New basal media for half-anther culture of Anthurium andreanum

The success of plant tissue culture as a means for plant propagation is greatly influenced by the culture medium, which provides not only inorganic nutrients, but usually also a carbohydrate (sucrose being most common) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. To stimulate healthy and vigorous growth, many media also include trace amounts of certain organic compounds, notably vitamins, and plant growth regulators, or PGRs (George et al. 2007). Further- more, how rapidly a tissue grows and the extent and quality of morphogenetic responses are strongly influenced by the type and concentration of nutrients supplied (Niedz and Evens 2007). In conjunction with the culture medium, each plant species has its own characteristic composition of elements and trace compounds to stimulate high growth responses in tissue culture (George et al. 2007).

The formulae of several basal media such as Chu (N_6) (Chu et al. 1975), Gamborg's B5 (Gamborg et al. 1968);

Lichter (NLN) (Huang and Keller 1989); Murashige and Skoog (MS) (Murashige and Skoog 1962), Murashige and Tucker (MT) (Murashige and Tucker 1969), and Nitsch and Nitsch (NN) (Nitsch and Nitsch 1969), among others, were successfully established not only for the tissue culture of various plants and explants, and research objectives (George et al. 2007),

but also for developing plant anther cultures (Arzate-Ferna´ndez et al. 1997; Ishizaka 1998; Nomizu et al. 2004; Segu´ı-Simarro and Nuez 2007; Cao et al. 2010; Doi et al. 2010; Sayem et al. 2010), the most commonly used being the MS medium formulation (Thengane et al. 1994; Han et al. 1997; Saji and Sujatha 1998; Metwally et al. 1998; Rimberia et al. 2006; Wang and Bao 2007; Fu et al. 2008; Sayem et al. 2010). This medium was previously developed for optimal growth of tobacco callus but is now applied in many plants by adapting the strength of macro-nutrients, or minor changes in the concentrations of minerals (Bouman and Tiekstra 2001). Practically, each and every medium composition is based on different combinations of micro and macronutri- ents but historically, even though there may appear to be similarities, media with new names have been established when representing a new class of group of plants, as is the case in this study for *Anthurium*.

MS media with some modifications have also been applied in the tissue culture of *Anthurium*. Recent research applications utilizing MS medium for the in vitro culture of *Anthurium* are summarized in Table 1. These studies indicate that, to date, all studies on in vitro culture of *Anthurium* used MS basal medium with all sorts of PGR variations; no other basal medium has served this purpose. The basal media were generally applied for somatic tissues such as apical shoots, leaves, petioles, spadices and roots. However, there are no specific MS basal medium formu- lations for anther culture of *Anthurium*.

Finding an appropriate basal medium is one of the important keys to address the development of plant anther culture protocols. Several basic media were tested to obtain the best response during every step in the establishment of plant anther culture protocols for callus induction, embryo formation, proliferation, and plantlet preparation. For example, in the anther culture of *Helianthus annuus* L.,

Thengane et al. ([1994](#)) found that MS was the best basal medium to obtain a high embryogenic response. MS was the most effective for callus induction in *Lycopersicon esculentum* Mill. (Shtereva et al. [1998](#)), liquid PG-96 for timothy (Guo et al. [1999](#)), N_6 for *Echinacea purpurea* (Zhao et al. [2006](#)), Linsmeir and Skoog (LS, Linsmaier and Skoog [1965](#)) and NN for pepper (Koleva-Gudeva et al. [2007](#)), and PGR-free B5 medium for carrot (Gorecka et al. [2009](#)). These studies indicate that anther culture is still dependent on the plant genotype and selection of basal medium.

In preliminary studies, some basal media, namely B5, MS, NLN and NN with various combinations and con- centration of 2,4-D (0.0–1.0 mg/l), NAA (0.0–0.5 mg/l) and BAP (0.0–2.0 mg/l) were investigated to stimulate callus induction during anther culture of *Anthurium*, but most of these basal medium-PGR combinations did not have a positive effect on callus formation. Most anthers cultured on those media browned, became necrotic and died, while in MS medium 50–70% of anthers remained viable but with no callus formation. The potential for callus formation was improved on half-strength MS containing

0.1 mg/l 2,4-D, 0.5 mg/l BAP and 0.5 mg/l Kin but preliminary results were still meager. Several cultivars of *A andreanum* i.e., 'Tropical', 'Carnaval', 'Amigo', 'Casino', 'Laguna', and 'Safari' were also selected in a preliminary study. From these, we determined that 'Tropical' was the most responsive cultivar in terms of callus formation with faster callus proliferation and shoot regeneration than others (unpublished data). This study thus concentrated on formulating a new basal medium suitable for developing a protocol of half-anther culture of *Anthurium* based on the potential media and selected-cultivar tested previously. In this process, which lead to the successful development to of a callus-induction medium for *Anthurium* half- anthers, a unique combination of micro- and macro- nutrients was hence assembled, and that

medium was henceforth termed Winarto–Teixeira (WT) medium, already recognized by the plant science peer community (Winarto et al. 2010b, 2011). Reliable induction and proliferation of callus derived from half-anther culture, followed by shoot regeneration and root formation, are the main objectives to develop an anther culture protocol for *Anthurium*. Survival, plant morphology, cytology and ploidy variation were assessed in acclimatized plantlets to confirm the suitability of the protocols.

Materials and methods

Plant material and explant preparation

Anthurium andreanum Linden ex Andre´ cv 'Tropical', a very popular cultivar in international markets, was the target plant material for our study. These tropical 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 cm^3 of potting medium, 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 15–20°C at night (temperature assessed by a thermo-hygrometer,

515

1 3

Table 1 Recent research using MS medium (or variants) in in vitro culture of *Anthurium*

Anthurium species	Explant source	Modification	Response/objective	Reference

A. scherzerianum Schott	Leaf	MS ? 18 lM 2,4-D, 6% sucrose	Embryo induction	Hamidah et al. ([1997](#))
		MS ? 0.46 lM kinetin	Embryo germination	
A. andreanum Hort	Root	MS ? 2.2 lM BA	Multiple shoots	Chen et al. ([1997](#))
	Leaf Leaf Leaf	MS ? 2.2–4.4 lM BA, 0.9 lM 2,4-D ½ MS ? 1.11 lM BA, 1.14 lM IAA, 0.46 lM Kin ½ MS ? 0.44 lM BA ½ MS ? 0.54 lM NAA, 0.93 lM Kin ¼ MS ? 0.88 lM BA, 0.9 lM 2,4-D, 0.46 lM Kin ¼ MS ? 0.88 lM BA, 0.54 lM NAA, 0.46 lM Kin ½ MS ? 0.44 lM NAA	Adventitious shoots Shoot induction Multiple shoots Roots Callus Multiple shoots Roots	Teng ([1997](#)) Martin et al. ([2003](#)) Joseph et al. ([2003](#))

		MS ? 4.4 lM BA, 0.05 lM NAA ½ MS ? 0.08 mg/l 2,4-D, 1.0 mg/l BAP, 1.0 mg/l 2-iP	Multiple shoots Callus	Vargas et al. ([2004](#)) Vie´gas et al. ([2007](#))
	Seed Leaf			
		½ MS ? 0.5 mg/l BAP	Shoots	
	Leaf	½ MS ? 0.5 mg/l 2,4-D, 1.0 mg/l BAP	Adventitious shoots	Bejoy et al. ([2008](#))
	Apical shoot	MS ? 0.1 mg/l NAA, 0.25 mg/l BAP	Multiple apical shoots	Gantait et al. ([2008](#))
		MS ? 0.5 mg/l BAP, 60 mg/l AS	Multiple shoots	
		MS ? 0.5 mg/l IAA, 2 g/l AC	Roots	
	Leaf	½ MS basal salt with 0.6 mg/l 2,4-D, 1 mg/l BA	Callus induction	Atak and C¸ elik ([2009](#))

		½ MS salt with 250 mg/l NH_4NO_3, 0.1 mg/l 2,4-D, 1 mg/l BA ½ MS ? 1 mg/l IBA, 0.04% AC	Shoot regeneration Roots	
	Leaf, spadix	¼ MS ? 1.0 mg/l BA	Multiple shoots	Jahan et al. ([2009](#))
		¼ MS ? 1.0 mg/l IBA	Roots	
	Seed	MS ? 2 mg/l BA and 0.5 mg/l NAA	Callus proliferation	Maira et al. ([2009](#))
	Leaf, petiole	½ MS ? 0.90 lM 2,4-D and 8.88 lM BA ½ MS ? 0.90 lM 2,4-D and 4.44 lM BA ½ MS ? 4.44 lM BA ½ MS PGR-free	Callus induction Callus proliferation PLB production PLB germination	Yu et al. ([2009](#))

BAP 6-benzylaminopurine, *BA* N^6-benzyladenine, *2,4–D* 2,4-dichlorophenoxy acetic acid, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *2–iP* N^6-[2-isopentenyl]adenine, *Kin* kinetin, *NAA*

a-naphthalene acetic acid, *TDZ* thidiazuron, *AC* activated charcoal, AS adenine sulphate, *MS* Murashige and Skoog ([1962](#)) medium, *PGR* plant growth regulator, *PLB* protocorm-like body

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 lmol/m^2/s light intensity during the dry season

Table 2 Two Winarto–Teixeira (WT) basal medium compositions (WT-1 and WT-2) and modified half-strength Murashige and Skoog (WT-3) tested for callus initiation in half-anther culture of *Anthurium*

Medium component Basal medium compositions tested (WT)

No pesticides, either as spray or as soil drench, were applied during maintenance of donor *Anthurium* plants to minimize the reduction of microspore vitality. Spadices with 50% of their stigmas in a receptive condition—indicated by high secretion of a sticky substance at the tip of the stigma (see Fig. 1 in Winarto et al. [2011])—were harvested from

(April–October) and 37–111 lmol/m^2/s in the rainy season (November–March). Light intensity was measured using a		WT-1	WT-2	WT-3
Digital Lux Meter, Lutron LX 101 (Lutron Electronic	Macronutrients			
Enterprise Co., Ltd, Taiwan). Measurement of data using the	NH_4NO_3	550	650	825
Lutron LX 101 was originally in lux but was then converted	KNO3	1,250	1,100	900
to lmol/m^2/s by multiplying each data point with a conver-	$Ca(NO_3)_2 \cdot 4H_2O$	–	392.5	–

sion factor for sunlight i.e., 0.0185 (Thimijan and Heins	$MgSO_4$	180	195.3	195
[1982](#)). The plants were watered with liquid fertilizer (2 g/l of	$CaCl_2$	300	–	220
N:P:K, 20:15:15; Nusa Tani, Ltd, Jakarta) at 3-day intervals.	$NaH_2PO_4 \cdot H_2O$	200	–	–

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[®] 50 WP, 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 hypo- chloride (NaOCl, Bayclin- Johnson Home Hygiene Prod-

ucts 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) (Winarto [2009](#)).

Anthers were isolated from the transition area of the spadix (approximately 2 cm in length). This area was cut from each spadix using a tissue culture blade (BB510, Aesculap AG & CO. KG AM Aesculap-Platz 78532, Tut- lingen, Germany) and scale-like petals were then carefully removed. The top to middle part of 3–4 anthers were sliced. Part of the anther (referred to throughout the rest of this manuscript as the half-anther) was cultured directly on WT-1 to WT-3 and modifications of these media (Table [2](#)) in the first experiment and NWT-1 to NWT-3 (Table [3](#)) in the second experiment. Half-anthers were

used since, in previ- ous trials (Winarto 2009) these explants were shown to form more callus than intact anthers. Two different types of calli i.e., fast- and slow-growth types derived from half-anthers cultured on WT-1 and NWT-3 media in the first and second experiments were multiplied by sub-culturing them sepa- rately in NWT-3 (for medium composition see Table 3) after approximately 4 months' incubation (Fig. 1c). Four to five sub-cultures were required for callus to grow and regenerate in sufficient amounts for experimental purposes. The

KH_2PO_4 150 165 85

Micronutrients

H_3BO_3 5.7 6.2 6.2

KI 0.65 − 0.83

$MnSO_4 \cdot H_2O$ 15.5 2.85 16.9

$ZnSO_4 \cdot 7H_2O$ 7.5 5.35 10.6

$Na_2MoO_4 \cdot 2H_2O$ 0.2 0.2 0.25

$CuSO_4 \cdot 5H_2O$ 0.02 0.025 0.025

$CoCl_2 \cdot 6H_2O$ 0.02 0.025 0.025

$Na_2EDTA \cdot 2H_2O$ 37.3 37.3 37.3

$FeSO_4 \cdot 7H_2O$ 27.5 27.5 27.5

Vitamins

Glycine	−	−	2.0
Myo-inositol	110.0	50.0	100.0
Nicotinic acid	−	0.5	0.5
Pyridoxine-HCl	−	0.3	0.5

Thiamine-HCl	0.5	1.0	0.1
PGRs			
2,4-D	—	—	0.1
NAA	0.01	0.2	—
TDZ	0.5	—	0.5
BAP	1.0	0.5	—
Kin	—	—	0.5

All values in mg/l

BAP 6-benzylaminopurine, *2,4–D* 2,4-dichlorophenoxy acetic acid, *Kin* kinetin, *NAA* a-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator

fast-growth type of callus could be multiplied faster than the slow-growth type, doubling in size (relative to slow-type) within 1.5–2.0 months after sub-culture; the slow-type took 4–5 months. Fast-type callus was generally green to light-green while the slow-type was reddish-yellow to light reddish-yellow. The fast type could produce a high number of regenerated shoots (up to 12 shoots per explant) in 4–5 months after the first subculture while the slow type took 9–12 months to produce 1–3 regenerated-shoots per explant (Rachmawati 2005; Winarto et al. 2011). Callus clusters were then sliced into 27 mm^3 ''blocks'' (3 9 3 9 3 mm,

Table 3 New Winarto–Teixeira (NWT) basal media, derived from WT-1, tested for callus initiation of half-anther culture of *Anthurium*

Medium component New Winarto–Teixeira media (NWT)

WT-1 NWT-1 NWT-2 NWT-3

l 9 w 9 h). These calli ''blocks'' were then cultured on callus growth and regeneration (CGR) media (Table 4) in culture bottles (jam jars 11.5 cm in height, 7 cm in diameter

and with 40 ml media) for ±4.0 months until shoots multi-

plied. For the rooting experiment, ±2.5 cm long shoots with

Macronutrients

NH_4NO_3	550	–	–	750
$(NH_4)_2SO_4$	–	750	500	–
KNO_3	1,250	1,300	1,500	1,750
$Ca(NO_3)_2 \cdot 4H_2O$	–	250	300	250
$MgSO_4$	180	195	215	200
$CaCl_2$	300	50	–	–
$NaH_2PO_4 \cdot H_2O$	200	195	150	150
KH_2PO_4	150	165	180	125
Micronutrients				
H_3BO_3	5.7	5.6	4.75	4.75
KI	0.65	0.65	0.55	0.45
$MnSO_4 \cdot H_2O$	15.5	15.5	14.75	12.5
$ZnSO_4 \cdot 7H_2O$	7.5	7.5	6.5	6.5
$Na_2MoO_4 \cdot 2H_2O$	0.2	0.2	0.15	0.1
$CuSO_4 \cdot 5H_2O$	0.02	0.02	0.015	0.01
$CoCl_2 \cdot 6H_2O$	0.02	0.02	0.015	0.01
$Na_2EDTA \cdot 2H_2O$	37.3	37.3	37.3	37.3
$FeSO_4 \cdot 7H_2O$	27.5	27.5	27.5	27.5
Vitamins				
Myo-inositol	110.0	125.0	130.0	125.0
Thiamine-HCl	0.5	0.65	0.75	0.55
PGRs				
2,4-D	–	0.75	0.75	0.25

NAA	0.01	0.05	0.01	0.02
TDZ	0.5	2.0	1.0	1.5
BAP	1.0	1.0	–	0.75

All values in mg/l

BAP 6-benzylaminopurine, *2,4–D* 2,4-dichlorophenoxy acetic acid, *Kin* kinetin, *NAA* a-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator

2–3 leaves that had regenerated successfully in the regeneration experiment were used. In this experiment, shoots were cultured in different rooting media (Table 5) in culture bottles as for CGR.

All media used in these studies contained 3% sucrose (Merck, Darmstadt, Germany) and 2.0 g/l gelrite (Duchefa- Biochemie, RV Harleem, The Netherlands). The pH of

media was adjusted to 5.8 (Model 420A pH meter, Thermo Orion, Beverly, USA) and sterilized for 20 min at 121°C and 15 kPa (Pressure Steam Sterilizer Vertical Cylindrical LS. 001, SMIC, Shanghai, China).

In a series of four experiments, half-anthers were incu- bated in the dark for ±2 months to induce callus; there-

after, callus cultures were placed under fluorescent lamps

(TL-Philips, The Netherlands) under *13.5 lmol/m^2/s in a 12-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8% rela- tive humidity for CGR. Callus cultures were maintained in

these conditions until shoots and roots formed.

Experiment 1: Development and selection of medium suitable for callus initiation

In the first experiment, two different medium compositions (Winarto and Teixeira medium, WT-1 and WT-2) were developed

for callus initiation in half-anther culture of *Anthurium* (Table 2). The development and application of WT-1 and WT-2 were based on preliminary research in which numerous medium compositions i.e., modification of MS basal medium containing 0.1 mg/l 2,4-D, 0.5 mg/l TDZ and

0.5 Kin; Che´e and Pool basal medium (Che´e and Pool 1987) supplemented with 0.2 mg/l NAA, 0.5 mg/l BAP and
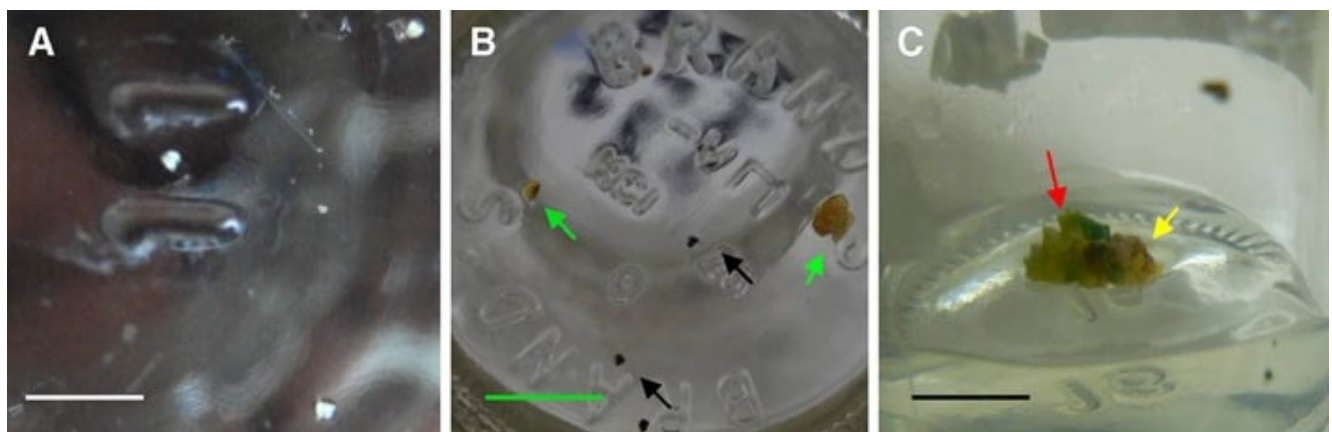
0.5 mg/l TDZ; and Miller and Murashige Syngonium

Fig. 1 Formation of callus in half-anther culture of anthurium. a Half-anthers in initial culture. b Half-anthers with callus produced approximately 60 days after culture initiation; *green arrows* indicate regenerated half-anther and callus produced while *black arrows* show dead half-anthers after browning. c Regenerated callus derived from

half-anthers approximately 4.0 months after culture initiation; *yellow arrow* indicates fast growth type of callus and *red arrow* shows slow growth type of callus. *White bar* = 0.6 cm, *green bar* = 0.45 cm, *black bar* = 0.75 cm. (Color figure online)

Table 4 Combination and concentration of PGRs in WT-1 and NWT-3 for callus growth and regeneration (i.e., shoot production) in half-anther culture of *Anthurium*

Basic media Callus growth and regeneration media (CGR) PGR combination and concentration (mg/l)

		2,4-D	NAA	TDZ	BAP
WT-1	CGR-1	—	0.01	0.5	1.0
	CGR-2	—	0.05	0.5	—
	CGR-3	—	0.01	1.0	—
	CGR-4	—	0.02	1.5	—
NWT-3	CGR-5	1.0	0.01	1.5	—
	CGR-6	0.75	0.05	2.0	—
	CGR-7	0.5	0.01	1.5	0.75
	CGR-8	0.25	0.02	1.5	0.75

BAP 6-benzylaminopurine, *2,4—D* 2,4-dichlorophenoxy acetic acid, *NAA a*-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator,

WT—1 Winarto—Teixeira basal medium-1, *NWT—3* New Winarto—Teixeira basal medium-3

Table 5 Combination and concentration of PGRs in WT-1 and NWT-3 for root formation from shoots derived from half-anther culture of

Anthurium

Basic media Rooting media (RM) Combination and concentration of PGR (mg/l)

		2,4-D	NAA	TDZ	BAP	Kin
WT-1	CGR-1	—	0.01	0.5	1.0	—
	CGR-2	—	0.2	—	—	1.0
	CGR-3	—	0.1	—	—	0.5
	CGR-4	—	—	—	—	—
NWT-3	CGR-5	0.25	0.02	1.5	0.75	—
	CGR-6	—	0.2	—	—	1.0
	CGR-7	—	0.1	—	—	0.5
	CGR-8	—	—	—	—	—

BAP 6-benzylaminopurine, *2,4*-D 2,4-dichlorophenoxy acetic acid, *Kin* kinetin, *NAA* a-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator, *WT–1* Winarto–Teixeira basal medium-1, *NWT–3* New Winarto–Teixeira basal medium-3

stage I and II basal medium (Miller and Murashige [1976]) with

1.0 mg/l 2,4-D, 0.01 mg/l NAA and 1.5 mg/l TDZ were tested to assess their positive response to half-anther survival and the capacity of these explants to stimulate callus formation (Winarto and Rachmawati [2007]). WT-3 is half-strength MS medium but modified by supplementing with 50 ppm Cefo- taxime (Cef) (Phytotec, Shawnee Mission, USA; ½ MS ? Cef) and 2 mg/l panthotenic acid (PA) (Sigma-Aldrich, Germany; ½ MS ? PA) and (½ MS ? Cef ? PA). WT-3 medium composition was shown to be the most appropriate medium for callus formation and adventitious shoot formation of different explants (shoot tips, young stems, leaves and petioles) of different *Anthurium* accessions in the Indonesian Ornamental Crops Research Institute collection (Winarto [2007]), and due to the explant-independent superiority of this medium, it was also applied for half-anther culture in this study. The ability of WT-1, WT-2, WT-3 and three modifi- cations of WT-3 containing Cef and PA in several combina- tions (Table [2]) to maintain surviving half-anthers and induce callus, were tested.

Experiment 1 was arranged in a complete randomized block design (CRBD) with four replications. Each treat- ment consisted of four bottles. Each bottle contained five half-anthers. Bottles were 5 cm in diameter and 7 cm in height, and each contained 5 ml of semi-solid media.

Experiment 2: Optimization of selected medium for callus initiation

Based on the results of Experiment 1, WT-1 was consid- ered to be the best medium for callus initiation. In the second experiment, the medium was improved by increasing or lowering

concentrations or by adding and omitting select medium components to stimulate higher callus initiation (Table 3). In doing so, three new medium compositions were selected, i.e., NWT-1, NWT-2 and NWT-3, based on their superior ability to induce callus.

This experiment was performed in an identical way to Experiment 1.

Table 6 Effect of medium (WT) composition on callus formation in *Anthurium* half-anther culture

Medium composition (WT) Percentage of callus regeneration (%)

Number of half-anthers producing callus

Percentage of callus formation (%)

Medium composition (WT)	Percentage of callus regeneration (%)	Number of half-anthers producing callus	Percentage of callus formation (%)
WT-1	11.3 a	2.3 a	44.5 a
WT-2	0.0 c	0.0 c	0.0 c
WT-3	0.0 c	0.0 c	0.0 c
WT-3 ? C	0.0 c	0.0 c	0.0 c
WT-3 ? P	0.0 c	0.0 c	0.0 c
WT-3 ? C ? P	7.5 b	1.5 b	19.4 b
Coefficient of variation (%)	11.39	10.78	19.44

C—50 ppm cefotaxime (Phytotec, USA), P—2 mg/l panthotenic acid (Sigma-Aldrich, Germany). Callus formation: 0 = no callus formed, 1 = little callus formed (\25% of total of explant size), 2 = moderate callus formed (25–50% of total of explant size), and 3 = abundant callus formed ([50% of total of explant size). Mean values followed by the same letter in the same column are not significantly different based on DMRT (*P* = 0.05)

Three growth parameters were observed in Experiments 1 and 2: (1) percentage half-anther regeneration (PAR, %);

(2) number of half-anthers producing callus; (3) callus formation score (0–3, where 0 = no callus formation, 1 = little callus formed (\25% of total explant surface area), 2 = moderate amount of callus formed (25–50% of total explant surface area), 3 = abundant callus formation ([50% of total explant surface area). Observations were made 2.5 months after dark incubation of half-anthers.

Experiment 3: Modification of WT-1 and NWT-3

to stimulate growth and regeneration of callus derived from half-anther culture

In a third experiment, select media, i.e., WT-1 and NWT-3, were modified by changing the combination and concen- trations of PGRs to further stimulate CGR. The modifica- tions in PGRs are summarized in Table 4.

The performance of CGR was assessed in two ways, i.e., as fast- and slow-growth calli types. The experiment was arranged as a CRBD with four replications. Each treatment consisted of 3 bottles, each of which contained 4 callus clusters (3 9 3 9 3 mm, l 9 w 9 h), selected based on their colour, from Experiments 1 and 2.

Parameters observed in Experiment 3 were: (1) volume of callus (cm^3); (2) callus growth rate (cm^3/month); (3) number of adventitious shoots/callus cluster; (4) shoot height (cm). Observations were made 4.0 months after initial culture.

Experiment 4: Modification of WT-1 and NWT-3 to stimulate roots on adventitious shoots derived from half-anther culture

In the fourth experiment, WT-1 and NWT-3 were modified by changing the PGR combinations and concentrations to stimulate root formation, as summarized in Table 5.

The experiment was arranged as a CRBD with four repli- cations. Each treatment consisted of 4 bottles, each of which

contained 5 shoots (±2.5 cm in length with 2–3 leaves) obtained from the regeneration experiment. A total of 80 uniform shoots were used per treatment.

Parameters observed in the experiment were: (1) root initiation period (days after culture), (2) number of roots produced/shoot, (3) root length (cm), (4) growth rate 1 (as root number/month) and (5) growth rate 2 (as root length/month). Frequent observations were made to record initial root formation and the final observation was made

2.5 months after culture.

Acclimatization of plantlets derived from half-anther culture

Plantlets approximately 3.0 cm in length with 2–3 leaves and 2–4 roots were removed carefully with forceps from the culture bottles. The roots of plantlets were washed gently under tap water to remove agar clinging to roots.

Roots of plantlets were then immersed in a 1% pesticide solution of 50% benomyl (Benlox[®] 50 WP, Dharma Guna Wibawa Ltd, Jakarta, Indonesia) and 20% streptomycin sulphate (Agrept[®] 20WP, Mastalin Mandiri Ltd, Jakarta, Indonesia) for 1 min, dried for 1–2 min then planted in a plastic box (30 9 20 9 15 cm, l 9 w 9 h) containing burned-rice husk, raw rice husk and organic manure (2:2:1, v/v/v) and watered sufficiently. Each plastic box contained

25 evenly-spaced plantlets. The box was covered with transparent plastic containing small holes for 7 days and was gradually acclimatized to ex vitro conditions by

placing rooted plantlets in a glasshouse under low light intensity (37–74 lmol/m^2/s). After 7 days, the plastic cover was removed. A total of 200 plantlets originating

from the rooting experiment and derived from plantlets induced

from both slow- and fast-growth type of calli were acclimatized.

Plantlets were then moved to an uncovered plastic box under the same conditions for 20–25 days. One month later, plants were replanted to fresh medium then repotted after 2–3 months of acclimatization and after 1 month acclimatization, plantlets were re-planted in a similar plastic box with a mixed medium of burned-rice husk, raw rice husk, bamboo peat, and organic manure (1:1:1, v/v/v)

to stimulate growth. Boxes were placed under natural light inside a glasshouse (35–40°C during the day and 15–20°C at night; 50–90% relative humidity during the day and 25–60% at night). 2–3 months after replanting, the adapted plantlets were potted individually in similar medium. The

percentage survival was recorded 2.5 months after the initiation of acclimatization.

Cytological analysis

Cytological variation of all regenerants was evaluated by using the modified-root tip chromosome counting method of Darnaedi ([1991](#)). Actively growing roots were randomly harvested from plantlets of both in vitro experiments and ex vitro acclimatization. Root tips were trimmed to 0.5–1.0 cm and then treated with 0.002 M 8-hydroxy-

quinoline (Sigma-Aldrich, Germany) for 3–5 h at 20°C.

After treatment, root tips were rinsed with sterile distilled water, immersed in 45% acetic acid glacial (Merck) solu- tion for 10 min. Following maceration in 1 N HCl (Merck): 45% acetic acid glacial (3:1, v/v) for 10 min in a water bath (Memmert WB7, Memmert GmbH ? Co. KG,

Germany) at 60°C, the explants were placed on a glass

object (China Sail Brand No. 23 Cat. No. 7101), the root cap

was removed, trimmed to *ca*. 1 mm and stained with 3–5 drops of 2% aceto-orcein (Sigma-Aldrich) for 15 min. Stained root tips were covered with a 22 9 22 mm cover slip, which was firmly and uniformly pressed to produce a thin layer of cells. Edges of cover slips were covered with entelan (Merck). Specimens were observed under a bio- logical microscope Labophot-2 (Nikon Corp., Tokyo, Japan) at 400–1,0009 magnification. Clear and informa- tive chromosomes were photographed with a digital camera Nikon DX 40; AF-S DX Zoom Nikkor 18–55 mm f/3.5–5.6 G ED II (Nikon Corp.). Chromo- some numbers were calculated from 10 cells per speci- men of each different acclimatized and adapted-plantlet derived from slow- and fast-growth type calli. The acclimatized and adapted-plantlets were ± 5.0 months- old after acclimatization.

Data analysis

Quantitative data in all experiments were analyzed by analysis of variance (ANOVA). Significant differences

between means were assessed by Duncan's multiple range test (DMRT) at *P* = 0.05 (Westfall et al. [1999](#)).

Results

Development and selection of medium suitable for callus initiation

Half-anthers cultured on medium remained in a lag growth phase until 15 days after culture initiation. Those half- anthers that stayed fresh and did not show browning symptoms usually had a high chance of producing callus. These were easy to detect by eye and thus easy to select. After 15 days' incubation, half-anther wall cells dediffer- entiated in response to medium components and PGRs. The cells become competent, and meristematic cells actively divided 20–35 days after culture, as reported previously in a histological study (Winarto et al. [2010a](#)). Visually, half- anthers became swollen and began to produce callus. Callus without browing continued

to grow and develop. Callus 0.15–0.50 cm long was easily observed approxi- mately 3.0 months after culture initiation (Fig. 1b). Approximately 4.0 months after culture, two different types of callus that differed in performance and color were obviously observed (Fig. 1c). The green-yellow callus (Fig. 1c, yellow arrow) had faster growth than the light reddish-yellow callus (Fig. 1c, red arrow). These were termed fast-type and slow-type callus, respectively. The green-yellow callus developed into two types of callus:

(1) green callus with fastest growth and the easiest to regenerate into shoots; (2) greenish-yellow callus with fast growth and rapid ability to regenerate into shoots. The light reddish-yellow callus grew continually and produced light reddish callus with slow growth and poor shoot regenera- tion; reddish callus showed the slowest growth and the lowest regeneration capacity.

Explant browning was an important problem in the half- anther culture of *Anthurium*, ranging from 19 to 100%. The problem normally emerged 5–15 days after culture initia- tion. Half-anthers cultured on medium changed from white to light brown (approximately 10 days), then turned dark brown (approximately 20 days) followed by necrosis approximately 25 days after culture initiation, and dead half-anthers were clearly observed 1–2 months after cul- ture initiation (Fig. 1b; black arrows). The problem, in fact, did not only occur in half-anther cultures, but also on subcultured calli.

Different medium compositions significantly affected callus formation in half-anther explants. Although most media were unable to stimulate a high percentage of callus on half-anthers, WT-1 was the most suitable medium, with up to 11% of half-anthers inducing callus. The callus score

Table 7 Effect of new Winarto–Teixeira basal media in callus formation during half-anther culture of *Anthurium*

New Winarto–Teixeira medium (NWT)

Percentage of callus regeneration (%)

Number of half-anthers producing callus

Percentage of callus formation (%)

New Winarto–Teixeira medium (NWT)	Percentage of callus regeneration (%)	Number of half-anthers producing callus	Percentage of callus formation (%)
WT-1 (control)	12.5 b	2.5 b	55.6 b
NWT-1	0.0 c	0.0 c	0.0 c
NWT-2	0.0 c	0.0 c	0.0 c
NWT-3	20.0 a	4.0 a	75.7 a
Coefficient of variation (%)	9.39	8.64	9.64

Callus formation: 0 = no callus formed, 1 = little callus formed (\25% of total of explant size), 2 = moderate callus formed (25–50% of total of explant size), and 3 = abundant callus formed ([50% of total of explant size). Mean values followed by the same letter in the same column are not significantly different based on DMRT (P = 0.05)

for WT-1 was moderate (2) to abundant (3) (Table 6). The second best composition medium was WT-3. Half-anthers could not survive on any other media, becoming brown 5–15 days after culture initiation and then turning black (=necrosis).

Optimization of selected media for callus formation

Although modification of WT-1 components was not able to improve the capacity of this medium to initiate callus, NWT-3 exhibited better results than all other medium formulations (Table 2), inducing callus in up to 20% of half-anthers; the callus score was abundant (3) (Table 7).

From Experiments 1 and 2, new media suitable for optimized callus formation in half-anther culture of *Anthurium* were established: WT-1 and NWT-3. Both media were then applied to all steps of in vitro *Anthurium* half-anther culture.

Modification of WT-1 and NWT-3 to stimulate growth and regeneration of callus derived from half-anther culture of *Anthurium*

Callus cultured on WT-1 and NWT-3 media clearly started to grow 15–20 days after culture initiation. Callus continued to grow and develop, easily observed by alteration of callus dimensions. Shoot initials, which developed and were observed 1.5–2.0 months after culture initiation (Fig. 2b), continued to grow, producing normal shoots (range = 2–13/ callus cluster) with 2–3 fully expanded leaves 3.5–4.5 months after culture (Fig. 2c). Shoot development was influenced by growth type of callus (i.e., fast- or slow-type), 6–13 shoots/callus cluster in the former and 0–6 shoots/ callus cluster in the latter. Regenerated callus, when sub- cultured, formed as many as 20 shoots/callus cluster (Fig. 2d).

Callus and media type had a high significantly effect on CGR, the effect of the former being stronger than the effect of the latter. Fast-type callus reached 1.58 cm^3 after 1 month and had a growth rate of up to 0.64 cm/month

while the slow-type callus was 1.34 cm^3 with a growth rate of only 0.52 cm/month (Table 8). Fast-type callus pro- duced as many as 9.0 shoots/callus cluster, 2.5 cm in height on CGR-8 (Table 8) while slow-type callus produced a maximum of 3.3 shoots/callus cluster on CGR-4.

Modification of WT-1 by varying the combination and concentrations of PGRs successfully improved CGR. CGR-4 (WT-1 ? 1.5 mg/l TDZ ? 0.02 mg/l NAA) was the most appropriate medium for CGR, followed by CGR-8 (NWT-3 ? 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ, and 0.75 mg/l BA) and was significant better than other CGR media. Altering the TDZ concentration from

0.5 to 1.5 mg/l in CGR-4 and CGR-8 improved CGR, while 2,4-D,

BA, NAA at any concentration had a poor effect on CGR.

Both fast- and the slow-type callus cultured on CGR-4 and CGR-8 resulted in higher CGR than other combina- tions, assessed by callus volume, callus growth rate, number of shoots/callus cluster and shoot height. However, for practical purposes, CGR-4 was suggested since it is cheaper than CGR-8, while still giving significantly posi- tive CGR results.

Modification of WT-1 and NWT-3 to induce root formation on adventitious shoots derived from half- anther culture of *Anthurium*

Modification of WT-1 and NWT-3 also significantly affected root form of adventitious shoots derived from half- anther culture of *Anthurium*. Roots initially formation 15–30 days after culture initiation on the base of the petiole attached to the stem, i.e., stem internodes. The initial root then increased in size (0.3–1.5 cm) and number (1–6).

RM-8 (PGR-free NWT-3) was the most suitable med- ium for root formation, being significantly different to others: it initiated roots in the shortest period of time (22.8 days) with the highest number of roots/shoot (3.8), the longest roots (1.28 cm), and the fastest growth rate (Table 9). The second best results were for RM-4 (PGR- free WT-1). WT-1 and NWT-3 thus had a good effect on
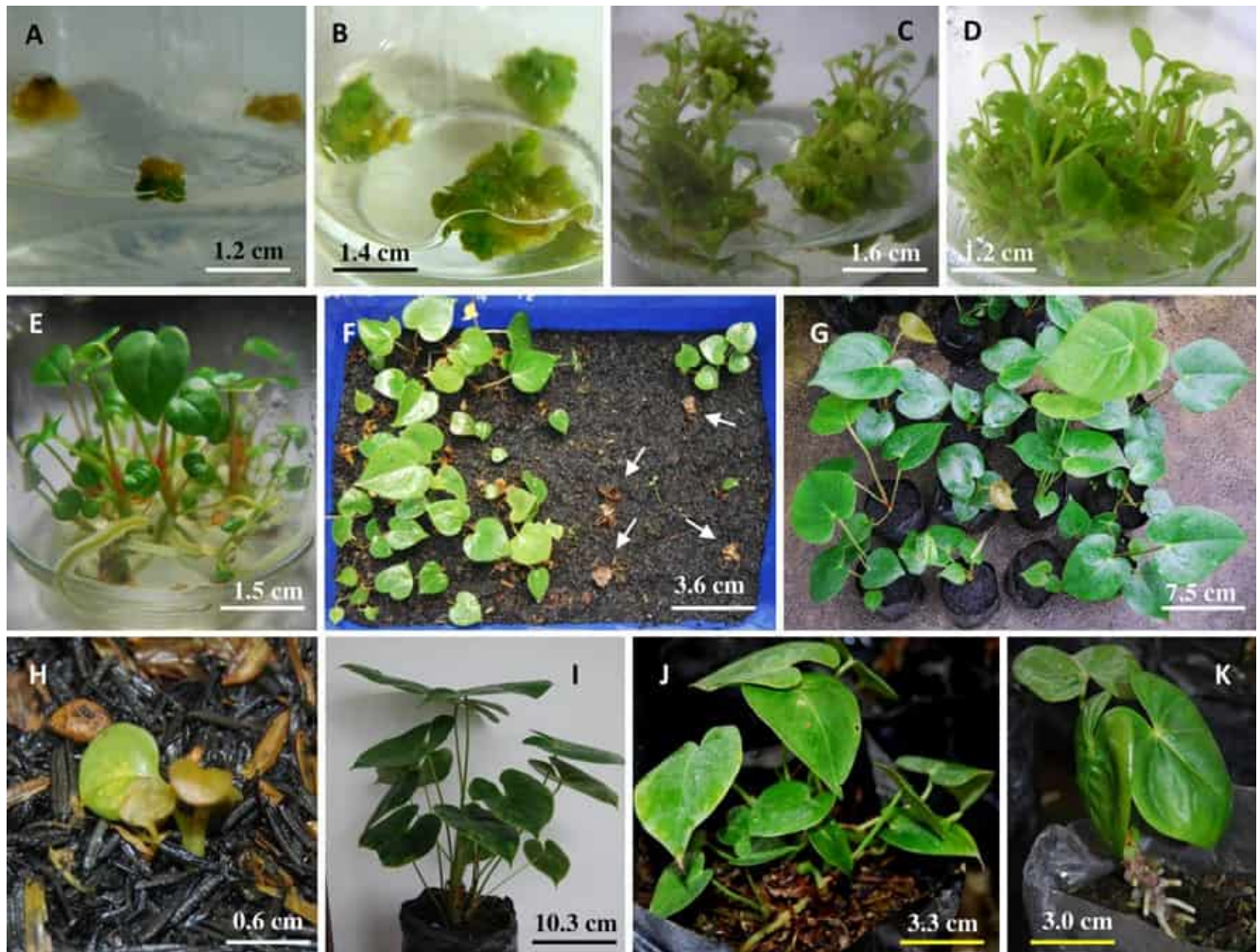
Fig. 2 Successive process of half-anther culture of anthurium from regeneration of callus up until ex vitro acclimatization. a Subcultured- calli for regeneration purposes 10 days after culture initiation. b Regenerated and initial shoots 1.5 months after culture. c Regen- erated shoots approximately 4.0 months after culture. d Multiple shoots approximately 4.0 months after culture in the multiplication stage. e Well rooted-shoots approximately 2.5 months after culture.

f Varied response of plantlets in the acclimatization stage; *white arrows* indicate dead plantlets. g Repotted and varied performance of plantlets adapted ex vitro. h Browning of un- healthy plantlets 20 days after acclimatization. i Normal plantlet approximately 8 months after repotting. j–k Abnormal plantlets derived from anther culture of anthurium approximately 8 months after repotting

root formation, although shoot performance was poor, but this

could be improved by modifying to RM-4 and RM-8, respectively (Fig. 2e).

Acclimatization of plantlets derived from half-anther culture of *Anthurium*

Acclimatization of plantlets to ex vitro conditions ranged from 64 to 100% (average = 82.5 ± 12.1%). After plants were individually repotted, acclimatized plantlets increased in size as did the size and number of leaves (Fig. 2f–g), although there were obvious differences and variation in size and performance (Fig. 2f–g).

During acclimatization, several plantlets which did not appear healthy were unable to survive ex vitro. These plantlets usually had yellowish-green leaves (Fig. 2h), which turned brown; the plants finally died 15–30 days after acclimatization (Fig. 2f, white arrows). Those plants

that did survive had abnormal growth: they were short with many leaves and buds (Fig. 2j–k).

Cytological analysis

Approximately 400 plantlets showing different survival and regeneration performance were successfully acclimatized and adapted well ex vitro under glasshouse conditions. Of these, 180 acclimatized plantlets approximately 5.0 months- old that had successfully acclimatized and adapted (Fig. 2g) were sampled randomly and used for cytological analysis to explore differences in ploidy level. Cytological analysis of donor plants revealed that they had $2n$ = 29–33 (i.e., chro- mosome number), although within the 180 plantlets a wide range of ploidy regenerants derived from half-anther culture of *Anthurium* was shown: 34 were haploid, 15 aneuploid, 126 diploid and 5 triploid. The number of chromosomes in hap- loid regenerants was 15.8 chromosomes/cell (n = 14–18),

Table 8 Interaction effect of different types of callus and

growth on callus growth and regeneration media during half-anther culture of

Anthurium

Callus growth and regeneration medium (CGR)

Explant response

Callus volume (cm^3) Callus growth

rate (cm/month)

Number of shoots per callus cluster

Height of shoots (cm)

Fast-type Slow-type Fast-type Slow-type Fast-type Slow-type Fast-type Slow-type

	Callus volume (cm^3)		Callus growth rate (cm/month)		Number of shoots per callus cluster		Height of shoots (cm)	
	Fast-type	Slow-type	Fast-type	Slow-type	Fast-type	Slow-type	Fast-type	Slow-type
CGR-1	0.97 b	0.46 b	0.34 b	0.08 b	5.0 c	1.3 b	1.55 bc	0.70 b
CGR-2	0.87 bc	0.65 b	0.29 bc	0.18 b	4.8 c	1.0 b	1.00 d	0.60 b
CGR-3	0.87 bc	0.65 b	0.29 bc	0.18 b	6.5 b	1.5 b	1.40 c	0.65 b
CGR-4	1.58 a	1.34 a	0.64 a	0.52 a	8.3 a	3.3 a	2.40 a	1.15 a
CGR-5	0.91 b	0.59 b	0.32 b	0.15 b	5.0 c	1.0 b	1.08 d	0.63 b
CGR-6	0.68 c	0.60 b	0.19 c	0.15 b	5.3 c	1.5 b	1.45 bc	0.65 b
CGR-7	1.03 b	0.63 b	0.37 b	0.17 b	6.8 b	1.8 b	1.68 b	0.73 b
CGR-8	1.50 a	1.35 a	0.60 a	0.53 a	9.0 a	3.0 a	2.50 a	1.25 a

Coefficient of variation (%)	13.07	18.80	18.43	30.26	9.60	22.92	9.81	15.75

CGR-1 = WT-1 ? 0.01 mg/l NAA, 0.5 mg/l TDZ, and 1.0 mg/l BAP (control); CGR-2 = WT-1 ? 0.05 mg/l NAA and 0.5 mg/l TDZ; CGR-3 = WT-1 ? 0.01 mg/l NAA and 1.0 mg/l TDZ; CGR-4 = WT-1 ? 0.02 mg/l NAA and 1.5 mg/l TDZ; CGR-5 = NWT-3 ? 1.0 mg/l 2,4-D,

0.01 mg/l and 1.5 mg/l TDZ; CGR-6 = NWT-3 ? 0.75 mg/l 2,4-D, 0.05 mg/l NAA and 2.0 mg/l TDZ; CGR-7 = NWT-3 ? 0.75 mg/l 2,4-D,

0.01 mg/l NAA, 1.0 mg/l TDZ, and 1.0 mg/l BAP; CGR-8 = NWT-3 ? 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ, and 0.75 mg/l BAP. Means followed by the same letter in the same column are not significantly different based on DMRT at $P = 0.05$

BAP 6-benzylaminopurine, *2,4–D* 2,4-dichlorophenoxy acetic acid, *Kin* kinetin, *NAA* a-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator, *WT–1* Winarto–Teixeira basal medium-1, *NWT–3* New Winarto–Teixeira basal medium-3

Table 9 Effect of different rooting medium on root formation of shoots derived from half-anther culture of *Anthurium*

Rooting medium (RM) Root initiation period (days)[a]

Number of roots/shoot

Root

length (cm)

Growth rate 1

(root number/month)

Growth rate 2

(root length/month)

RM-1	70.3 b	2.3 de	0.38 c	0.8 e	0.60 d
RM-2	46.3 c	2.8 bcd	0.80 b	2.5 cd	1.48 ab
RM-3	32.3 d	2.5 cde	0.78 b	1.8 d	1.50 a
RM-4	26.8 ef	3.3 abc	1.00 ab	2.8 bc	1.10 c
RM-5	80.3 a	1.8 e	0.28 c	0.5 e	0.58 bc
RM-6	30.5 de	3.0 abcd	1.08 ab	3.3 abc	1.18 bc
RM-7	28.5 de	3.5 ab	0.93 b	3.8 a	1.30 abc
RM-8	22.8 f	3.8 a	1.28 a	3.5 ab	1.23 abc
Coefficient of variation (CV, %)	7.74	17.42	24.28	23.57	7.49

RM-1 = WT-1 ? 0.01 mg/l NAA, 0.5 mg/l TDZ, and 1.0 mg/l BAP (control); RM-2 = WT-1 ? 0.2 mg/l NAA and 1.0 mg/l Kin; RM- 3 = WT-1 ? 0.1 mg/l NAA and 0.5 mg/l Kin; RM-4 = WT-1 PGR-free; RM-5 = NWT-3 ? 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ,

and 0.75 mg/l BAP; RM-6 = NWT-3 ? 0.2 mg/l NAA and 1.0 mg/l Kin; RM-7 = NWT-3 ? 0.1 mg/l NAA, and 0.5 mg/l Kin; RM- 8 = NWT-3 PGR-free. Means followed by the same letter in the same column are not significantly different based on DMRT at *P* = 0.05

BAP 6-benzylaminopurine, *2,4–D* 2,4-dichlorophenoxy acetic acid, *Kin* kinetin, *NAA* a-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator, *WT–1* Winarto–Teixeira basal medium-1, *NWT–3* New Winarto–Teixeira basal medium-3

[a] The period of time from shoot initiation until roots were clearly observed to have emerged

23.8 for aneuploids (*n* = 20–26), 31.7 for diploids (*n* = 28–34) and 49.2 for triploids (*n* = 45–57).

Discussion

From this study new basal media and a simple protocol for half-anther culture of *Anthurium* were successfully

established. Through successive tests on different media, those media that did not result in favourable callus for-mation were eliminated. The first optimal medium was half-strength MS containing 0.1 mg/l 2,4-D, 0.5 mg/l BA and 0.5 mg/l Kin, termed WT-1 and from which NWT-3 was established. WT-1, the origin basal medium formula- tion (Table 2) and NWT-3, the new improved formula (Table 3), based on WT-1 medium components, were most

suitable media to induce callus formation compared to other media. Gradual improvement of results in the anther culture of *Anthurium* from half-strength MS (WT-3) to WT-1 and from WT-1 to NWT-3 were obtained by gradual modification of macro- and micro-nutrients, vitamins and PGRs. A set of factors, namely by reducing NH_4NO_3 to 550 mg/l, increasing KNO_3 to 1,250 mg/l and *myo*-inositol and thiamine HCl, supplementing $NaH_2PO_4 \cdot H_2O$ at 200 mg/l, removing nicotinic acid and pyridoxine HCl, and combining NAA, TDZ and BA (Table 2) presumably resulted in a better effect than WT-2 and WT-3 in terms of callus initiation. The callus-inducing ability of NWT-3 was further improved by: (1) increasing the concentration of ammonium and potassium nitrate, magnesium sulphate, and vitamins, and by adding TDZ and NAA; (2) elimi- nating calcium chloride; (3) reducing the concentration of sodium and potassium dihydrogen phosphate, micro nutrients, and BA (Table 3). All these alterations presum- ably enhanced and produced a balanced basal medium composition that was high suitable for callus formation. Obtaining a balanced medium composition was also nec- essary to successfully establish an in vitro culture of *Gatharanthus roseus* (Morard and Henry 1998), callus growth of *Prunus domestica* L. (Nowak et al. 2007), regeneration of *Dianthus henteri* (Cristea et al. 2010), and callus induction and regeneration of *Foeniculum vulgare* (Khorami and Safarnejad 2011). Specific basal medium for callus initiation in anther

culture was also reported on other plants. In *Lycopersicon esculentum* Mill, MS basal medium was a more appropriate basal medium than N, LS and GD basal media (Shtereva et al. [1998]). N6 basal medium was superior to Mo, MS and NN basal media for *Linum usi- tatissimum* (Bohusˇ et al. [2004]). LS and NN basal media were better than MS, N, Che´e and Pool (Che´e and Pool [1987]) basal media in the anther culture of *Capsicum ann- uum* L. (Koleva-Gudeva et al. [2007]) and PGR-free B5 basal medium for the anther culture of *Daucus carota* L. (Gorecka et al. [2009]). In hybrid *Cymbidium*, MS with or without Gamborg's micronutrients, and Gamborg's B-5 basal media promoted strong callus formation than Vacin and Went (Vacin and Went [1949]), Phytamax, Knudson C (Knudson [1946]), White (White [1963]), Hoagland's No. 2 (Hoagland and Arnon [1950]), N_6, Schenk and Hildebrand (Schenk and Hildebrandt [1972]), Woody Plant Medium (Lloyd and McCown [1981]), and Quoirin and Lepoivre (Quoirin and Lepoivre [1977]) (Teixeira da Silva et al. [2005]). These studies mostly indicate that basal medium is a key factor in developing plant anther culture protocols and maximize the response of anther explants cultured for organogenic events including callus and embryo induction, shoot and root regeneration, and embryo germination (Zhao et al. [2006]; Koleva-Gudeva et al. [2007]; Gorecka et al. [2009]; Sutan et al. [2010]).

In separate, independent studies on the anther culture of

A. andreanum cv. Carnaval (Winarto and Mattjik [2009a]) and local *Anthurium* cultivars (Winarto and Mattjik [2009b]), WT-1 and NWT-3 were better than several other basal media (CP, B5, K, LS, MS, MM, NN, W, VW, inter alia). In those studies, WT-1 containing 0.5 mg/l TDZ and 0.01 mg/ l NAA was best for callus induction while WT-1 supple- mented with 1.5 mg/l TDZ and 0.02 mg/l NAA produced the highest number of shoots. NWT-3 supplemented with

0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ and

0.75 mg/l BAP was also successfully applied to callus induction from the anther culture of *A. andreanum* Linden ex Andre´ cv. Casino, Laguna and Safari (unpublished data). NWT-3 basal medium was successfully applied to the tissue culture of *Rosa hybrida* L. cv. Kiss to induce callus, to regenerate and multiply shoots and to form roots (Winarto 2006), *Anthurium* clones, *Dendrobium* and *Phalaenopsis* clones, and anther culture of *Dianthus chinensis* (unpub- lished data). In a latest study investigating basal salt com- position, it was revealed that the basal salt of WT-1 and NWT-3 significantly affected shoot regeneration and mul- tiplication of *Rumohra adiantiformis* and *Ruscus hypo- phyllum* L. (unpublished data). It is thus evident that with minor and specific modifications, WT-1 and NWT-3 basal media have a high potential to be applied for different purposes in in vitro plant propagation such as callus induction, shoot regeneration, adventitious shoot produc- tion and proliferation, root induction and proliferation and embryogenesis, not only for *Anthurium* spp. but also for several other ornamentals of unrelated families.

The *Anthurium* anther culture protocol in this study was established by application of WT-1 and NWT-3, newly designed media, for callus initiation and proliferation, shoot regeneration and root formation. Modification of type, combination and concentration of PGRs in the media (Tables 4, 5) had a highly positive effect on callus growth, shoot regeneration and root formation (Tables 8, 9), indi- cating that each step of the anther culture protocol needs an optimal basal medium with appropriate modifications. Other detailed anther culture protocols with varied basal media and their modifications are listed in Table 10.

Acclimatization in this study was successful (approxi- mately 83%). Haploid *Anthurium* plantlets tend to die (Winarto 2009) during acclimatization, reaching as many as 90% in haploid plants (Winarto et al. 2011). The low level of acclimatization in haploid *Anthurium* plants derived from anther culture during

the acclimatization stage has also been extensively reported or observed: 15% for *A. andreanum* cv. 'Tropical' (Rachmawati [2005]), 8% for 'Carnaval' (Winarto and Mattjik [2009a]), 20% for 'Casino' and 12% for 'Laguna' (Winarto, unpublished data).

Morphological and cytological variations of plantlets derived from *Anthurium* half-anther culture were evident in

Table 10 A simple protocol for anther culture of *Anthurium* and other popular plant anther culture protocols

Plant species Basal medium and its modification Response/objective Reference

Ornamental plants

Anthurium andreanum Linden ex Andre´ cv.

WT-1 ? 0.01 mg/l NAA, 0.5 mg/l TDZ, 1.0 mg/l BAP Callus induction This study

Tropical

NWT-3 ? 0.25 mg/l 2,4-D, 0.02 mg/l NAA, 1.5 mg/l TDZ, 0.75 BAP

Callus induction and multiple shoot regeneration

NWT-3 ? 0.2 mg/l NAA, 1.0 mg/l Kin Roots

Helianthus annuus L. 1.0 MS ? 1.0 mg/l 2,4-D, 0.5 mg/l BAP, 40 g/l sucrose Embryo induction Thengane et al. ([1994])

MS ? 0.5 mg/l Kin, 0.5 mg/1 BAP, 100 mg/l CH, 2.5 mg/l $AgNO_3$

Embryo germination

MS ? 0.1 mg/l NAA, 0.5 mg/l BA Callus induction and embryo formation Saji and Sujatha ([1998])

MS ? 0.5 mg/l BA Multiple shoots and roots

Lilium longiflorum Thunb. cv. N6 medium, dark incubation

Callus induction Arzate-Ferna´ndez et al.

'Wase Teppo Yuri' ½ MS ? 1.5% sucrose, 2.5 g/l gelrite Callus regeneration

([1997](#))

Asiatic hybrid lily 'Connecticut MS ? 2 mg/1 picloram, 2 mg/1 zeatin Callus induction Han et al. ([1997](#)) King' MS ? 0.1 or 0.5 mg/l picloram, 0.01 mg/1 BA Callus regeneration

Cyclamen persicum 9 *C. purpurascens* B5 ? 0.1 or 1.0 mg/l NAA or 0.1 mg/l 2,4-D Embryoid production Ishizaka ([1998](#)) B5 ? 30 g/l sucrose Embryo germination

Hepatica nobilis L. NN ? 1% AC Embryo induction Nomizu et al. ([2004](#)) NN without AC Embryo germination

Viola wittrockiana 'Caidie' ½ MS ? 0.01 mg/l 2,4-D, 2.0 mg/l BA Callus formation Wang and Bao ([2007](#)) MS ? 1.0 mg/l GA_3, 4.0 mg/l $AgNO_3$, 0.02% AC, 1.0 mg/l TDZ Callus regeneration

MS ? 0.2 mg/l NAA, 2.0 mg/l TDZ, 3.0 mg/l GA_3 Shoot multiplication

Dianthus chinensis L. MS ? 2.0 mg/l 2,4-D, 1.0 mg/l BA, 394.6 mg/l glutamine Embryo induction Fu et al. ([2008](#)) 400 mg/l CH, and 10 mg/l proline, 7.5 g/l agar, 30 g/l sucrose

½ MS ? 0.1 mg/l NAA Root formation

½ NLN ? 130 g/l sucrose Embryo induction Doi et al. ([2010](#))

Gentiana triflora ½ MS ? 30 g/l sucrose Embryo regeneration Vegetable plants

Cucurbita pepo MS ? 5 mg/l 2,4-D, 150 g/l sucrose Embryo induction and germination Metwally et al. ([1998](#))

Capsicum annuum L. Nitsch ? 0.55 mg/l zeatin, 0.88 mg/l IAA, 2% maltose, 1% AC Embryo induction Supena et al. ([2006](#))

½ MS ? 0.02 mg/l BA, 2% sucrose, 0.6% plant agar Embryo germination

Solanum lycopersicum L. NLN ? 0.5 mg/l BAP, 0.5 mg/l NAA, 130 g/l sucrose Callus and embryo Segu´ı-Simarro and Nuez

MS ? 0.25 mg/l zeatin riboside, 20 g/l sucrose Callus regeneration

([2007](#))

Brassica spp. MS ? 0.5 mg/l 2,4-D or B5 ? 0.5 mg/l 2,4-D Callus induction Sayem et al. ([2010](#)) MS ? 1.0 mg/l BAP 0.3 mg/l 2,4-D Callus maintenance

MS ? 2.0 mg/l BAP, 0.5 mg/l NAA Callus regeneration

this study. Morphological variation was clearly observed in in vitro regenerants and in ex vitro plantlets derived from half-anther culture of *Anthurium*. Variation in callus color, deviation from standard potted growth and regeneration (= shoot) capacity are strongly evident in anther or half-anther culture of *Anthurium* (Rachmawati [2005](#); Winarto et al. [2010a](#); Winarto et al. [2011](#)). In addition, strong variation in acclimatized plantlets are also evident: plant size, leaf size, shape and length (Fig. [2](#)g), as well as spathe and spadix color, shape and size (Winarto et al. [2011](#)).

Reference

Embryo induction Embryo multiplication

Embryogenic callus induction Embryo [maturation](#)

Embryo enlargement and regeneration

Rimberia et al. (2006)

Cao et al. (2010)

BAP 6-benzylaminopurine, *BA* N^6-benzyladenine, *CPPU* *N*-(2-chloro-4-pyridyl)-N^0-phenylurea, *2,4–D* 2,4-dichlorophenoxy acetic acid, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *2–iP* N^6-[2-isopentenyl]adenine, *Kin* kinetin, *NAA* a-naphthalene acetic acid, *TDZ* thidiazuron, *AC* activated charcoal, *B5* Gamborg's medium (Gamborg et al. 1968), *CH* casein hydrolysate, N_6 Chu (N_6) medium (Chu et al. 1975), *ME* malt extract, *MS* Murashige and Skoog (1962) medium, *MT* Murashige and Tucker (1969) medium, *NN* Nitsch and Nitsch (1969), *NLN* Lichter (NLN) medium (Huang and Keller 1989), *WT–1* Winarto–Teixeira basal medium-1 (Winarto et al. 2011), *NWT–3* New Winarto–Teixeira basal medium-3 (Winarto et al. 2011)

Cytological variation revealed different ploidy levels of acclimatized plantlets: haploid, diploid, triploid or aneuploid (Winarto et al. 2010b, 2011). The different ploidy levels resulted from cytological variation in anther wall cells (Winarto et al. 2010a), which in other plants, have been shown to become competent and meristematic, divide actively and overcome alterations caused by the applica- tion of PGRs such as 2,4-D, TDZ and BAP during tissue culture (Dolezel and Novak 1984; Rodrigues et al. 2004; Jin et al. 2008). The application of PGRs generally causes an imbalance in mitotic activity of cells (Cella´rova´ et al. 2004) that leads to increasing frequency of mitotic aber- rations in *Allium sativum* L. (Dolezel and Novak 1984), reduction in chromosomes in *Arabidopsis thaliana* (Fras and Maluszynska 2004), endopolyploidy in hybrid *Cym- bidium* (Teixeira da Silva and Tanaka 2006), and loss of chromosomes 4 and 5 in *Gossypium hirsutum* (Jin et al. 2008), although, simultaneously, there were small but statistically significant increases in SCE frequencies with 5

Response/objective

MS ? 0.01 mg/l CPPU, 0.1 mg/l NAA MS ? 0.0025 mg/l CPPU

MT ? 0.5 g/l ME, 40 g/l sucrose, 8 g/l agar MT ? 1.5 g/l ME, 40 g/l sucrose, 8 g/l agar

MT ? 0.5 mg/l BA, 0.5 mg/l kin, 0.1 mg/l NAA, 25 g/l sucrose, 8 g/l agar

½ MT ? 0.5 mg/l NAA, 0.1 mg/l IBA, 25 g/l sucrose, 0.5 g/l AC, 8 g/l agar

Roots

and 15 lM 2,4-D.

Basal medium and its modification

Although the level of doubled-haploid regenerants for *Anthurium* is not as successful as other plant anther cultures of some well established model plants—90% in *Hordeum vulgare* (Szarejko et al. 1997), 70% in *Capsicum annuum* L. (Supena et al. 2006) and 65% in Asiatic hybrid lily (Han et al. 1997)—the 40 (22%) acclimatized haploid plantlets obtained in this study is a remarkable and suc- cessful indicator that successful haploid technology has been developed in this study for *Anthurium*. Moreover, the result of our protocol was better than haploid plantlet recovery in *Lilium longiflorum* Thunb. (Arzate-Ferna´ndez et al. 1997), *Helianthus annuus* L (Saji and Sujatha 1998), and *Dianthus cinensis* L. (Fu et al. 2008). In *Spatiphyllum wallisii* (Araceae), few haploid plants could be success- fully regenerated. The plants were not produced by gynogenesis (Eeckhaut et al. 2001).

Citrus sinensis [L.] Osbeck cv. Rohde Red

Variation was also observed in regenerants derived from plant anther cultures of *Solanum commersonii* (Cardi et al. 1993), *Lilium longiflorum* (Arzate-Ferna´ndez et al. 1997), Asiatic

hybrid lily (Han et al. [1997]), *Helianthus annuus* L. 'Morden' (Thengane et al. [1994]; Saji and Sujatha [1998]), *Dianthus chinensis* L. (Fu et al. [2008]), *Daucus carota* (Kozik et al. [2002]), *Citrus clementina* Hort. ex Tan

Table 10 continued

Plant species

Fruit plants

Carica papaya L.

(Chiancone et al. [2006]), *Carica papaya* L. (Rimberia et al. [2006]), and *Brassica oleracea* var. *botrytis* (Ockendon [2008]) (details in Table [10]). The morphological changes were in the size of plants, leaves, flowers, fruits, parthe- nocarpic ability, fruit yield, albino, vigor, etc. In this and other studies by our group on *Anthurium*, cytological and morphological variations were wide-spread.

References

Arzate-Ferna´ndez AM, Nakazaki T, Yamagata H, Tanisaka T (1997) Production of double haploid plants from *Lilium longiflorum* Thunb. anther culture. Plant Sci 123:179–187

Atak C¸, C¸ elik O¨ (2009) Micropropagation of *Anthurium andraeanum*

from leaf explants. Pak J Bot 41(3):1155–1161

Bejoy M, Sumitha VR, Anish NP (2008) Foliar regeneration in *Anthurium andreanum* Hort. cv. Agnihothri Biotech 7(1): 134–138

Bohusˇ O, Dedicˇova´ B, Hricova´ A, Sˇamaj J, Pretrova´ A (2004) Flax

anther culture: effect of genotype, cold treatment and media. Plant Cell Tissue Organ Cult 79(2):233–238

Bouman H, Tiekstra A (2001) Mineral nutrition in tissue culture: influence on propagation and quality of the plantlets. In: Horst WJ et al (eds) Plant nutrition—food Security and sustainability of agro-ecosystems. Kluwer, The Netherlands, pp 316—317

Cao H, Biswas MK, Lu Y, Amar MH, Tong Z, Xu Q, Juan Xu J, Guo W, Deng X (2010) Doubled haploid callus lines of Valencia sweet orange recovered from anther culture. Plant Cell Tissue Organ Cult. Published online: 17 Oct 2010

Cardi T, Iannamico V, D'ambrosio F, Filippone E, Lurquin PF (1993) In vitro regeneration and cytological characterization of shoots from leaf explants of three accessions of *Solanum commersonii*. Plant Cell Tissue Organ Cult 34(1):107—114

Cella´rova´ E, Rychlova´ M, Seidelova´ A, Honriv R (2004) Comparison of mitotic activity and growth in two long term callus cultures of *Matricaria recutita* L. Acta Biotechnol 10(3):245—251

Che´e R, Pool RM (1987) Improved organic media constituents for in vitro shoot multiplication of *Vitis*. Sci Hort 32:85—95

Chen FC, Kuehnle AR, Sugii N (1997) *Anthurium* roots for micropropagation and *Agrobacterium*-mediated gene transfer. Plant Cell Tissue Organ Cult 49:71—74

Chiancone B, Tassoni A, Bagni N, Germana MA (2006) Effect of polyamines on *in vitro* anther culture of *Citrus clementina* Hort. ex Tan. Plant Cell Tissue Organ Cult 87:145—153

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FI (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sinica 18:659—668

Cristea V, Brummer AT, Jarda L, Micla˘us¸ M (2010) In vitro culture initiation and phytohormonal influence on *Dianthus*

henteri—a Romanian endemic species. Rom Biotechnol Lett 15(1):25–33

Darnaedi D (1991) Informasi Kromosom. Pelatihan Sitogenetika.

PAU Ilmu Hayat, IPB, 5 Nov 1991. Bogor

Doi H, Takahashi R, Hikage T, Takahata Y (2010) Embryogenesis and doubled haploid production from anther culture in gentian (*Gentiana triflora*). Plant Cell Tissue Organ Cult 102:27–33

Dolezel J, Novak FJ (1984) Cytogenetic effect of plant tissue culture medium with certain growth substances on *Allium sativum* L. meristem root tip cells. Biol Plant 26(4):293–298

Eeckhaut T, Werbrouck S, Dendauw J, van Bockstaele E, Debergh P (2001) Induction of homozygous *Spatiphyllum wallisii*

genotypes through gynogenesis. Plant Cell Tissue Organ Cult 67:181–189

Fras A, Maluszynska J (2004) The correlation between the chromosome variation in callus and genotype of explants of *Arabidopsis thaliana*. Genetica 121(2):145–154

Fu XP, Yang SH, Bao MZ (2008) Factors affecting somatic embryogenesis in anther cultures of Chinese pink (*Dianthus chinensis* L.). In Vitro Cell Dev Biol Plant 44:194–202

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gantait S, Mandal N, Bhattacharyya S, Das PK (2008) *In vitro* mass multiplication with pure genetic identity in *Anthurium andrea- num* Lind. Plant Tissue Cult Biotech 18(2):113–122

George EF, Hall MA, De Klerk GJ (2007) Plant propagation by tissue culture. In: The background. exegetic, vol 1, 3rd edn, Basingstone

Gorecka K, Krzyzanowska D, Kiszczak W, Kowalska U (2009) Plant regeneration from carrot (*Daucus carota* L.) anther culture derived embryos. Acta Physiol Plant 31:1139–1145

Guo YD, Sewon P, Pulli S (1999) Improved embryogenesis from anther culture and plant regeneration in timothy. Plant Cell Tissue Organ Cult 57:85–93

Hamidah M, Karim AGA, Debergh P (1997) Somatic embryogenesis and plant regeneration in *Anturium scherzerianum*. Plant Cell Tissue Organ Cult 48:189–193

Han DS, Niimi Y, Nakano M (1997) Regeneration of haploid plants from anther cultures of the Asiatic Irbid lily 'Connecticut King'. Plant Cell Tissue Organ Cult 47:153–158

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circ Univ Calif Agric Exp Station Berkley, p 347

Huang B, Keller WA (1989) Microspore culture technology. J Tissue Cult Methods 12:171–178

Ishizaka H (1998) Production of microspore-derived plants by anther culture of an interspecific F1 hybrid between *Cyclamen persicum* and *C. purpurascens*. Plant Cell Tissue Organ Cult 54:21–28

Jahan MT, Islam MR, Khan R, Mamun ANK, Ahmed G, Hakim L (2009) *In vitro* clonal propagation of anthurium (*Anthurium andraeanum* L.) using callus culture. Plant Tissue Organ Cult Biotech 19(1):61–69

Jin S, Mushke R, Zhu H, Tu L, Lin Z, Zhang Y, Zhang X (2008) Detection of somaclonal variation of cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. Plant Cell Rep 27(8):1303–1316

Joseph D, Martin KP, Madassery J, Philip VJ (2003) *In vitro* propagation of three commercial cut flower cultivars of

Anthurium andraeanum L. Hort. Indian J Exp Biol 41:154–159

Khorami R, Safarnejad A (2011) *In vitro* selection of *Foeniculum vulgare* for salt tolerance. Not Sci Biol 3(2):90–97

Knudson K (1946) A new nutrient solution for germination of orchid seeds. Am Orchid Soc Bull 15:214–217

Koleva-Gudeva LR, Spasenoski M, Trajkova F (2007) Somatic embryogenesis in pepper anther culture: the effect of incubation treatments and different media. Sci Hort 111(2):114–119

Kozik EU, Nowak R, Kłosin˜ska U, Go´recka K, Krzyz_ anowska D, Go´recki R (2002) Morphological diversity of androgenic carrot plants. J Appl Genet 43(1):49–53

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. Physiol Plant 18:100–127

Lloyd G, McCown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Comb Proc Int Plant Propag Soc 30:421–427

Maira O, Alexander N, Vargas TE (2009) Micropropagation and organogenesis of *Anthurium andreanum* Lind cv Rubrun. Plant Sci 585:3–14

Martin KP, Joseph D, Madassery J, Philip VJ (2003) Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraeanum* L. Hort. In Vitro Cell Dev Biol Plant 39(5):500–504

Metwally EI, Moustafa SA, El-Sawy BI, Shalaby TA (1998) Haploid plantlets derived by anther culture of *Cucurbita pepo*. Plant Cell Tissue Organ Cult 52:171–176

Miller LR, Murashige T (1976) Tissue culture propagation of tropical foliage plants. In Vitro 12(12):797–813

Morard P, Henry M (1998) Optimization of the mineral composition of *in vitro* culture media. J Plant Nutr 21(8):1565–1576

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497 Murashige T, Tucker DPH (1969) Growth factor requirement of citrus tissue culture. In: International citrus symposium, 1, Riverside, Proceedings Riverside, vol 3. University of California,

pp 1155–1169

Niedz RP, Evens TJ (2007) Regulating plant tissue growth by mineral nutrition. In Vitro Cell Dev Biol Plant 43:370–381

Nitsch JP, Nitsch C (1969) Haploid plant from pollen grains. Science 169:85

Nomizu T, Niimi Y, Han DS (2004) Haploid plant regeneration via embryogenesis from anther cultures of *Hepatica nobilis*. Plant Cell Tissue Organ Cult 79:307–313

Nowak BK, Miczyn´ski K, Hudy L (2007) The effect of total inorganic

nitrogen and the balance between its ionic forms on adventitious bud formation and callus growth of 'We̜gierka Zwykła' plum (*Prunus domestica* L). Acta Physiol Plant 29(5):479–484

Ockendon DJ (2008) The ploidy of plants obtained from anther culture of cauliflowers (*Brassica oleracea* var. *botrytis*). Ann Appl Biol 113(2):319–325

Quoirin M, Lepoivre P (1977) Improved medium for *in vitro* culture of *Prunus* sp. Acta Hort 78:437–442

Rachmawati F (2005) Kultur anther pada anthurium (*Anthurium andreanum* Linden ex Andre´). Thesis. Departemen Agronomi dan Hortikultura. Fakultas Pertanian. Institut Pertanian Bogor, 133 Halaman, Indonesia

Rimberia F, Adaniya S, Etoh T, Ishimine Y (2006) Sex and ploidy of anther culture derived papaya (*Carica papaya* L.) plants. Euphytica 149(1–2):53–59

Rodrigues IR, Forte BC, Olivera JMS, Mariath JEA, Bodanese-Zanettini MH (2004) Effects of light conditions and 2, 4-D concentration in soybean anther culture. Plant Growth Regul 44:125–131

Saji KV, Sujatha M (1998) Embryogenesis and plant regeneration in anther culture of sunflower (*Helianthus anuus* L.). Euphytica 103:1–7

Sayem MA, Maniruzzaman M, Siddique SS, Al-Amin M (2010) *In vitro* shoot regeneration through anther culture of *Brassica* spp. Bangladesh J Agric Res 35(2):331–341

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199

Segu´ı-Simarro JM, Nuez F (2007) Embryogenesis induction, callogenesis, and [plant regeneration by *in vitro* culture](#) of tomato isolated microspores and whole anthers. J Exp Bot 58(5): 1119–1132

Shtereva LA, Zagorska NA, Dimitrov BD, Kruleva MM, Oanh HK (1998) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill). II. Factors affecting induction of androgenesis. Plant Cell Rep 18:312–317

Supena EDJ, Suharsono S, Jacobsen E, Custers JBM (2006) Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper

(*Capsicum annuum* L.). Plant Cell Rep 25:1–10

Sutan AN, Popescu A, Isac V (2010) *In vitro* culture medium and explant type effect on callogenesis and shoot regeneration in two genotypes of ornamental strawberry. Rom Biotech Lett 15(2):12–18

Szarejko I, Falk DE, Janusz A, Nabialkowska D (1997) Cytological and genetic evaluation of anther culture-derived doubled haploids in barley. J Appl Genet 38(4):437–452

Teixeira da Silva JA, Tanaka M (2006) Embryogenic callus, PLB and TCL paths to regeneration in hybrid *Cymbidium* (Orchidaceae). J Plant Growth Reg 25(3):203–210

Teixeira da Silva JA, Yam T, Fukai S, Nayak N, Tanaka M (2005) Establishment of optimum nutrient media for *in vitro* propagation of *Cymbidium* Sw. (Orchidaceae) using protocorm-like body segments. Propag Ornam Plants 5(3):129–136

Teng WL (1997) Regeneration of anthurium adventitious shoots using liquid or raft culture. Plant Cell Tissue Organ Cult 49:153–156 Thengane SR, Joshi MS, Khuspe SS, Mascarenhas AE (1994) Anther culture in *Helianthus annuus* L., influence of genotype and culture conditions on embryo induction and plant regeneration.

Plant Cell Rep 13:222–226

Thimijan RW, Heins HD (1982) Photometric, radiometric, and quantum light units of measure: a review of procedures for interconversion. HortSci 18:818–822

Vacin EF, Went EW (1949) Some pH changes in nutrient solution.

Bot Gaz 110:605–613

Vargas TE, Mejias A, Oropeza M, de Garcia E (2004) Plant regeneration of *Anthurium andraeanum L.* cv. Rubrun. Electron J Biotechnol 7(3):5

Vie´gas J, da Rocha MTR, Ferreira-Moura I, Correˆa MGS, da Silva

JB, dos Santos NC, Teixeira da Silva JA (2007) *Anthurium andraeanum* (Linden ex Andre´) culture: *in vitro* and *ex vitro*. Floric Ornam Biotechnol 1(1):61–65

Wang J, Bao MZ (2007) Plant regeneration of pansy (*Viola wittrockiana*) 'Caidie' via petiole-derived callus. Sci Hort 111(3):266–270

Westfall PH, Tobias RD, Rom D, Wolfinger RD, Hochberg Y (1999) Multiple comparisons and multiple tests: using the SAS system. SAS Publishing, SAS Institute Inc, Cary

White PR (1963) The cultivation of animal and plant cells. Ronald Press, New York

Winarto B (2006) The effect of explant types and culture media on the *in vitro* regeneration of adventitious shoots of rose. J Agrotrop 11(2):67–73

Winarto B (2007) Response of axillary and adventitious shoot formation of *in vitro* culture of *Anthurium*. J Hortic 17(1):17–25 Winarto B (2009) Androgenesis: a breakthrough effort for preparing haploid or double-haploid plants in *Anthurium*. PhD Disserta- tion. Department of Agronomy and Horticulture, Faculty of

Agriculture. Bogor Agriculture Institute, 235 pp

Winarto B, Mattjik NA (2009a) Response of *Anthurium andreanum* Linden ex Andre´ cv. Carnaval anther in medium with various combinations of plant growth regulator concentrations. J Agron Indonesia 37(2):138–144

Winarto B, Mattjik NA (2009b) Studies on anther culture of local accessions of *Anthurium*. J Hortic 19(4):386–395

Winarto B, Rachmawati F (2007) Preliminary study of anther

culture of *Anthurium*. J Hortic 17(2):127–137

Winarto B, Mattjik NA, Purwito A, Marwoto B (2010a) Improvement of selected induction culture media on callus induction in anther culture of *Anthurium* and a histological study on its callus formation. J Nat Indonesia 12(2):93–101

Winarto B, Mattjik NA, Teixeira da Silva JA, Purwito A, Marwoto B (2010b) Ploidy screening of anthurium (*Anthurium andreanum* Linden ex Andre´) regenerants derived from anther culture. Sci Hortic 127:86–90

Winarto B, Rachmawati F, Pramanik D, Teixeira da Silva JA (2011) Morphological and cytological diversity of regenerants derived from *Anthurium* half-anther culture. Plant Cell Tissue Organ Cult 105(3):375–382

Yu YX, Liu L, Liu JX, Wang J (2009) Plant regeneration by callus- mediated protocorm-like body induction of *Anthurium andraea- num* Hort. Agric Sci China 8(5):572–577

Zhao FC, Nilanthi D, Yang YS, Wu H (2006) Anther culture and haploid plant regeneration in purple coneflower (*Echinacea purpurea* L.). Plant Cell Tiss Organ Cult 86:55–62