

Disinfection procedures for *in vitro* propagation of *Anthurium*

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ABSTRACT

Disinfection of plant material is the most important step of the tissue culture protocol. In this process, an attempt is made to eliminate microbial contaminants from the surface and interior of plant material, thus giving the explant a fighting chance at survival *in vitro*. Initial cultures of *Anthurium* species and cultivars, which are usually established from *ex vitro* material grown in a greenhouse, pots or in the field, easily contaminate the *in vitro* milieu. This review highlights the differences in disinfection protocols that exist for different species or cultivars of *Anthurium*. The protocol needs to be adjusted based on the material used: spadices, spathes, seeds, leaves, or roots. Regrettably, most of the currently published protocols, derived from a literature that spans over 100 published papers, have numerous weaknesses and flaws in the information provided pertaining to disinfection and infection levels. Advice for future *Anthurium* researchers should thus be followed cautiously.

Key words: contamination, seed, sodium hypochlorite, spadix, spathe, tissue culture

EXPLANT DISINFECTION FOR THE *IN VITRO* ENVIRONMENT

In the first stage (Stage I) of plant tissue culture, explants that are processed from *ex vitro* are freed from microbial contamination and transferred to an *in vitro* environment (George 1993, George and Debergh 2008). Explant disinfection is the first and most challenging step of establishing an aseptic culture and is therefore a prerequisite for the successful tissue culture of any plant. During disinfection, all microorganisms such as bacteria or fungi that contaminate the explant exogenously

and endogenously have to be removed (Buckley and Reed 1994), without destroying and killing the plant tissues. Since disinfection agents used for surface disinfection of explants can also be toxic to plant tissues (George 1993), a balance between the level of contamination and explant survival should always be considered when using disinfection agents. Stage I can be regarded as complete if explants are able to survive and grow in an *in vitro* environment without microbial contamination.

Successful disinfection depends on several plant-related and environmental factors. Plant-

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related factors include plant species and cultivar, as well as the age and type of explant. The *ex vitro* growth environment (grown in a pot, field or greenhouse, watering, season when explants are collected, etc.) of the mother plant, which furnishes the explant (George 1993, Barrett and Cassells 1994, Niedz and Bausher 2002, Te-Chato et al. 2006), affects the level of contamination and the success of the disinfection procedure. *Anthurium* is easily contaminated *in vitro* (Brunner et al. 1995) by bacteria (Winarto and Rachmawati 2007, Winarto et al. 2009) such as blight pathogen (*Xanthomonas campestris* pv. *dieffenbachiae*) (Norman and Alvarez 1994) and fungi (Winarto and Rachmawati 2007, Winarto 2009, 2010). Several explant types in *Anthurium* can be used to establish an *in vitro* culture (Teixeira da Silva et al. 2015). Disinfection of plant material for *in vitro* culture generally occurs in three steps (Pierik 1997): (1) processes of initial washes and disinfection; (2) additional disinfection steps after rinsing; (3) final rinses.

In this review, all known disinfection procedures that have been applied for the disinfection and *in vitro* culture establishment of various explant types of *Anthurium* are summarized. These explants then serve for pure and applied purposes *in vitro* during tissue culture and micropropagation (Teixeira da Silva et al. 2015).

SURFACE DISINFECTION OF SEED, SPADIX AND SPATHE EXPLANTS

Germination *in vitro* of *Anthurium* seeds is possible but the method to achieve it depends on the cultivar. In most cases, the authors did not indicate the success of disinfection and its impact on survival and/or germination frequency (Tab. 1), thus all available protocols have been described. Alves Dos Santos et al. (2005) first washed *A. andraeanum* 'Canaanéia' spathes with tap water and 2% chlorhexidine digluconate (a liquid soap) then sprayed them with an antibiotic with a bactericidal effect (12.1 mM sodium rifampicin) for 2 h. Thereafter, spathes were disinfested with 70% (v/v) ethanol for 2 min, 2.5% (w/v) calcium hypochlorite ($\text{Ca}(\text{OCl})_2$) for 10 min and 50% (w/v) carbendazim for 2 h. Fruits were excised from spathes on a horizontal laminar air flow cabinet and decontaminated with 70% ethanol for 2 min and 2.5% $\text{Ca}(\text{OCl})_2$ for 10 min. Vargas et al. (2004) indicated that *A. andraeanum* 'Rubrun' fruits, once separated from spadices, could be surface disinfected in 3% (v/v) sodium hypochlorite (NaOCl) for 15 min and rinsed with sterile distilled water (SDW) for 30 min. Using

a modified protocol of Pierik et al. (1974), seeds, in batches of 100, were then disinfected for 20 min in 1% NaOCl and rinsed twice in SDW for 30 min. In that study, 74% of seeds germinated in the light as opposed to only 30% in the dark, but only in the presence of 6-benzyladenine (BA). Wang et al. (1998) used a different protocol for four *Anthurium* spp. (*A. pittieri*, *A. ravenii*, *A. antioquiense*, *A. aripoense*) and three cultivars (Midori, Ozaki, and Mauna Kea): seeds were removed from the mesocarp and disinfected with 10% (v/v) Clorox® plus one drop of Tween-20 per 100 ml (a surfactant) for 30 min. Seeds were then treated once more with 5% Clorox® plus one drop of Tween-20 per 100 ml for 30 min with gyratory shaking. Seeds were rinsed three times with SDW on a laminar flow bench. Ancy et al. (2012) washed dry *A. bicolor* seeds in running tap water (RTW), soaked them for 5-8 min in a mild liquid detergent (10% (v/v) Teepol), then washed seeds thoroughly with RTW. Seeds were surface disinfected by dipping them in 0.1% (w/v) mercuric chloride (HgCl_2) solution for 10-15 min, washed 4-5 times with SDW inside a laminar flow bench, dipped in hydrogen peroxide (H_2O_2) for 30 s then rinsed with SDW. Maira et al. (2010) used 3% NaOCl for 15 min then three rinses in SDW (5 min each) for *A. andraeanum* 'Rubrun' fruits separated from the spadix while seeds were dipped in 1% NaOCl for 20 min then rinsed twice in SDW for 10 min each rinse.

Eapen and Rao (1985) used 70% ethanol for 30 s followed by 0.1% HgCl_2 solution containing 0.25 ml l⁻¹ Tween-20 for 5 min to surface sterilize *A. patulum* spathes. Finnie and Van Staden (1986), on an unspecified *A. andraeanum* cultivar, used a simple method for five explant types (leaves, petioles, spathes, spadices, and roots): a swab with 80% ethanol, a 30 min dip in 1% NaOCl plus two drops of Tween-20 and three 30-min rinses in SDW. Even so, the authors observed 100% contamination of spathe and spadix explants. Winarto (2009), Winarto et al. (2011 a, b) and Winarto and Teixeira da Silva (2012 c) disinfected the spadices of *A. andraeanum* 'Tropical' as follows: a wash under tap water for 30-60 min, immersion in a 1% solution of 50% benomyl (Benlox® 50 WP, a pesticide) and 20% streptomycin sulphate (Agrept® 20 WP) for 30 min and five rinses with SDW (5 min each rinse). Spadices were then immersed in 1% NaOCl for 10 min, 2% NaOCl for 5 min, 80% alcohol for 30 s, followed by 5-6 rinses in SDW (5 min each rinse). For an unspecified *A. andraeanum* cultivar, Jahan et al. (2009) used

Table 1. Effectiveness of disinfection protocols for *Anthurium* species and hybrids

<i>Anthurium</i> species or cultivar	Superficial disinfection procedures	Infection/contamination	Reference
<i>A. andraeanum</i>	Seeds, in batches of 100, were disinfected for 20 min in 1% NaOCl and rinsed twice in SDW* for 30 min	INR**	Pierik et al. 1974
<i>A. patulum</i>	Spathes, leaves, petioles and pedicels were disinfected with 70% ethanol for 30 s followed by 0.1% HgCl ₂ solution containing 0.25 ml l ⁻¹ Tween-20 for 5 min, then rinsed in sterile demineralized water three times	INR	Eapen and Rao 1985
<i>A. andraeanum</i>	Five explant types (leaves, petioles, spathes, spadices, and roots) were disinfected with a swab with 80% ethanol, 30 min dip in 1% NaOCl + two drops of Tween-20 and three 30 min rinses in SDW	100% contamination of spathe and spadix explants	Finnie and Van Staden 1986
<i>A. scherzerianum</i> (18 cultivars)	Entire leaves were disinfected in 70% ethanol for a few seconds then in a solution of NaOCl containing 15 g l ⁻¹ active chlorine and a few drops of Tween-20 for either 10-15 min (young leaves) or 15-25 min (older leaves). Leaves were rinsed with three rinses with SDW for 10, 30 and 60 min	10-30% of contamination	Geier 1986
<i>A. andraeanum</i>	Young, unexpanded leaves 2-3 cm in size were exposed to 1% NaOCl for 15 min then a thorough rinse in SDW	INR	Teng 1997
<i>Anthurium</i> spp. (<i>A. pittieri</i> , <i>A. ravenii</i> , <i>A. antioquiense</i> , <i>A. aripoense</i>) and cultivars: Midori, Ozaki, Mauna Kea	Seeds were removed from the mesocarp and disinfected with 10% Clorox® plus one drop of Tween-20 per 100 ml (a surfactant) for 30 min. Seeds were then treated once more with 5% Clorox® + 1 drop of Tween-20 per 100 ml for 30 min with gyrotory shaking. Seeds were rinsed three times with SDW	INR	Wang et al. 1998
<i>A. andraeanum</i> 'Lima White', 'Tropical White', 'Tropical Red'	Leaves was washed under running tap water, then dipped for 5 min in 5% detergent, washed thoroughly with double SDW, dipped in a solution of 0.1% HgCl ₂ for 8-10 min, then a wash with double SDW	INR	Joseph et al. 2003
<i>A. andraeanum</i>	Procedure 1: leaves were rinsed with 0.01% KMnO ₄ for 10 min, 0.05% streptomycin for 20 min, 0.05% nystatin for 20 min, 0.05% cefazolin sodium for 20 min, 75% alcohol for 30 s, and then 0.01% HgCl ₂ for 2 min Procedure 2: leaves were rinsed with 75% alcohol for 30 s, then soaking in 0.01% HgCl ₂ solution for 2 min	Explant contamination was 0% in Procedure 1 and 25% in Procedure 2	Lan et al. 2003
<i>A. andraeanum</i> 'Tinora Red', 'Senator'	Leaves was washed under running tap water, and then dipped for 5 min in detergent, washed thoroughly with SDW, dipped in a solution of 0.1% HgCl ₂ for 10-12 min, then one wash with SDW	INR	Martin et al. 2003
<i>A. andraeanum</i> 'Rubrun'	Fruits separated from spadices were surface disinfected in 3% NaOCl for 15 min and rinsed with SDW for 30 min. Then seeds were isolated and sterilized for 20 min in 1% NaOCl, afterwards they were washed two times with sterile water for 30 min	INR	Vargas et al. 2004
<i>A. andraeanum</i> 'Canaanía'	Spathes with tap water and 2% chlorhexidine digluconate (a liquid soap) then sprayed them with an antibiotic (12.1 mM sodium rifampicin) for 2 h. Thereafter, spathes were disinfested with 70% ethanol for 2 min, 2.5% calcium hypochlorite (Ca(OCl) ₂) for 10 min and 50% (w/v) carbendazim for 2 h. Fruits were excised from spathes on a horizontal laminar air flow cabinet and decontaminated with 70% ethanol for 2 min and 2.5% Ca(OCl) ₂ for 10 min	INR	Alves dos Santos et al. 2005

Table 1. Effectiveness of disinfection protocols for *Anthurium* species and hybrids (continued)

<i>Anthurium</i> species or cultivar	Superficial disinfection procedures	Infection/contamination	Reference
<i>A. andraeanum</i> 'Osaki', 'Nitta', 'Anouchka'	Leaves was dipped in 0.6% Benlate for 30 min, a wash with soapy water, rinse in tap water, then a dip in 70% ethanol for 30 s, then in 1.5% NaOCl containing two drops of Tween-20 for 20 min with gentle agitation. Surface-disinfected leaves were rinsed three times with SDW at 15 min each rinse and a final 30 min rinse in SDW	Pre-sterilization and sterilization reduced the contamination level to <10%. Fungal contamination appeared during the first week of inoculation while contamination due to the presence of internal contaminants, appeared after three weeks in culture	Puchoo 2005
<i>A. andraeanum</i> 'Carnaval', 'Neon', 'Choco', 'Sonate', 'Midori', 'Pistache', 'Tropical', 'Safari', 'Arizona', 'Cancan'	A simple disinfection procedure with 0.1% HgCl ₂ then sectioned to about 1 cm ² pieces, but no rinses were indicated	INR	Nhut et al. 2006
<i>A. andraeanum</i> 'Flamingo'	Leaves was washed under running tap water for 20 min, then passed them through 70% ethanol, then immersed them for 20 min in NaOCl (with 1.4% active chlorine) to which two drops of Tween-20 were added. Finally, leaves were washed thoroughly with SDW then dipped for 20 min in a unique antioxidant solution (to avoid explant oxidation and browning) containing 150 mg l ⁻¹ citric acid, 100 mg l ⁻¹ ascorbic acid and 200 mg l ⁻¹ cysteine	INR	Viégas et al. 2007
<i>A. andraeanum</i> 'Agnihothri'	Leaves were dipped in 15% commercial bleach for 20 min followed by 7 min in 0.1% HgCl ₂ and thorough washes with SDW	INR	Bejoy et al. 2008
<i>A. andraeanum</i>	Apical shoot buds were surface sterilized with Teepol, washed with sterile water, treated with antifungal solution Cetrimide for 5 min. Then treated with NaOCl for 5 min, washed and treated again with 0.1% HgCl ₂ for 5 min. Explants then thoroughly washed 4-5 times in SDW	INR	Gantait et al. 2008
<i>A. andraeanum</i> 'Arizona', 'Sumi'	Leaf explants were surface disinfected for 1 min in 70% (v/v) ethanol and soaked in gentamicin solution for 30 min then soaked in 20% bleach (5% NaOCl) for 12 min. Leaves were rinsed three times in SDW and then cut to 1 cm ² explants	INR	Atak and Çelik 2009
<i>A. andraeanum</i>	Spadix sections and leaves was rinsed under running tap water for 30 min, then dipped briefly in 0.5% Trix, a commercial detergent, in 70% ethanol for 1 min, in 1.5% NaOCl with 0.01% Tween-20 for 8 min, then rinsed thoroughly with SDW	INR	Jahan et al. 2009
<i>A. andraeanum</i> 'Nicoya'	<i>In vitro</i> -derived shoot tip, with consecutive 10 min dips in 10% betadine, 3 g l ⁻¹ Benomyl, 4 ml l ⁻¹ kasumin, 500 mg l ⁻¹ bixdroxyl and 5.25% NaOCl, each dip being followed by three washes with SDW	INR	Liendo and Mogollón 2009
<i>A. andraeanum</i> 'Red Hot'	Young leaf laminae washed with detergent, surface disinfected by soaking in 80% (v/v) ethanol for 2 min, followed by 20% Clorox® solution with a drop of Tween-20 for 15 min, with constant agitation. The lamina segments were then rinsed five times with autoclaved water	INR	Seah 2009
<i>A. andraeanum</i> 'Nitta'	Young, unfolded greenhouse-derived leaves were then cut into small pieces (size not indicated), disinfected with 70% ethanol for 30 s, washed three times with DSW, disinfected with 0.1% HgCl ₂ for 10 min and rinsed several times, supposedly in SDW	INR	Islam et al. 2010

Table 1. Effectiveness of disinfection protocols for *Anthurium* species and hybrids (continued)

<i>Anthurium</i> species or cultivar	Superficial disinfection procedures	Infection/contamination	Reference
<i>A. andreanum</i> 'Rubrun'	Fruits separated from the spadix were used 3% NaOCl for 15 min then three rinses in SDW (5 min each), for seeds, 1% NaOCl for 20 min then two rinses in SDW for 10 min each	INR	Maira et al. 2010
<i>A. andraeanum</i> 'Pumasillo', 'Corallis'	Young leaf was pre-treated with a fungicide (0.25% Bavistin) and a bactericide (0.2% 8-hydroxyquinoline citrate) for 2 h with constant orbital shaking (80 rpm), then transferred to a detergent (10% Teepol) for 10 min then washed twice in SDW. Finally, treated with 5% NaOCl, wiped with 70% ethanol, dipped in 0.1% HgCl ₂ for 2-5 min, then washed four times with SDW	6.5% contamination, but as high as 100% for other protocols	Kumari et al. 2011
<i>A. digitatum</i>	Leaves in which a 0.1% solution of HgCl ₂ was initially used followed by five washes with SDW. Then dipped in H ₂ O ₂ for 15 s, then rinsed in SDW	INR	Reddy et al. 2011
<i>A. andreanum</i> 'Tropical'	Spadices were washed under tap water for 30-60 min, immersion in a 1% solution of 50% benomyl (Benlox® 50 WP, a pesticide) and 20% streptomycin sulphate (Agrept® 20 WP) for 30 min and a rinse 5 times with SDW (5 min each rinse). Spadices were then immersed in 1% NaOCl for 10 min, 2% NaOCl for 5 min, 80% alcohol for 30 s, followed by 5-6 rinses in SDW (5 min each rinse)	INR	Winarto 2009, Winarto et al. 2011 a, b, Winarto and Teixeira da Silva 2012 c
<i>A. bicolor</i>	Seeds were washed by in running tap water, soaked for 5-8 min with a mild liquid detergent (10% Teepol), then washed thoroughly with running tap water. Seeds were surface disinfected by dipping them in 0.1% HgCl ₂ solution for 10-15 min, a wash with SDW 4-5 times, dipped in H ₂ O ₂ for 30 s then rinsed with SDW	INR	Ancy et al. 2012
<i>A. andraeanum</i> 'Terra'	Leaves were disinfected for 20 min in 3% NaOCl and three rinses with SDW was sufficient for <i>in vitro</i> culture	INR	Farsi et al. 2012
<i>A. andraeanum</i> 'CanCan'	Shoot tips derived from greenhouse-grown plants first were washed for 5 min in tap water then dipped for 5 min in 1% Tween-20 in a glass beaker for 5 min. Then the shoot tips exposed sequentially to 70% ethanol for 30 s, 3% H ₂ O ₂ for 5 min and a 20% Clorox® (1.2% NaOCl) solution for 10 min with vigorous shaking. Surface-disinfected shoot tips were thoroughly rinsed three times in SDW and transferred to sterile Petri dishes for 5 min to dry, then trimmed to 1 cm to serve as the explant	INR	Gantait et al. 2012
<i>A. andraeanum</i> 'Casino', 'Antadra'	Lamina and petioles was washed under running tap water for 30 min with some drops of dishwashing liquid, dipped them in 70% (v/v) ethanol for 30-40 s, then surface disinfected them with 1% (w/v) NaOCl containing 2-3 drops of Tween-20 for 10 min followed by three rinses with SDW for 2-10 min. Margins of explants were cut before plating	INR	Raad et al. 2012

*SDW – sterile distilled water

**INR – information not reported

the same disinfection protocol, not only for spadix sections, but also for leaves. After rinsing under RTW for 30 min, explants were dipped briefly in 0.5% (v/v) Trix, a commercial detergent, in 70% ethanol for 1 min, in 1.5% NaOCl with 0.01% Tween-20 for 8 min, then rinsed thoroughly with SDW prior to plating *in vitro*.

SURFACE DISINFECTION OF LEAF, PETIOLE AND PEDICEL EXPLANTS

Eapen and Rao (1985) surface sterilized *A. patulum* leaves, petioles and pedicels in 70% ethanol for 30 s, in 0.1% HgCl₂ containing 0.25 ml l⁻¹ Tween-20 for 5 min, then rinsed explants three times in sterile deionized water. Geier (1986) described a protocol for the surface sterilization of entire leaves of 18 cultivars of *A. scherzerianum* in 70% ethanol for a few seconds then in a solution of NaOCl containing 15 g l⁻¹ active chlorine and a few drops of Tween-20 for either 10-15 min (young leaves) or 15-25 min (older leaves). Leaves were rinsed three times with SDW for 10, 30 and 60 min. Puchooa (2005) dipped the leaves of three *A. andraeanum* cultivars (Osaki, Nitta, and Anouchka) in 0.6% Benlate for 30 min, a wash with soapy water, a rinse in tap water, a dip in 70% ethanol for 30 s, then a dip in 1.5% NaOCl containing two drops of Tween-20 for 20 min with gentle agitation. Surface-disinfected leaves were rinsed three times with SDW (15 min each rinse) and a final 30 min rinse in SDW. Joseph et al. (2003), after washing folded, half-folded and unfolded green and brown leaves of three *A. andraeanum* cultivars (Lima White, Tropical White, and Tropical Red) under RTW, dipped leaves for 5 min in 5% detergent, washed them thoroughly with double SDW, dipped them in a solution of 0.1% HgCl₂ for 8-10 min, then washed them with double SDW. Martin et al. (2003) first washed the leaves of two *A. andraeanum* cultivars (Tinora Red and Senator) under RTW, dipped them for 5 min in detergent, washed them thoroughly with SDW, dipped them in a solution of 0.1% HgCl₂ for 10-12 min, then washed them once with SDW.

Nhut et al. (2006) used a simple disinfection procedure with 0.1% HgCl₂ but no rinses were indicated. Viégas et al. (2007) first washed *A. andraeanum* 'Flamingo' leaves under RTW for 20 min, passed them through 70% ethanol, then immersed them for 20 min in NaOCl (with 1.4% active chlorine) to which two drops of Tween-20 were added. Finally, leaves were washed thoroughly with SDW then dipped for 20 min in a unique antioxidant solution containing 150 mg l⁻¹ citric acid,

100 mg l⁻¹ ascorbic acid and 200 mg l⁻¹ cysteine to avoid explant oxidation and browning. Explants were then plated directly from this antioxidant solution. For *A. andraeanum* 'Agnihothi' leaves, Bejoy et al. (2008) recommended 20 min in 15% commercial bleach followed by 7 min in 0.1% HgCl₂ and thorough washes with SDW. Islam et al. (2010) used young, unfolded greenhouse-derived leaves (age of mother plants not described) of *A. andraeanum* 'Nitta' which were then cut into small pieces (size not indicated), disinfected with 70% ethanol for 30 s, washed three times with DSW, disinfected with 0.1% HgCl₂ for 10 min and rinsed several times, supposedly in SDW. Reddy et al. (2011) described a protocol for the surface disinfection of greenhouse-derived *A. digitatum* leaves in which a 0.1% solution of HgCl₂ was initially used followed by five washes with SDW. Explants were then dipped in H₂O₂ for 15 s, then rinsed in SDW. However, several important aspects and details of the protocol were not disclosed or were inaccurately described.

A much simpler disinfection procedure was used by Teng (1997) for an unspecified cultivar of *A. andraeanum*, in which young, unexpanded leaves 2-3 cm in size were exposed to 1% NaOCl for 15 min then a thorough rinse in SDW. Jahan et al. (2009) washed leaf and spadix segments of an unspecified *A. andraeanum* cultivar under RTW for 30 min, treated them with 0.5% (v/v) commercial detergent, five rinses with DW, then surface disinfected them for 1 min in 70% ethanol, then for 8 min in 1.5% NaOCl plus 0.01% Tween-20. Details about final rinses were not indicated. Atak and Çelik (2009) used a different approach for two *A. andraeanum* cultivars (Arizona and Sumi). Leaf explants were surface disinfected for 1 min in 70% ethanol, soaked in gentamicin for 30 min then soaked in 5% NaOCl for 12 min. Leaves were rinsed three times in SDW and then cut into 1 cm² explants. Seah (2009) washed young leaf laminae of *A. andraeanum* 'Red Hot' with detergent, then surface disinfected them by soaking in 80% ethanol for 2 min, followed by 20% (v/v) Clorox[®] solution with a drop of Tween-20 for 15 min, with constant agitation. The lamina segments were then rinsed five times with autoclaved water.

Kumari et al. (2011) first pre-treated young leaf explants of two *A. andraeanum* cultivars (Pumasillo and Corallis) with a fungicide (0.25% Bavistin) and a bactericide (0.2% 8-hydroxyquinoline citrate) for 2 h with constant orbital shaking (80 rpm). Leaves were then transferred to a detergent (10%

Teepol) for 10 min then washed twice in SDW. Leaves were then treated with 5% NaOCl, wiped with 70% ethanol, dipped in 0.1% HgCl₂ for 2-5 min, then washed four times with SDW. Farsi et al. (2012) claimed that disinfection of *A. andraeanum* ‘Terrai’ leaves for 20 min in 3% NaOCl and three rinses with sterile water was sufficient for *in vitro* culture. Raad et al. (2012) first washed lamina and petioles of two *A. andraeanum* cultivars (Casino and Antadra) under RTW for 30 min with some drops of dishwashing liquid, dipped them in 70% ethanol for 30-40 s, then surface disinfected them with 1% NaOCl containing 2-3 drops of Tween-20 for 10 min followed by three rinses with SDW for 2-10 min. Margins of explants were cut before plating.

Lan et al. (2003) used a complicated procedure to disinfect the leaf explants of an unspecified cultivar of *A. andraeanum*: A rinse with 0.01% KMnO₄ for 10 min, 0.05% streptomycin for 20 min, 0.05% nystatin for 20 min, 0.05% cefazolin sodium for 20 min, 75% alcohol for 30 s, then 0.01% HgCl₂ for 2 min, although rinses were not described. The level of explant contamination was zero; in contrast, contamination was 25% when another procedure was used: a rinse with 75% alcohol for 30 s, then soaking in 0.01% HgCl₂ solution for 2 min.

Winarto (2010) pre-treated the young leaf explants of *A. andraeanum* ‘Tropical’ by placing them under RTW for 1 h, immersing them in 1% detergent for 30 min, followed by a 1% solution of 50% benomyl and 20% streptomycin sulphate for 30 min and rinsing five times with SDW (5 min each rinse). The young leaf explants were then disinfected in 2% NaOCl for 5 min, 80% alcohol for 3 min, followed by 5-6 rinses in SDW (5 min each rinse). In each stage of pre-treatment and disinfection, solutions were always shaken manually. At the end of the disinfection procedure, the damaged surfaces of explants were sliced off, then cultured in initiation medium. This procedure would result in an average of 25% contamination of young leaf explants while substitution of 2% NaOCl and 80% alcohol for 3 min with 0.05% HgCl₂ for 7 min and 0.01% HgCl₂ for 3 min resulted in 0-3% contamination (Winarto, unpublished data).

SURFACE DISINFECTION OF SHOOT TIPS

Gantait et al. (2008, 2012) indicated that the shoot tips of *A. andraeanum* ‘CanCan’ derived from greenhouse-grown plants should first be washed for 5 min in RTW then dipped for 5 min in 1%

Tween-20 (dissolved in SDW) in a glass beaker for 5 min. Shoot tips were then exposed sequentially to 70% ethanol for 30 s, 3% H₂O₂ for 5 min and a 20% Clorox® (1.2% NaOCl) solution for 10 min with vigorous shaking under a laminar flow bench. Surface-disinfected shoot tips were thoroughly rinsed three times in SDW and transferred to sterile Petri dishes for 5 min to dry, then trimmed to 1 cm to serve as the functional explant. In a rather unorthodox procedure, Liendo and Mogollón (2009) surface disinfected ‘Nicoya’ *in vitro*-derived shoot tips (which should have already been sterile), with consecutive 10 min dips in 10% betadine, 3 g l⁻¹ Benomyl, 4 ml l⁻¹ kasumin, 500 mg l⁻¹ bidroxyl and 5.25% NaOCl, each dip being followed by three washes with SDW.

OTHER ALTERNATIVES FOR ESTABLISHING ASEPTIC *IN VITRO* CULTURES OF *ANTHURIUM*

Cardoso (2009) used a novel way of propagating *A. andraeanum in vitro* by disinfecting the medium with 0.005% chlorine dioxide (ClO₂) (i.e., without the use of autoclaving) and plantlet growth was as effective or even better than plants grown in medium disinfected by conventional autoclaving. Similar success of the use of ClO₂ had also been observed for gerbera greenhouse material that was acclimatized to *in vitro* conditions in medium containing ClO₂ (Cardoso and Teixeira da Silva 2012). This procedure increases the possibility for the direct acclimatization of *ex vitro* anthurium explants to *in vitro* conditions. Unlike ClO₂, peracetic acid (0.01%) as a medium sterilant was ineffective. Peiris et al. (2012) showed that the growth of *A. andraeanum* shoots in glass vessels disinfected with 5-15% NaOCl was as effective as growth in autoclaved glassware.

CONCLUSIONS

Although the disinfection procedures applied in different laboratories for *Anthurium* differed somewhat, they depended on the species/cultivar and on the explant type used for establishing the *in vitro* culture. Sodium or calcium hypochlorite was one of the most frequently chosen surface disinfection agents, applied either alone (Teng 1997, Wang et al. 1998, Maira et al. 2010, Farsi et al. 2012) or in combination with ethanol (70%) (Vargas et al. 2004, Alves Dos Santos et al. 2005, Puchooa 2005, Viégas et al. 2007, Kumari et al. 2011, Raad et al. 2012) for seeds, spadices or spathes and leaves.

However, the percentage of active chlorine was often not indicated, simply the concentration of the commercial bleach solution. Lack of such details reduces repeatability of the protocol. The time period applied for disinfection protocols was different for seeds, spadices and spathes (20-30 min) compared to leaves (12-25 min). The application period could be reduced to 15 min (Seah 2009) or to 8 min (Jahan et al. 2009) if it was applied after washing explants in tap water and using some detergent, or

adding some drops of Tween-20, or to 12 min when explants were first soaked in gentamicin solution (Atak and Çelik 2009).

The application of 0.1% HgCl_2 solution for 8-15 min alone (Joseph et al. 2003, Martin et al. 2003, Reddy et al. 2011) or combined with 70% ethanol for a few seconds (Eapen and Rao 1985, Islam et al. 2010) or with H_2O_2 for 15-30 s (Reddy et al. 2011, Ancy et al. 2012) were also suitable. Since HgCl_2 is extremely toxic to all living organisms, it is

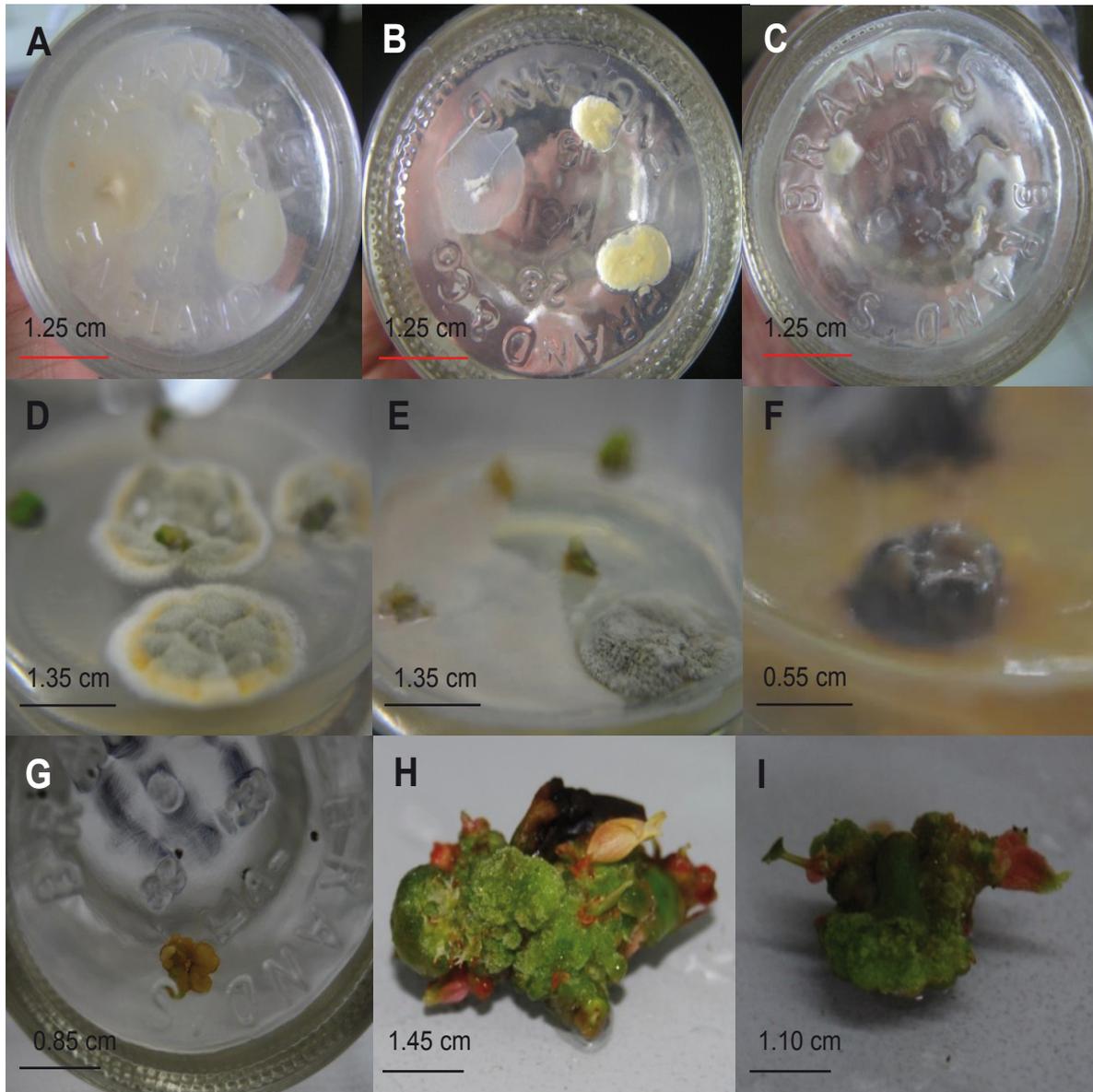


Figure 1. The importance of the disinfection process for *in vitro* culture establishment in *Anthurium*. (A-C) Half-anthers of *A. andraeanum* ‘Tropical’ contaminated by bacteria (Winarto and Rachmawati 2007, Winarto 2009). (D) Leaf segments of *A. andraeanum* ‘Tropical’ contaminated by fungi and bacteria during aseptic culture (Winarto 2010). (E-F) *A. andraeanum* ‘Tropical’ petiole explants contaminated by fungi and bacteria during aseptic culture (Winarto 2010). Callus derived from half-anther (G) of *A. andraeanum* ‘Carnaval’ (Winarto and Mattjik 2009), leaf (H) and petiole (I) of *A. andraeanum* ‘Tropical’ (Winarto 2010). All photos are unpublished derived from Winarto and Rachmawati (2007), Winarto 2009, 2010) and Winarto and Mattjik (2009). All studies using a similar sterilization protocol are clearly described in Winarto (2009), Winarto et al. (2011 a, b) and Winarto and Teixeira da Silva (2012 c).

Table 2. Generalized ‘ideal’ surface disinfection protocol of a range of explants for the *in vitro* culture of *Anthurium*

Disinfection process	Pre-treatment and disinfection agents	Time of application
Pre-treatment	running tap water	30-60 min
	1% detergent	30 min
	1% pesticide solution (fungicide + bactericide)	30 min
	4-5 rinses with distilled water	3 min each
Surface disinfection	0.05% HgCl ₂	5-7 min
	0.01% HgCl ₂	1-3 min
	5-6 rinses with sterile distilled water	3 min each

Note: This protocol, devised by Budi Winarto, results in low contamination (~10%), but not in completely infection-free explants. The protocol was successfully applied to different explants (seeds, leaves, petioles, spadices, shoot tips) and was also successful for the *in vitro* culture of other ornamental plants

very important and critical that explants be rinsed carefully after use to ensure the survival of plant material.

There are other possibilities for the surface disinfection of explants which were successfully applied in the tissue culture of other plant species but which not widely tested for *Anthurium*. H₂O₂ is a strong oxidizing and disinfecting agent due to its oxygen-oxygen single bond and can be used also for the surface disinfection of different explants from various plant species, such as sugar-apple, water lettuce, sour cherry or cotton (Farooq et al. 2002, Aasim et al. 2013, Mihaljević et al. 2013, Barampuram et al. 2014). When it was applied to the disinfection of non-seed explants (nodal explants, buds, leafy shoots), 50-60% of the explants became contamination-free (Farooq et al. 2002, Aasim et al. 2013, Mihaljević et al. 2013). However, H₂O₂ was more effective than chlorine gas or bleach when it was applied for the surface disinfection of cotton seeds (Barampuram et al. 2014). The level of contamination-free seeds was 98.88% compared to 54.44-77.77% and 77.77-85.55% achieved with chlorine gas and bleach, respectively. In *Anthurium* species H₂O₂ was not applied alone, only as one part of the disinfection process of seeds (Ancy et al. 2012), leaves (Reddy et al. 2011) and shoot tips (Gantait et al. 2008, 2012).

Another recently used possibility for the removal of bacterial contamination of single-node explants of *Valeriana officinalis* L. is the application of nano-silver (Abdi et al. 2008). Nano-silver treatment (100 mg l⁻¹, for 180 min) was applied after surface sterilization (70% ethanol for 1 min and 10% Clorox[®] for 1 min) resulting in 89% contamination-free explants.

Very few studies indicated the level of contamination following the disinfection protocol (e.g., Fig. 1), the protocols that did not work (negative

results), or the impact of the disinfection protocol (if more than one) on explant survival and regeneration (Tab. 1). Detailed information on these factors is essential for scientists who wish to establish a new protocol and who do not have experience with the plant material. Such details are also essential for other laboratories to be able to replicate an *in vitro* protocol. A broad, generalized protocol for the effective (<10% contamination) disinfection of a range of explants in *Anthurium* is provided in Table 2. Since the first step of the *in vitro* establishment protocol involves the transfer of *ex vitro* material to *in vitro* conditions (Teixeira da Silva et al. 2015), disinfection remains the most important first step of a tissue culture or micropropagation protocol, especially in *Anthurium*, which infects easily. It is not uncommon for journals to downplay, or ignore negative results, or lack-luster ones, but in plant tissue culture, such results are essential and should be published even though they are usually not. The importance of indicating the fine-scale details of such steps and trials related to disinfection is exemplified by leatherleaf fern: first the disinfection trials (Winarto and Teixeira da Silva 2012 a), then the resulting tissue culture protocol that employed optimized sterilization protocols (Winarto and Teixeira da Silva 2012 b).

The *Anthurium* literature has considerable errors and problems, including the lack of details related to disinfection and tissue culture-related protocols, broadly described as snub publishing (Teixeira da Silva 2013 a, 2014 a). The need to correct the *Anthurium* literature, whose errors may have arisen for a number of possible reasons, including incomplete traditional peer review, editorial oversight (Teixeira da Silva and Dobránszki 2015), or incomplete or inaccurate information by authors, is essential. For information to be useful for future generations of *Anthurium* scientists, the literature

needs to be as correct as possible. That can only be achieved through a process of rigorous and qualified post-publication peer review (Teixeira da Silva, 2013 b, 2014 b), including the analysis of retractions related to *Anthurium* (Teixeira da Silva 2014 c), or even challenging neologisms in the *Anthurium* literature (Teixeira da Silva 2014 d).

AUTHOR'S CONTRIBUTIONS

All four authors contributed equally to the development and writing of this review. All four authors take public responsibility and abide by the regulations of authorship as stipulated by the four clauses of the ICMJE.

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