PHAPC and 1.1 NHAPC (Table 2); TA-3 showed the poorest callus formation.

Effect of isolation technique and Gelrite concentration on callus formation

The choice of isolation technique and a reduction in Gelrite concentration had a significant effect on callus formation although there was no interaction effect. When IT-1 and IT-2 were re-tested, IT-1 (the control) showed to be the most appropriate isolation technique in anthurium half-

Table 1 Effect of isolation technique on callus induction in anther culture of *Anthurium andreanum* Linden ex André cv. 'Tropical'

Isolation technique (IT)	Percentage of half-anthers producing callus (PHAPC, %)	Number of half-anthers producing callus per treatment (NHAPC)
IT-1	6.8 a	1.1 a
IT-2	3.7 b	0.6 b
IT-3	0.0 b	0.0 b

IT-1, half-anthers without treatment (=control); IT-2, half-anthers whose anther wall was pressed lightly to release some microspores; IT-3, microspore clusters. Means followed by the same letter in the same column are not significant different (Tukey's test; $P = 0.05$). Data reflects and is derived from single effect of isolation technique. PHAPC was assessed after 2 months while NHAPC was assessed after 3 months

Table 2 Effect of agar type on callus induction of anther culture of *Anthurium andreanum* Linden ex André cv. 'Tropical'

Type of agar (TA)	Percentage of half-anthers producing callus (PHAPC, %)	Number of half-anthers producing callus per treatment (NHAPC)
TA-1	7.1 a	1.1 a
TA-2	6.3 a	1.0 a
TA-3	2.4 b	0.4 b

TA-1, 2.0 g/l Gelrite; TA-2, 3.0 g/l Phytagel; TA-3, 7.0 g/l Swallow agar. Means followed by the same letter in the same column are not significant different (Tukey's test; $P = 0.05$). Data reflects and is derived from single effect of types of agar. PHAPC was assessed after 2 months while NHAPC was assessed after 3 months

anther culture (Table 3). IT-1 stimulated more callus than IT-2 with 8 % PHAPC and 1.3 NHAPC for the former. When Gelrite concentration was reduced from 2.0 g/l (GC-1) to 1.5 g/l (GC-2), callus formation improved (Table 4) and resulted in 9 % PHAPC and 1.5 NHAPC. The treatment also stimulated faster callus growth (unquantified observation).

Effect of anther culture position and induction culture media on callus formation

Changing the position of anther culture and adding 2,4-D in two potential induction culture media, in fact, significantly affected callus formation. Both treatments also stimulated an interaction effect in all parameters observed, although ACP had a greater effect than the addition of 2,4-D in induction culture media. Among the three anther culture positions, ACP-3, i.e., the culture of half-anthers adaxial side down onto medium, stimulated callus formation significantly more than other positions. The treatment induced 19 % PHAPC and 1.2 NHAPC. The addition of 0.5 mg/l 2,4-D to WT medium (WT + D) stimulated more callus formation although there was no significant difference between NWT medium without 2,4-D (NWT-D) and NWT with 0.5 mg/l 2,4-D (NWT + D). WT + D medium was

Table 3 Effect of isolation technique on callus formation in *Anthurium andreanum* Linden ex André cv. 'Tropical'

Isolation technique (IT)	Percentage of half-anthers producing callus (PHAPC, %)	Number of half-anthers producing callus per treatment (NHAPC)
IT-1	8.2 a	1.3 a
IT-2	4.3 b	0.6 b

IT-1, half-anther without treatment (=control); IT-2, half-anther with a lightly pressed half-anther wall releasing some microspores. Means followed by the same letter in the same column are not significant different (Tukey's test; $P = 0.05$). Data reflects and is derived from single effect of isolation technique. PHAPC was assessed after 2 months while NHAPC was assessed after 3 months

Table 4 Effect of Gelrite concentration on callus formation in *Anthurium andreanum* Linden ex André cv. 'Tropical'

Gelrite concentration (GC)	Percentage of half-anthers producing callus (PHAPC, %)	Number of half-anthers producing callus per treatment (NHAPC)
GC-1	7.1 ab	1.1 a
GC-2	9.4 a	1.5 a
GC-3	5.5 ab	0.9 ab
GC-4	3.2 b	0.4 b

GC-1, 2.0 g/l, GC-2, 1.5 g/l, GC-3, 1.0 g/l; GC-4, 0.5 g/l. Means followed by the same letter in the same column are not significant different (Tukey's test; $P = 0.05$). Data reflects and is derived from single effect of gelrite concentration. PHAPC was assessed after 2 months while NHAPC was assessed after 3 months

able to induce 21 % PHAPC and 1.3 NHAPC while NWT-D was the second best induction culture medium in terms of callus formation. In terms of PHAPC and NHAPC, both media had a significant effect compared to other media.

Based on the interaction effect between ACP and induction culture media in terms of callus formation, ACP-1 in combination with WT + D was the most appropriate combination treatment for callus formation and gave significantly more callus than other combinations. The combination stimulated 42 % PHAPC and 3.4 NHAPC (Tables 5 and 6). The second best combination was ACP-1 in combination with NWT-D. In ACP-2, good results were shown by ACP-2 combined with WT-D. The poorest results in callus formation were normally recorded in all combinations between ACP-1 and any induction culture media.

Regeneration of callus derived from half-anthers, plantlet preparation and acclimatization

Callus derived from all three experiments were subcultured regularly on NWT medium containing 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ and 0.75 mg/l BA (regeneration medium; Winarto et al. 2011b) every 1.5-2.0 months. Subculturing callus regularly onto fresh regeneration medium stimulated callus growth (Fig. 2b, c). Initial shoots were clearly observed 5.5 months after the third subculture of half-anther-derived callus (Fig. 2d). By subculturing callus containing initial shoots onto the same medium induced shoots (as many as 9/callus clump) (Fig. 2e). Repeated subculture of callus onto the same medium gradually enhanced the number of shoots produced, with an average of up to 15 shoots/callus cluster (Fig. 2f). After the seventh subculture, several axillary roots (1-3/shoot) were easily observed one month after culture.

Well-rooted shoots were easily induced from callus derived from the eighth subculture on hormone-free WT medium. Initial roots clearly formed 15–20 days after culture, with an average of 2.3 roots/shoot (Fig. 2g). The

Table 5 Interaction effect of anther culture position (ACP) and induction culture media on percentage of half-anthers producing initial callus (i.e., PHAPC) per treatment in *Anthurium andreanum* Linden ex André cv. 'Tropical' half-anther culture

Induction culture medium	Anther culture position		
(WT/NWT)	ACP-1	ACP-2	ACP-3
WT-D	0.0 b	4.2 b	4.2 c
NWT-D	6.3 a	16.7 ab	29.1 b
WT + D	8.4 a	20.9 a	41.7 a
NWT + D	0.0 b	12.6 a	6.3 c

ACP-1, abaxial side down on medium; ACP-2, upright in medium; ACP-3, adaxial side down on culture medium. WT-D, WT (Winarto-Teixeira; Winarto et al. 2011b) medium containing 0 mg/l 2,4-D, 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ; NWT-D, NWT (New Winarto-Teixeira; Winarto et al. 2011b) medium containing 0 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA, 1.5 mg/l TDZ; WT + D, WT medium containing 0.5 mg/l 2,4-D, 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ; NWT + D, NWT containing 0.5 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA and 1.5 mg/l TDZ. Means followed by the same letter in the same column are not significant different (Tukey's test; $P = 0.05$). PHAPC was assessed after 2 months

Table 6 Interaction effect of anther culture position (ACP) and induction culture media on number of half-anthers producing callus (i.e., NHAPC) per treatment in *Anthurium andreanum* Linden ex André cv. 'Tropical' half-anther culture

Induction culture medium	Anther culture position		
(WT/NWT)	ACP-1	ACP-2	ACP-3
WT-D	0.0 b	0.3 b	0.3 c
NWT-D	0.4 a	0.8 ab	2.3 b
WT + D	0.5 a	1.3 a	3.4 a
NWT + D	0.0 b	1.0 a	0.4 c

ACP-1, abaxial side down on medium; ACP-2, upright in medium; ACP-3, adaxial side down on culture medium. WT-D, WT (Winarto-Teixeira; Winarto et al. 2011b) medium containing 0 mg/l 2,4-D, 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ; NWT-D, NWT (New Winarto-Teixeira; Winarto et al. 2011b) medium containing 0 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA, 1.5 mg/l TDZ; WT + D, WT medium containing 0.5 mg/l 2,4-D, 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ; NWT + D, NWT containing 0.5 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA and 1.5 mg/l TDZ. Means followed by the same letter in the same column are not significant different (Tukey's test; $P = 0.05$). NHAPC was assessed after 3 months

well-rooted shoots (n = 150) harvested from the preparation stage were easily acclimatized ex vitro with 100 % survival when transplanted to a mixed substrate of raw rice-husk, burned-rice husk, and organic manure (1:1:1, v/v/v) (Fig. 2h). The plants derived from half-anther culture, despite the high level of survival, showed considerable variation in terms of plant size, leaf shape, leaf length–width ratio, and petiole length (Fig. 2i).

Discussion

Unlike other ornamentals, the culture of entire anthers in anthurium is not possible or shows great difficulties. Whole anthers have been cultured on semi-solid medium in *Lilium longiflorum* (Arzate-Fernandez et al. 1997; Han et al. 1997; Niimi et al., 2001; Han et al. 2006), *Helianthus annuus* (Thengane et al. 1994; Saji and Sujatha 1998), *Cyclamen persicum* and *C. purpurascens* (Ishizaka 1998), and *Dianthus chinensis* (Fu et al., 2008). However, the use of whole anthers resulted in high levels of contamination and browning in anthurium (Winarto and Rachmawati 2007; Winarto and Mattjik 2009a, b; Winarto et al. 2011a). In a very preliminary study conducted by Van der Burg and Everaarts (2006), the whole anthers of *A. andreanum* cv. 'Tropical' were cultured with their filaments, and even though the filaments swelled, no callus formed. Use of the basal part of half-anthers also showed poor results (Winarto 2009), and eventually it was established that the apical part of half-anthers (Fig. 1) was the most receptive part for callus induction in anthurium (Winarto and Rachmawati 2007; Winarto and Mattjik 2009a, b; Winarto et al. 2010a, b, 2011a, b). The low regeneration capacity of *Anthurium* anthers is main problem in the establishment of an anther culture system for this ornamental. Building on that base, this study further focused on the culture conditions to improve callus induction, with the objective of reducing contamination and achieving a high level of survival of final regenerants.

Microspores that were squeezed out of half-anthers onto culture initiation media did not form callus, possibly due to short-lived cell viability (5–10 days) after culture initiation (Winarto et al. 2010a). Similarly, in potato (*Solanum tuberosum*) microspore culture, microspores lost viability within 2 days and did not develop further (Íhová and Tupý 1999). The reduction of cell viability in *Zea mays* microspore cultures was caused by a lack of osmoticum in the medium (Obert et al. 2000). Although the potential of isolated microspores to grow could still be observed one month after culture, microspores did not respond (embryogenesis or callogenesis) right until the end of the observation period i.e., 2 months after culture. In other studies, the lack of a response by microspores in vitro was caused by the absence of a nurse culture, but this effect depended on the availability of sucrose and glutamine, both in tobacco (*Nicotiana tabacum*) (Aruga and Nakajima 1985) and *Lilium* (Clement and Audran 1995) anther culture. In *Triticum aestivum*, the failure to release microspores from cell cycle control can block morphogenesis (Zeng 2003), and this is true in tobacco and snapdragon (*Antirrhinum majus*) as a result of high starch accumulation (Barinova et al. 2004).

Untreated half-anthers that were cultured abaxial-side down on culture medium formed a high level of callus, confirming our previous reports (Winarto and Rachmawati 2007; Winarto and Mattjik 2009a, b; Winarto et al. 2010a, b, 2011a, b). However, by changing the position of half-anthers from abaxial side down to adaxial side down on the culture medium improved callus formation significantly (Table 5). According to Welander (1988), when the position is changed, the ability of the palisade parenchyma to transport nutrients and growth regulators from the medium into the explant increases, as does oxygen exchange on the explant due to a higher number of stomata located abaxially (Blancke and Belcher 1989), although these facts relate to leaf explants and are thus not necessarily applicable to anthers. Histologically, anther wall cells in the adaxial position had a high morphogenic capacity and produced callus easily in response to medium components and plant growth regulators (Winarto et al. 2010a). A high response of explants in terms of morphogenesis, callus and/ or embryo formation, proliferation and regeneration, when cultured adaxial side down on culture medium, was also reported in *Dionaea muscipula* (Teng 1999), apricot (*Prunus armeniaca*) (Perez-Tornero et al. 2000), *Oncidium* 'Gower Ramsey' (Chen and Chang 2000), *Picea abies* (Ramarosandratana and Van Staden 2003), *Dianthus caryophyllus* (Winarto et al. 2005), *Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf. (García-Luis et al. 2006), *Cicer arietinum* (Naz et al. 2008), *Phalaenopsis*, *P. amabilis* and *P. nebula* (Gow et al. 2009), and *Medicago trunculata* (Wang et al. 2011).

The effect of agar on explant growth is influenced by gel strength, mineral composition, mineral availability, inhibitory compounds and water availability (Scholten and Pierik 1998; Cardoso et al. 2007). Agar concentration and type influence medium efficiency and thus explant growth (George et al. 2007). In half-anther culture of *Anthurium*, 2.0 g/l Gelrite in WT medium was suitable for callus formation compared to Swallow agar (7 g/l), while the speed and quality of callus growth could be further enhanced by reducing the concentration of Gelrite to 1.5 g/l. In *Lilium longiflorum* anther culture, 2.5 g/l Gellan gum in N6-medium was successful (Arzate-Fernandez et al. 1997), although the same concentration in MS medium containing 2 mg/l picloram and zeatin (Han et al. 1997), MS medium supplemented with 4 µM picloram (Han et al. 2000), or half-strength MS macronutrients, full micronutrients, and MS vitamins, 0.01 mg/l NAA and 5 % sucrose (Han et al. 2006) were all as equally successful. B5 medium (Gamborg et al. 1968) containing 0.1 and 1.0 mg/l NAA, 0.1 mg/l 2,4-D and 90 g/l sucrose was utilized for anther culture of *Cyclamen persicum* and *C. purpurascens* (Ishizaka 1998).

2,4-D is one of the most effective auxins in callus and/or embryoid formation; however, the optimum concentration applied in anther culture varies from one plant to another (Thengane et al. 1994; Arzate-Fernández et al. 1997; Han et al. 1997; Ishizaka 1998; Han et al. 2000; Zhao et al. 2006; Fu et al. 2008). According to Rodrigues et al. (2004), the presence of a plant growth regulator induces the morphogenic response of the anther wall and connective tissue in light while an optimum concentration induces subsequent cell division (Leguay and Guern 1977). In the anther culture of *Helianthus annuus*, application of 1.0 mg/l 2,4-D in combination with 0.5 mg/l BA in MS medium could effectively induce a high number of embryos (Thengane et al. 1994). A parallel response (i.e. high levels of callus) was observed when *Lilium longiflorum* anthers were cultured in the dark on N6 medium supplemented with 2 mg/l 2,4-D (Arzate-Fernández et al. 1997). Similarly, 4.52, 9.05 and 13.57 μM 2,4-D in MS medium was suitable for callus induction of *Echinacea purpurea* (Zhao et al. 2006), and 9.0 μM 2,4-D, 4.44 μM BA, 2.7 mM glutamine, 400 mg/l casein hydrolysate, 0.9 mM proline, 7.5 g/l agar, and 30 g/l sucrose in MS medium was suitable for embryogenic callus formation of *Dianthus chinensis* (Fu et al. 2008). In our study, 0.5 mg/l 2,4-D in combination with 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ in WT medium strengthened the capacity for callus to form and grow.

Callus derived from half-anthers in this study were also easily regenerated by sub-culturing them regularly onto NWT medium supplemented with 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 0.75 mg/l BA and 1.5 mg/l TDZ, as reported previously (Winarto et al. 2011a, b). In other *Anthurium* studies, regeneration was possible using MS medium containing 0.5 mg/l BA (Viégas et al. 2007), MS fortified with 1.0 mg/l BA (Jahan et al. 2009), MS with 0.5 mg/l BA and 60 mg/l adenine sulphate (Gantait et al. 2008); MS medium with 0.3 mg/l BAP (Bejoy et al. 2008); half-strength MS salts with NH_4NO_3 lowered to 250 mg/l, 0.1 mg/l 2,4-D and 1 mg/l BA (Atak and Çelik 2009). Adventitious roots formed simply from shoots cultured in hormone-free NWT medium (Winarto et al. 2011b), as occurred in this study. The results differed slightly from our previous study in which well-rooted shoots could be established on WT medium containing 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l TDZ (Winarto et al. 2011a). In other studies, root formation was positively established in MS medium supplemented with 0.0, 0.5 and 1.0 mg/l BA (Viégas et al. 2007), 0.02 mg/l NAA and 0.2 mg/l BA (Winarto et al. 2010c); MS with 0.5 mg/l IAA and 2 g/l activated charcoal (Gantait et al. 2008), MS fortified with different concentrations (0.2–2.0 mg/l) of IBA and IAA singly (Jahan et al. 2009), modified half-strength MS salts supplemented with 1 mg/l IBA and 0.04 % active charcoal (Atak and Çelik 2009).

Well-rooted shoots could be simply acclimatized in this study by culturing plantlets in a mixed substrate of raw rice husk, burned-rice husk, and organic manure (1:1:1, v/v/v) with high survival (100 %), which was also reported in previous studies (Viégas et al. 2007; Winarto et al. 2010c, b). A survival <100 % during the acclimatization of anthurium regenerants was established by culturing rooted plantlets in small pots containing sand, loamy soil and coco-peat (1:1:1, v/v/v) with 85 % survival (Jahan et al. 2009); sand, soil, charcoal and coconut fibre (1:1:1:1, v/v/v/v) with 85 % survival (Gantait et al. 2008); coarse river sand and charcoal (3:1, v/v) with 89 % survival (Bejoy et al. 2008).

Acclimatized plantlets derived from half-anther culture varied in terms of plant size, leaf shape, leaf length–width ratio, petiole length, spathe and spadix color, and spadix length. The regenerants that varied cytologically showed variation in the number of chromosomes i.e. 17.5 chromosomes/cell for haploid regenerants (n = 15–20), 23.8 for aneuploids (n = 21–26), 31.7 for diploids (n = 28–34) and 49.2 for triploids (n = 45–57). The ploidy ratio of the variant regenerants was 23 % haploid, 5 % aneuploid, 69 % diploid and 3 % triploid (Winarto et al. 2010c, 2011a).

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