Establishment of an Efficient Micropropagation System in *Anthurium* Hybrids Through *In Vitro* Callogenesis and Suspension Culture

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The development of new *Anthurium* cultivars relies on efficient micropropagation, which is highly dependent on genotypes, to provide enough young plants for cultivation. Microbial contamination is the critical factor for successful initiation of aseptic culture of *Anthurium* cultivars. The aims of this study were to investigate surface disinfection with either sodium hypochlorite or a fungicide Plant Preservative Mixture™ (PPM), and supplemental PPM in the medium to evaluate their effect on reducing contamination and tissue browning. Next, a liquid suspension culture system was developed to regenerate the adventitious shoots efficiently in a short time for subsequent establishment of young plantlets for transplanting. Disinfecting newly developed leaves of ‘Kaohsiung No. 1’ with 0.6% sodium hypochlorite was more effective than other disinfection treatments even though only 5% of leaf explants produced callus. However, disinfecting the leaves of ‘Kaohsiung No. 2’ with 0.15% sodium hypochlorite was more effective than other treatments, with a callus induction rate up to 100%. No callus was induced on ‘Orange Hot’ at any hypochlorite concentration. Disinfection using PPM could reduce the contamination rate. By supplementing 0.01% PPM to the culture medium, the callus induction rate of ‘Kaohsiung No. 2’ was up to 55%, and the browning rate was lower than the control with hypochlorite disinfection. However, surface disinfection using 25% and 50% PPM did not lead to any callus formation in any of the three cultivars. Calluses of *A.* ‘Kaohsiung No. 2’ induced after 60 days of culture were transferred onto liquid suspension after 60 days for further proliferation of adventitious shoots and subsequent plantlet regeneration. Young plantlets could be successfully transplanted in peat moss and later flowered within 16 months.

**Key Words:** adventitious shoot regeneration, *Anthurium* spp., leaf explant, surface disinfection.

*Introductions*

*Anthurium andraeanum* and its related species belong to Araceae, with their origin distributed in Central and South America (Croat, 1983, 1986; French and Hegnauer, 1997), including about 1000–1500 tropical species (Farsi et al., 2012; Gantait et al., 2008). Numerous *Anthurium* cultivars are produced for sale as cut flowers, flowering potted plants, and landscape plants (Budiarto, 2008; Nowbuth et al., 2005). *Anthurium* plants can be produced year-round, with diverse flower colors, easy packing, and long shelf life. As result, they have become one of the important economic subtropical flower crops in Taiwan (Huang, 2013). We have developed several novel varieties through conventional cross hybridization and have been awarded variety rights for selected cultivars including ‘Kaohsiung No. 1’ (Happy Melody) and ‘Kaohsiung No. 2’ (Ruby) in recent years (Huang, 2013). The continuous release of new cultivars may help meet market demands, reduce the nursery production cost, and may increase the international competitiveness of the *Anthurium* industry (Huang, 2013; Kamemoto and Kuehnlne, 1996). Micropropagation of *Anthurium* cultivars through leaf explant culture and callus induction and subsequent plantlet regeneration is widely adopted for mass production worldwide (Kuehnlne and Sugii, 1991; Martin et al., 2003; Teng, 1997). The first important step in micropropagation is to establish an aseptic plant material culture in order to eliminate bacterial, fungal, and insect contaminants effectively (Atak and Özae, 2012; Chen and Yeh, 2007; Fang and Hsu, 2012; Gantait and Mandal, 2010). However, surface disinfection to establish clean, aseptic
stocks depends on the species/cultivar (Teixeira da Silva et al., 2015a, b).

Sodium hypochlorite (NaClO) is the most frequently used disinfection chemical to establish aseptic culture of Anthurium. The disinfection time of explants with sodium hypochlorite varies with concentration (Atak and Özue, 2012) and the sensitivity of explants to the chemical. Concentration ranges of sodium hypochlorite at 1–5% for 15–20 minutes have been generally adopted for disinfection (Atak and Özue, 2009; Farsi et al., 2012; Gantait et al., 2012; Raad et al., 2012a, b). The spathe and spadix of Anthurium could be disinfected in 0.1% sodium hypochlorite for two minutes, and in 1% or 2% of sodium hypochlorite for five minutes, establishing an aseptic culture (Budiarto, 2008; Winarto et al., 2010). Viégas et al. (2007) successfully obtained clean explants by treating leaves of A. andraeanum with 1.43% sodium hypochlorite. Either single or combination treatments with the disinfection chemicals were used to sterilize explants ready to start aseptic culture.

Regardless of the form of chlorine used, hypochlorous acid (HOCl), the primary sterilant, is formed during sterilization (Niedz and Bausher, 2002). Despite the use of hypochlorite for successful surface disinfection, endogenous contaminants in the explants remain a concern when trying to obtain higher percentages of clean stocks in many plants. Therefore, it is also necessary to examine alternative disinfectants to solve the problems of fungal and bacterial contaminants. Plant Preservative Mixture™ (PPM) is a relatively new and broad-spectrum preservative and biocide for use in plant tissue culture (George and Tripepi, 2001). PPM is composed of mainly two broad-spectrum industrial isothiazolone biocides, chloromethylisothiazolone and methylisothiazolone, as well as other chemicals (Guri and Patel, 1998; Lunghusen, 1998). It was evaluated as an alternative to conventional antibiotics and fungicides to control microbial contamination (Niedz, 1998). Before using PPM, its effect on explant disinfection and culture response must be evaluated for each species and usually each cultivar of interest (George and Tripepi, 2001). Effective control of microbial contamination using PPM was achieved as long as inoculum levels were low (Niedz and Bausher, 2002). PPM at 0.5 to 4.0 mL·L⁻¹ levels supplemented to the media were tested with Chrysanthemum leaf explants, and the results revealed 0.5 to 1.0 mL·L⁻¹ PPM is optimal to reduce contamination (George and Tripepi, 2001). Niedz and Bausher (2002) reported overcoming contaminants on bud explants of trifoliate oranges from greenhouse-grown trees that routinely controlled (95% clean) with the ‘standard’ disinfestation procedure and on culture medium containing 5 mL·L⁻¹ PPM.

The aims of this study were to investigate the effect of sodium hypochlorite and PPM used for both surface disinfection and as supplement to the culture medium on the performance in Anthurium callus induction and subsequent plantlet regeneration. The effect of sodium hypochlorite disinfection on subsequent callus growth was also evaluated. Once the aseptic culture was established, we used a liquid suspension culture to fasten the plantlet regeneration and subsequent establishment of potted flowering plants.

**Materials and Methods**

**Plant materials and culture conditions**

The pot plants of Anthurium ‘Kaohsiung No. 1’ and A. ‘Kaohsiung No. 2’ were newly selected cultivars, and received Taiwan new plant variety rights in a breeding program from the Kaohsiung District Agricultural Research and Extension (Pingtung, Taiwan). Anthurium ‘Orange Hot’, a mutant cultivar derived from micropropagated Anthurium ‘Red Hot’ (Henny et al., 2003), was used to compare the micropropagation response. The mother plants were grown in a shading-net house at Kaohsiung District Agricultural Research and Extension, and received a photosynthetic photon flux of 200 μmol·m⁻²·s⁻¹ during the summer at noon. Plants were fertigated biweekly with 1 g·L⁻¹ of Peters® 20N-20P₂O₅-20K₂O fertilizer (Scotts Co. LLC, USA).

**Surface disinfection with sodium hypochlorite and callus induction**

Newly extended young leaves of the above three Anthurium andraeanum cultivars were used in this study. Leaves excised from the mother plant were washed in tap water and two drops of detergent (Salatt, Taiwan). Subsequently, the leaves were disinfected with 0.15, 0.3, 0.45, 0.6, and 0.75% sodium hypochlorite (Clorox, Malaysia) respectively, with two drops of Tween-20. After 10 minutes, they were rinsed three times in sterile water. The leaves were cut into about 1 × 1 cm² segments containing leaf veins as explants and inoculated on half-strength MS (1/2 MS; Murashige and Skoog, 1962) containing 0.5 mg·L⁻¹ TDZ (designated the induction medium) to induce callus formation according to our previous report (Yang et al., 2003). The medium pH was adjusted to 5.6–5.8 before autoclaving and subsequently 25 mL aliquots were dispensed into plastic Petri dishes (Alpha Plus Scientific Corp., Taiwan). The leaf explants were incubated in a dark environment at 25 ± 2°C and 60% RH until callus formation. Data on the primary callus that developed on leaf explants after 10 weeks were collected. The percentages of callus formation, contamination and browning were investigated.

**Effect of leaf disinfection and medium supplementation with Plant Preservative Mixture™ on callus induction**

Leaves of the three cultivars were removed from potted mother plants and washed as described above. PPM was used and applied in two ways: for leaf disinfection and as a culture medium supplement. Leaves
excised from the mother plant were washed in tap water and two drops of detergent (Salatt), disinfected in 25% and 50% PPM solutions with two drops of Tween-20, respectively, for 10 minutes and then rinsed three times in sterile water. In addition, the 0.15% sodium hypochlorite-disinfected leaves were cut into 1 × 1 cm² segments and placed in the induction medium to which 0.01% and 0.05% PPM were supplemented, respectively. Surface disinfection with 0.15% sodium hypochlorite was used as a control. The leaf explants were excised as described above and incubated in the dark at 25 ± 2°C and 60% RH until callus formation, which took about 10 weeks. Data collection and analysis were as described above.

**Callus growth as affected by hypochlorite**

The purpose of this experiment was to examine whether disinfection with sodium hypochlorite affected callus growth and subsequent shoot regeneration. A leaf explant of *A. 'Kaohsiung No. 2’* was used in this experiment. Disinfection with different sodium hypochlorite concentrations was the same as in the callus induction experiment. In this experiment, the average callus weight was measured after 60 days.

**Establishing an efficient shoot regeneration procedure for mass production of plantlets**

For adventitious shoot regeneration, calluses induced from leaf explants in the dark after 60 days were transferred to a modified liquid 1/2 MS with other ingredients full strength, and 0.2 mg·L⁻¹ BA (Kuehnle et al., 2001) then cultured in 120 mL-flasks containing 25 mL medium at 100 rpm. Subcultures were carried out with fresh medium at an interval of 30 days. Cultures were maintained at 25 ± 2°C under a 16/8 h light and dark photoperiod with 23.2 μmol·m⁻²·s⁻¹ photosynthetic photon flux density (PPFD) (measured with LI-COR, Inc., USA) supplied by cool-white fluorescent lamps (TLD36W/54NS; Phillips).

**Experimental design and statistical analysis**

For the callus induction experiment, each Petri dish contained five explants as one replicate. For all experiments, the treatments were arranged in a completely randomized design (CRD) with four replications. The data were processed and analyzed using SAS v8.01 software (Statistical Analysis System Inst., USA) by analysis of variance (ANOVA) and the least significant difference (LSD) test.

**Results**

**Effect of disinfection with sodium hypochlorite on callus induction**

By increasing the sodium hypochlorite concentration, the contamination rate was reduced among the three cultivars, despite tissue browning also occurring (Table 1). Callus formation on ‘Kaohsiung No. 2’ leaf explants was observed after three weeks of culture in the induction medium with all sodium hypochlorite disinfection concentrations except the control. Callus formation continued up to seven weeks and after that there was no further callus growth. Table 1 shows the rate of callus induction of leaf explants after 10 weeks when disinfected with different sodium hypochlorite concentrations. The callus induction rate was up to 100% when the explants of ‘Kaohsiung No. 2’ were disinfected with 0.6% sodium hypochlorite, while only a 5% callus induction rate was obtained for ‘Kaohsiung No. 1’ disinfected with 0.6% sodium hypochlorite. All other treatments resulted in much lower callus formation rates for both cultivars. No calluses were induced in ‘Orange Hot’ by any hypochlorite treatments.

| Sodium hypochlorite concentration (%) | Contamination (%) | Browning (%) | Callus formation (%) |
|--------------------------------------|-------------------|--------------|---------------------|
| **Kaohsiung No. 1**                  |                   |              |                     |
| 0                                    | 100a              | 0d           | 0d                  |
| 0.15                                 | 15b               | 85ab         | 0d                  |
| 0.3                                  | 15bc              | 85ab         | 0d                  |
| 0.45                                 | 0c                | 100a         | 0d                  |
| 0.6                                  | 0c                | 95a          | 5d                  |
| 0.75                                 | 0c                | 100a         | 0d                  |
| **Kaohsiung No. 2**                  |                   |              |                     |
| 0                                    | 100a              | 0d           | 0d                  |
| 0.15                                 | 0c                | 0d           | 100a                |
| 0.3                                  | 0c                | 50d          | 50b                 |
| 0.45                                 | 5bc               | 65bc         | 30c                 |
| 0.6                                  | 5bc               | 55c          | 40bc                |
| 0.75                                 | 0c                | 90a          | 10d                 |
| **Orange Hot**                       |                   |              |                     |
| 0                                    | 100a              | 0d           | 0d                  |
| 0.15                                 | 5bc               | 95a          | 0d                  |
| 0.3                                  | 10bc              | 90ab         | 0d                  |
| 0.45                                 | 10bc              | 90a          | 0d                  |
| 0.6                                  | 20b               | 80ab         | 0d                  |
| 0.75                                 | 5bc               | 95a          | 0d                  |
| **Cultivar (CV)**                    | NS                | ***          | ***                 |
| **Concentration (Conc.)**            | ***               | ***          | ***                 |
| **CV × Conc.**                       | NS                | ***          | ***                 |

* The culture medium was 1/2 MS containing 0.5 mg·L⁻¹ TDZ and the leaves were disinfected for 10 minutes.

* For analysis of collected data, two-way ANOVA was performed. Each value represents the mean of four replications, and on each column the means followed by the same letter were not significantly different by LSD test (P ≤ 0.05). Abbreviation used, NS, *, **, ***: non-significant, significant difference at P ≤ 0.05, P ≤ 0.01, P ≤ 0.001, respectively (factorial experiment in CRD arrangement).
Effects of medium supplementation or surface disinfection with Plant Preservative Mixture \textsuperscript{TM} on callus induction

For both medium supplementation and surface disinfection treatments, the contamination percentage decreased with increasing PPM concentrations, but the browning percentage increased for Kaohsiung No. 1. (Table 2). The contamination rate was much lower when PPM was added to the culture medium as compared to the use of disinfection pretreatment. However, the disinfection by PPM (25\% and 50\%) had a detrimental effect on callus formation from Anthurium leaf explants after three weeks, as shown for both cultivars ‘Kaohsiung No. 1’ and ‘Orange Hot’. Only explants of ‘Kaohsiung No. 2’ responded to 0.01\% PPM supplementation to the induction medium, with up to a 55\% callus induction rate. Again, no further callus growth was observed beyond seven weeks. There was no significant difference in the callus induction rate for explants of ‘Kaohsiung No. 2’ disinfected with 0.15\% sodium hypochlorite as a control and with supplementation of 0.01\% and 0.05\% PPM in media, although 0.01\% PPM in media resulted in a slightly higher induction rate (Table 2).

Effect of sodium hypochlorite disinfection on callus growth in ‘Kaohsiung No. 2’

Since the explants of Anthurium ‘Kaohsiung No. 2’ responded better to sodium hypochlorite disinfection in terms of a lower tissue browning rate and optimal callus induction, a new batch of leaf explants was surface-disinfected with the same hypochlorite concentrations as described above to examine the effect on callus induction and subsequent growth. The fresh weight of each callus was measured after 60 days. The best sodium hypochlorite concentrations for disinfection and subsequent callus growth were observed at 0.15\%, 0.3\% and 0.45\% and 0.75\% rather than 0.6\% (Fig. 1). There was no significant difference among three sodium hypochlorite disinfection concentrations in terms of the callus weight of Anthurium ‘Kaohsiung No. 2’. The appearance of calluses induced from leaf explants, regenerated adventitious shoots and plantlets in Anthurium ‘Kaohsiung No. 2’ are shown in Figure 2. Calluses could be induced readily under dark incubation (Fig. 2A). However, light illumination was required for subsequent shoot regeneration and proliferation (Fig. 2B, C). With the above disinfection concentration, the average shoot number regenerated per explant was approximately 12–16 using 0.15\%–0.45\% sodium hypochlorite concentrations (data not shown), and higher concentrations resulted in much lower shoot regeneration.

Efficient adventitious shoot regeneration with a callus suspension culture in ‘Kaohsiung No. 2’

A mass of both new callus and adventitious shoots developed when calluses of Anthurium ‘Kaohsiung No. 2’ obtained from hypochlorite disinfection experi-

![Fig. 1. Effect of disinfection with different sodium hypochlorite concentrations on subsequent callus weight of Anthurium ‘Kaohsiung No. 2’ cultured for 60 days on half strength MS induction medium containing 0.5 mg L\textsuperscript{-1} TDZ.](image-url)
ments were transferred to a liquid regeneration medium (MS plus 0.2 mg·L\(^{-1}\) BA) by suspension culture in a shaker for 60 days under light. After 30 days in suspension, the adventitious shoots began to differentiate. After 60 days, adventitious roots emerged and could be regarded as plantlets at this stage.

Adventitious shoots were readily regenerated when calluses were transferred to modified liquid regeneration medium for 60 days (Fig. 2D). After the formation of adventitious roots, the plantlets were washed thoroughly in running tap water and transplanted to peat moss in 128 plug trays. Then, after 45 days, the plantlets were transplanted to 3.5-inch plastic pots. Regenerated plants were successfully established in finished 5-inch plastic pots containing coconut shells. Mature Anthurium ‘Kaohsiung No. 2’ plants established in the greenhouse grew normally and bloomed after 16 months (Fig. 2F).

**Discussion**

In this report, callus induction using three cultivars ranged between 0% and 100%, and varied largely depending on the cultivar. This result indicated different cultivars responded to different disinfection concentrations of sodium hypochlorite. Several reports indicated that many Anthurium cultivars are quite difficult to propagate vegetatively due in part to explant contamination and oxidative browning (Budiarto, 2008). In this study, we showed that sodium hypochlorite is a better disinfection agent as compared to PPM, although each genotype responded differently to surface disinfection. PPM is effective against both bacteria and fungi, is heat stable, and unlike conventional antibiotics, can be autoclaved in media. These PPM characteristics make it an attractive alternative to using conventional antibiotics and fungicides in plant tissue culture (George and Tripeti, 2001; Niedz, 1998). However, PPM’s effect on metabolic or transport pathways can also affect the ability of leaf explants from certain species to form adventitious shoots as demonstrated by the significant decrease in the regenerative ability of chrysanthemum explants. Therefore, the effects of PPM must be evaluated for each species of interest prior to use. Shoot regeneration was completely inhibited by 2.0 mL·L\(^{-1}\) PPM from chrysanthemum leaves (George and Tripeti, 2001). Little phytotoxicity was observed even at the highest concentrations tested of 2.0 mL·L\(^{-1}\) in citrus seeds (Niedz, 1998). Jimenez et al. (2006) reported giant bamboo was successfully propagated in vitro from axillary buds using sodium hypochlorite (1.5% w/v) for 10 min with culturing on MS medium containing 2 mL·L\(^{-1}\) of PPM. The recommended concentration of PPM for Anthurium to reduce or eliminate airborne contamination in the media is 0.01 to 0.05%. Selection of a suitable surface disinfectant with an optimal concentration sufficient to control any microbial contamination without harming the explant tissue is important (Elsheikh et al., 2013). A total of 13 different bacterial species were identified in Aglaonema and these were found to be mostly associated with soil and water. Three antibiotics, including gentamicin, tetracycline and chloramphenicol, were selected for their effectiveness at low concentrations (4–32 mg·L\(^{-1}\)) to inhibit bacterial growth in most of the bacterial species found (Fang and Hsu, 2012). Using PPM as the surface disinfectant did not have any significant effects and supplementation with PPM did not effectively increase the callus induction rate.

Callus induction is critical to achieve a micropropagation mass. In micropropagation studies, the success of the protocols depended on the variety of Anthurium, explant type, and the media components used for shoot and root regeneration (Atak and Özae, 2012; Budiarto, 2008). Leaf explants have been used for propagation by indirect organogenesis (Atak and Özae, 2009). Te-chato et al. (2006) used leaves, nodes and internodes of three Anthurium genotypes. The time to callus induction was
different with different Anthurium genotypes. Initially, callus formation on the leaf explants was observed three weeks after culture (Yu et al., 2009). Callus formation was observed 60 days after culture of Anthurium andreanum ‘Terra’ explants (Farsi et al., 2012). On the leaves of A. andreanum ‘Valentino’, callus formation was observed after about nine weeks of culture (Yu et al., 2009). Budiarto (2008) showed callus development from spathe explants in the dark after 60 days’ incubation, and after transferring to light conditions for a further 45 days, torpedo-shaped calluses were formed. Finally, after 75 days plantlet regeneration occurred. Bejoy et al. (2008) demonstrated that around 14,000 plantlets could be produced in 17 months from a single leaf using this protocol. In our study, callus formation was observed after about 10 weeks of culture. When calluses were transferred to a modified liquid regeneration medium for 60 days, adventitious shoots were formed. Subsequently, the plantlets were acclimated for one week and then transplanted to 128 insert cell trays with shading nets (Fig. 1). Using the disinfection procedure described in this report, we successfully established callus formation from leaf explants and established a rapid plantlet regeneration system, including callus proliferation and subculture, for Anthurium with a total duration of six months. Usually, it takes at least one year to achieve efficient plantlet regeneration and proliferation of Anthurium cultivars in local laboratories. Plant regeneration of Anthurium ‘Kaohsiung No. 2’ has been achieved through adventitious shoot formation of calluses from lamina explants. Our research also showed that the application of 2,4-D (0.02 mg·L⁻¹) with TDZ (1.0 mg·L⁻¹) could positively support the propagation of Anthurium spp. Also, by significantly reducing the concentration of sodium hypochlorite, the ‘Kaohsiung No. 2’ callus induction rate increased up to 100%. Atak and Özae (2009) found the callus induction rate for the Arizona variety was 80%, and was only 70% for the Sumi variety. The maximum callus induction rate with BA occurred at a concentration of 2.5 mg·L⁻¹ and was up to 90% in the Terra variety (Farsi et al., 2012).

In conclusion, we successfully established the micropropagation of two potted Anthurium cultivars, ‘Kaohsiung No. 1’ (Happy Melody) and ‘Kaohsiung No. 2’, (Ruby) by using leaf explants from mother plants and a TDZ-based callus induction medium (Yang et al., 2003) with subsequent plantlet regeneration in a liquid suspension system containing a low BA concentration (0.2 mg·L⁻¹). Some genotypes such as ‘Orange Hot’ have a weak organogenesis response, and this can probably be improved by optimizing suitable cytokinins and/or auxins in the callus induction stage and adjusting the composition of nutrients such as the ammonium ion concentration (Atak and Özae, 2009; Joseph et al., 2003). Using our optimized micropropagation system, we also regenerated another new cultivar, Anthurium ‘Kaohsiung No. 3’, with a tulip-like flower, which required only 10 months to grow from an in vitro plantlet to a finished flowering pot plant, with a flowering rate of 50% (Huang and Chen, 2016). Sodium hypochlorite between 0.15%-0.45% for surface disinfection was more suitable for callus induction and adventitious shoot regeneration from leaf explants of Anthurium ‘Kaohsiung No. 2’. Sodium hypochlorite concentrations higher than 0.6% had a negative effect on both callus induction and adventitious shoot regeneration.

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