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# New basal media for half-anther culture of *Anthurium andreanum* Linden ex André cv. Tropical

**Budi Winarto · F. Rachmawati · Jaime A. Teixeira da Silva**

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**Abstract** A successful protocol for high frequency callus induction and plant regeneration from *Anthurium andreanum* Linden ex André cv. Tropical half-anthers is described. Different variables using Winarto and Teixeira and Murashige and Skoog basal media supplemented with several plant growth regulators [2,4-dichlorophenoxy acetic acid (0.1–1.0 mg/l), α-naphthalene acetic acid (0.01–0.2 mg/l), thidiazuron (0.5–2.0 mg/l), 6-benzylaminopurine (0.5–1.0 mg/l), and kinetin (0.5–1.0 mg/l)] were tested for their ability to induce high frequency callusing in half-anthers, indirect regeneration and rooting of shoots. Basal medium, as well as the combination and concentration of hormones applied, had a significant effect on callus formation, shoot regeneration and adventitious root formation. Winarto and Teixeira-1, an original basal medium containing 0.01 mg/l α-naphthalene acetic acid, 0.5 mg/l thidiazuron and 1.0 mg/l 6-benzylaminopurine was suitable for callus formation while an improved basal medium i.e., New Winarto–Teixeira-3 supplemented with 0.25 mg/l 2,4-dichlorophenoxy acetic acid, 0.02 mg/l α-naphthalene acetic acid, 1.5 mg/l thidiazuron and 0.75 mg/l 6-benzylaminopurine enhanced callus formation. High shoot regeneration and multiplication was also possible on New Winarto–Teixeira-3. Shoots formed a strong adventitious root system on New Winarto–Teixeira-3 containing 0.2 mg/l α-naphthalene acetic acid and 1.0 mg/l kinetin. Plantlets that varied in size and performance were successfully acclimatized and adapted to ex vitro conditions. Cytological analysis of 180 acclimatized-plantlets ex vitro revealed that 34 were haploid ($n = 14$–18), 15 aneuploid ($n = 20$–26), 126 diploid ($n = 28$–34) and 5 triploid ($n = 45$–57). The potential use of this protocol for developing half-anther culture of other *Anthurium* species or cultivars is discussed.

**Keywords** Basal medium · Anther culture · Cytology · *Anthurium andreanum* · Linden ex André cv. Tropical · Tissue culture · Acclimatization

B. Winarto (✉) · F. Rachmawati
Tissue Culture Laboratory, Indonesian Ornamental Crops Research Institute, P.O. Box 8, Sindanglaya, Pacet-Cianjur 43253, West Java, Indonesia
e-mail: budi_winarto67@yahoo.com

J. A. Teixeira da Silva (✉)
Faculty of Agriculture and Graduate School, Kagawa University, Miki-cho, Kagawa 761-0795, Japan
e-mail: jaimetex@yahoo.com

## Introduction

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). Furthermore, 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-Fernández et al. 1997; Ishizaka 1998; Nomizu et al. 2004; Seguí-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 macronutrients 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 formulations 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 concentration 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 macronutrients 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 André 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,

**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 μM 2,4-D, 6% sucrose | Embryo induction | Hamidah et al. (1997) |
| | | MS + 0.46 μM kinetin | Embryo germination | |
| *A. andreanum* Hort | Root | MS + 2.2 μM BA | Multiple shoots | Chen et al. (1997) |
| | Leaf | MS + 2.2–4.4 μM BA, 0.9 μM 2,4-D | Adventitious shoots | Teng (1997) |
| | Leaf | ½ MS + 1.11 μM BA, 1.14 μM IAA, 0.46 μM Kin | Shoot induction | Martin et al. (2003) |
| | | ½ MS + 0.44 μM BA | Multiple shoots | |
| | | ½ MS + 0.54 μM NAA, 0.93 μM Kin | Roots | |
| | Leaf | ¼ MS + 0.88 μM BA, 0.9 μM 2,4-D, 0.46 μM Kin | Callus | Joseph et al. (2003) |
| | | ¼ MS + 0.88 μM BA, 0.54 μM NAA, 0.46 μM Kin | Multiple shoots | |
| | | ½ MS + 0.44 μM NAA | Roots | |
| | Seed | MS + 4.4 μM BA, 0.05 μM NAA | Multiple shoots | Vargas et al. (2004) |
| | Leaf | ½ MS + 0.08 mg/l 2,4-D, 1.0 mg/l BAP, 1.0 mg/l 2-iP | Callus | Viégas et al. (2007) |
| | | 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 Çelik (2009) |
| | | ½ MS salt with 250 mg/l $NH_4NO_3$, 0.1 mg/l 2,4-D, 1 mg/l BA | Shoot regeneration | |
| | | ½ MS + 1 mg/l IBA, 0.04% AC | 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 μM 2,4-D and 8.88 μM BA | Callus induction | Yu et al. (2009) |
| | | ½ MS + 0.90 μM 2,4-D and 4.44 μM BA | Callus proliferation | |
| | | ½ MS + 4.44 μM BA | PLB production | |
| | | ½ MS PGR-free | PLB germination | |

*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* α-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 μmol/m$^2$/s light intensity during the dry season (April–October) and 37–111 μmol/m$^2$/s in the rainy season (November–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/m$^2$/s by multiplying each data point with a conversion factor for sunlight i.e., 0.0185 (Thimijan and Heins 1982). The plants were watered with liquid fertilizer (2 g/l of N:P:K, 20:15:15; Nusa Tani, Ltd, Jakarta) at 3-day intervals. 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 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 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) (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, Tutlingen, 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 previous 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 separately 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 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 × 3 × 3 mm,

**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) | | |
|---|---|---|---|
| | WT-1 | WT-2 | WT-3 |
| Macronutrients | | | |
| $NH_4NO_3$ | 550 | 650 | 825 |
| KNO3 | 1,250 | 1,100 | 900 |
| $Ca(NO_3)_2{\cdot}4H_2O$ | – | 392.5 | – |
| $MgSO_4$ | 180 | 195.3 | 195 |
| $CaCl_2$ | 300 | – | 220 |
| $NaH_2PO_4{\cdot}H_2O$ | 200 | – | – |
| $KH_2PO_4$ | 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* α-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator

**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 |
| 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* α-naphthalene acetic acid, *TDZ* thidiazuron, *PGR* plant growth regulator

l × w × 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 multiplied. For the rooting experiment, ±2.5 cm long shoots with 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 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 in a 12-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8% relative 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; Chée and Pool basal medium (Chée 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

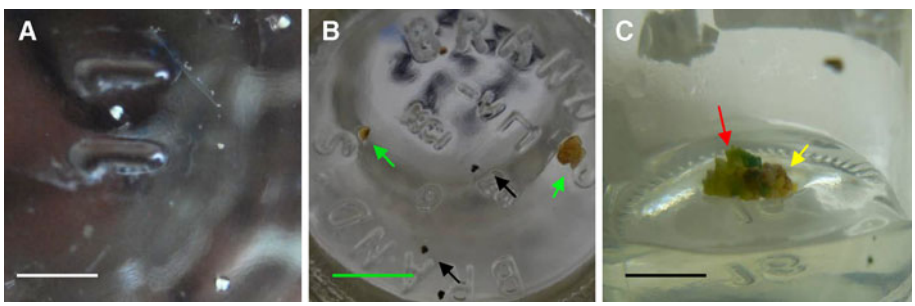


**Fig. 1** Formation of callus in half-anther culture of anthurium. **a** Half-anthers in initial culture. **b** Half-anthers with callus produced approximately 60 days after culture initiation; *green arrows* indicate regenerated half-anther and callus produced while *black arrows* show dead half-anthers 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* α-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* α-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 Cefotaxime (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 modifications of WT-3 containing Cef and PA in several combinations (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 treatment 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 considered 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 (%) |
|---|---|---|---|
| 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 concentrations of PGRs to further stimulate CGR. The modifications 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 × 3 × 3 mm, l × w × 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 replications. 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 × 20 × 15 cm, l × w × 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 μmol/$m^2$/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-hydroxyquinoline (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) solution 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 × 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 biological microscope Labophot-2 (Nikon Corp., Tokyo, Japan) at 400–1,000× magnification. Clear and informative 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.). Chromosome numbers were calculated from 10 cells per specimen 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 dedifferentiated 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 approximately 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 regeneration; 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 initiation. 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 culture 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 (%) |
|---|---|---|---|
| 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 subcultured, 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 produced 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 combinations, 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 positive 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 medium 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

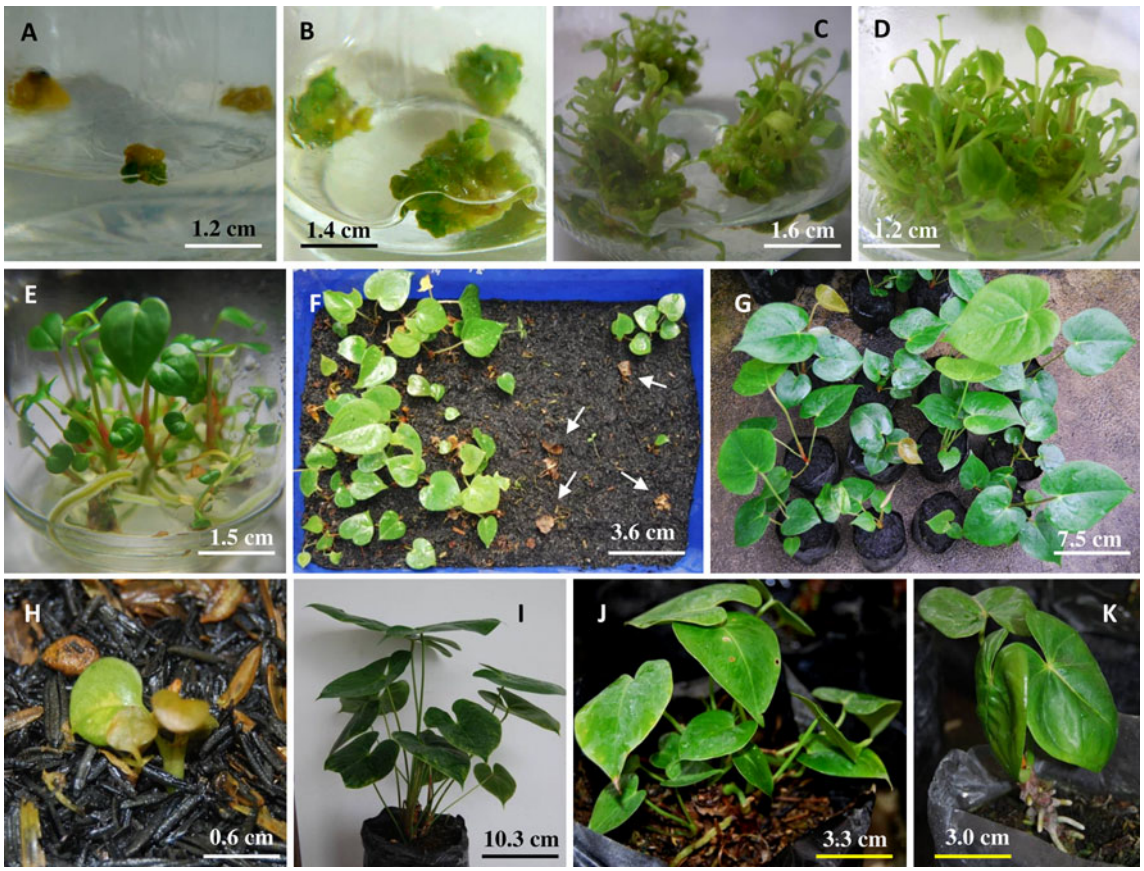


**Fig. 2** Successive process of half-anther culture of anthurium from regeneration of callus up until ex vitro acclimatization. **a** Subcultured-calli for regeneration purposes 10 days after culture initiation. **b** Regenerated and initial shoots 1.5 months after culture. **c** Regenerated shoots 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., chromosome 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 haploid 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 |
| 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* α-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* α-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 formation 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 formulation (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) eliminating calcium chloride; (3) reducing the concentration of sodium and potassium dihydrogen phosphate, micro nutrients, and BA (Table 3). All these alterations presumably enhanced and produced a balanced basal medium composition that was high suitable for callus formation. Obtaining a balanced medium composition was also necessary 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 usitatissimum* (Bohuš et al. 2004). LS and NN basal media were better than MS, N, Chée and Pool (Chée and Pool 1987) basal media in the anther culture of *Capsicum annuum* 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 supplemented 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 André 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* (unpublished data). In a latest study investigating basal salt composition, it was revealed that the basal salt of WT-1 and NWT-3 significantly affected shoot regeneration and multiplication of *Rumohra adiantiformis* and *Ruscus hypophyllum* 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 production 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), indicating 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 (approximately 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 André cv. Tropical | WT-1 + 0.01 mg/l NAA, 0.5 mg/l TDZ, 1.0 mg/l BAP | Callus induction | This study |
| | 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-Fernández et al. (1997) |
| 'Wase Teppo Yuri' | ½ MS + 1.5% sucrose, 2.5 g/l gelrite | Callus regeneration | |
| 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* × *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 | Seguí-Simarro and Nuez (2007) |
| | MS + 0.25 mg/l zeatin riboside, 20 g/l sucrose | Callus regeneration | |
| *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 | |

**Table 10** continued

| Plant species | Basal medium and its modification | Response/objective | Reference |
|---|---|---|---|
| Fruit plants | | | |
| *Carica papaya* L. | MS + 0.01 mg/l CPPU, 0.1 mg/l NAA | Embryo induction | Rimberia et al. (2006) |
| | MS + 0.0025 mg/l CPPU | Embryo multiplication | |
| *Citrus sinensis* [L.] Osbeck cv. Rohde Red | MT + 0.5 g/l ME, 40 g/l sucrose, 8 g/l agar | Embryogenic callus induction | Cao et al. (2010) |
| | MT + 1.5 g/l ME, 40 g/l sucrose, 8 g/l agar | Embryo maturation | |
| | MT + 0.5 mg/l BA, 0.5 mg/l kin, 0.1 mg/l NAA, 25 g/l sucrose, 8 g/l agar | Embryo enlargement and regeneration | |
| | 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 | |

*BAP* 6-benzylaminopurine, *BA* $N^6$-benzyladenine, *CPPU N*-(2-chloro-4-pyridyl)-N′-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* α-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)

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. 2g), as well as spathe and spadix color, shape and size (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 application 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 (Cellárová et al. 2004) that leads to increasing frequency of mitotic aberrations in *Allium sativum* L. (Dolezel and Novak 1984), reduction in chromosomes in *Arabidopsis thaliana* (Fras and Maluszynska 2004), endopolyploidy in hybrid *Cymbidium* (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 and 15 μM 2,4-D.

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 successful 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-Fernández 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 successfully regenerated. The plants were not produced by gynogenesis (Eeckhaut et al. 2001).

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

(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, parthenocarpic ability, fruit yield, albino, vigor, etc. In this and other studies by our group on *Anthurium*, cytological and morphological variations were wide-spread.

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