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IN VITRO SEED GERMINATION AND SEEDLING GROWTH OF *CALANTHE DISCOLOR* LINDL

ABSTRACT

We investigated the effects of sodium hypochlorite (NaOCl) and culture medium on embryo swelling and germination of *Calanthe discolor* Lindl., and established a method for determining the swelling and protocorm formation of *C. discolor* seeds *via* in vitro examination of immature seeds. Treatment of immature seeds with NaOCl greatly enhanced the extent of embryo swelling and protocorm formation of immature zygote embryos compared to seeds without NaOCl treatment. The effects of the culture media were also evaluated with regard to embryo swelling and protocorm formation of in vitro cultured seeds with and without NaOCl treatment. Additionally, the effects of white fluorescent light and red and blue LED lights on seedling growth in in vitro culture were examined. The most suitable condition for seedling growth after 12 weeks of culture was the red LED light with POM medium. These results show effective asymbiotic germination and growth of *C. discolor*.

Key words: *Calanthe discolor*, chlorophyll, germination, growth, seed, terrestrial orchid.

INTRODUCTION

Orchidaceae comprise a fascinating group of ornamental plants, and numerous novel cultivars have been produced by interspecific and intergeneric hybridization of plants with exotic and elegant flowers. However, many orchid species are endangered as a consequence of environmental disruption, succession of natural habitats, and overexploitation for horticultural purposes. In situ conservation by preservation and enhancement of dwindling populations of endangered orchid species is difficult because of the relatively slow growth and

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low germination rate, and because these processes require symbiotic relationships with mycorrhizal fungi in natural habitats (Bernard 1903; Warcup 1973; Rasmussen 1995).

The genus *Calanthe* is widely distributed in tropical and subtropical regions, including Africa, Middle and South America, Asia, and Pacific islands (Gale and Drinkell 2007; Lee *et al.* 2008). It contains approximately 171 species worldwide, of which approximately 29 inhabit Asian regions, such as Korea, Japan, and China (Lee *et al.* 2008). However, only four species (*Calanthe discolor* Lindl., *C. sieboldii* Ohwi, *C. reflexa* Maximowicz, and *C. aristulifera* Hayata) and two natural hybrids (*Calanthe* × *bicolor* and *Calanthe* × *kibanakirishima*) (Hyun *et al.* 1999a,b; Lee and Choi 2006; Lee *et al.* 2008) inhabit Korea. The genus *Calanthe* has beautiful flowers and high ornamental value.

Calanthe discolor has beautiful flowers that consist of a green perianth and red lips in raceme inflorescences, and is highly valued. Therefore, establishment of protocols for mature seed germination in this species is a prerequisite for ex situ conservation. Seed germination is the limiting step for the establishment of plantlets from the germination of terrestrial orchids because germination is more difficult than in epiphytic species (Arditti and Ernst 1984). Kano (1972) reported that *Calanthe* germination in temperate regions is the most difficult among all terrestrial orchid species studied. Several studies have reported propagation of *Calanthe* species by asymbiotic germination (Miyoshi and Mii 1988, 1995a,b; Park *et al.* 2000; Lee *et al.* 2007). Various strategies, such as presoaking of seeds in water (Mii and Kako 1974), organic solvent treatment (Mii 1978), phytohormones treatment (Miyoshi and Mii 1995a), pretreatment with ultrasonication (Miyoshi and Mii 1988), and the addition of a polyphenol absorbent to the medium (Miyoshi and Mii 1995b), have been used in efforts to improve the germination and/or protocorm formation of *Calanthe* species. Recently, Lee *et al.* (2007) collected seeds of *C. tricarinata* at various stages of seed formation and maturation, and the frequency of germination of immature seeds at 150 days postanthesis reached approximately 30%. The germination frequency of fully matured seeds was low and only a few seeds germinated. Lee *et al.* (2007) also noted that scarification, for example, by prolonged treatment with chemical solutions and ultrasound, stimulated the germination of fully matured seeds and resulted in frequencies of seed germination (32% to 39%) similar to that of immature seeds.

The effectiveness of seed disinfection reagents such as sodium hypochlorite (NaOCl) and calcium hypochlorite $Ca(OCl)_2$ for stimulation of orchid germination has been reported in *Dendrobium* species (Malmgren 1996). Previously, we reported that NaOCl treatment effectively stimulated the in vitro germination of seeds in the genus *Cypripedium* (Bae *et al.* 2009; Bae *et al.* 2010; Bae and Choi 2013). The mechanism underlying this stimulating effect on germination of orchids is not well understood (Harvais and Hadley 1967). Germination of epiphytic orchids and some terrestrial orchids has been observed under both dark

and light conditions (Werckmeister 1971; Ueda 1972; Arditti and Ernst 1984), whereas stimulation of seed germination in darkness has been described for many terrestrial species (Harvais 1973; Stoutamire 1974; Arditti and Ernst 1984; Kauth *et al.* 2006). Additionally, there is a need for an efficient light source to improve production efficiency, reduce costs, and improve the quality of micropropagated plants. The utilization of a more versatile, efficient light source for in vitro plant regeneration and growth could offer new and important possibilities to achieve success in commercial micropropagation. Light-emitting diodes (LEDs) have been proposed as a primary light source for space-based plant research chambers and bioregenerative life-support systems, and as a potential alternative light source for in vitro plant growth and development (Bula *et al.* 1991; Yeh and Chung 2009; Nhut and Nam 2010). However, most LED studies examined vegetables (pepper, tomato, and onion); only a few studies have evaluated orchid (Bae *et al.* 2014) and *Gerbera* spp. (Kwon *et al.* 2012).

In the present study, we evaluated the effects of NaOCl treatment and LED illumination on germination and seedling growth, respectively, for *C. discolor*. We also discuss the importance of a reproducible protocol for establishment of germination and seedling growth from seeds using asymbiotic culture.

MATERIAL AND METHODS

Plant materials and culture conditions

Capsules of *C. discolor* were collected from a single population near Youngarioreum in Jeju, Korea, in July 2013 (voucher specimen, NIBRVP0000439107 and seed number, KWPSGR0000141268). Following collection, the seeds were immersed in deionized, sterilized water and agitated for 30 min. The seeds were then treated with 30 mL of 1% NaOCl in deionized water (v/v) for 30 min, followed by three 30-s rinses in deionized, sterilized water. All media were supplemented with 20 g/L sucrose and the pH was adjusted to 5.5 with 0.1 M KOH before addition of 3.0 g/L gelrite. Media were autoclaved at 117.7 kPa and 121°C for 15 min. The cultures were maintained in a growth room at 20 ± 2°C.

Morphological observation on seed coat by Scanning Electron Microscopy (SEM)

For SEM observation, seeds were fixed by 2% glutaraldehyde for 1-2 days, and then were dehydrated with ethanol and acetone series, and critical point dried by liquid carbon dioxide. Finally, the seeds were coated with gold for 240-s by a sputter coater (Cressington Scientific Instruments Ltd, UK). The coated samples were examined by SEM (Jeol, Japan) at an accelerating voltage of 15 kV and digital images were recorded.

Effects of NaOCl and culture medium on embryo swelling and seed germination

Mature seed capsules were sterilized in 1% NaOCl for 30 min and then rinsed three times with sterile water. The capsules were then cleaved with a scalpel blade, and the seeds were scraped off. The seeds were treated with 1% NaOCl for 30 min. Subsequently, the seeds were left in the final rinse water until transfer to POM (Phytomax Orchid Maintenance, P6668; Sigma, USA) medium, SGM (Seed Germination Maintenance, P6543; Sigma, USA) medium, and MS (Murashige and Skoog 1962) medium after 10 weeks of culture. Two basal media were used in this study: POM and SGM media without plant growth regulators. Embryo swelling was defined as at least a doubling in size and formation of a protocorm. The number of swollen embryos and embryo diameter were recorded every 2 weeks by examination with a microscope.

Effects of LEDs on seedling growth and analysis of chlorophyll contents

Protocorms were transferred to POM and MS medium for seedling. Cultures were incubated in LED growth chambers (Sejong Scientific, Korea) at 23 ± 2°C and 60% relative humidity. Seedlings were subjected to three different culture treatments with the following sources of illumination: (1) fluorescent light (FL), (2) red LED (RL, peak wavelength: 660 nm), and (3) blue LED (BL, peak wavelength: 450 nm). The duration of lighting for all treatments was 16 h per day. Plantlet height was evaluated by measuring the average length of the shoots and roots and the weight per plantlet after 12 weeks of culture. The content of chlorophyll a and b and the total chlorophyll content were determined using methods developed by Hiscox and Israelsham (1979) with some modifications. The color intensity of the resulting extract was measured using a spectrophotometer (Shimadzu, Japan) at 645 nm and 663 nm, and the chlorophyll content (mg/g fresh weight) was calculated using the following formulae. Chl a (mg/g FW of leaf) = (OD_{663} 0.127 – OD_{645} 0.00269) × 100, Chl b (mg/g FW of leaf) = (OD_{645} 0.0229 – OD_{663} 0.00468) × 100, total chlorophyll content = (OD_{645} 0.0202 – OD_{663} 0.00802) × 100, and ratio of Chl a to Chl b = Chl a/Chl b.

Statistical analysis

All data were expressed as the mean ± standard error (SE) and analyzed using an analysis of variance (ANOVA). Each experiment was replicated three times with at least 200 seeds per replication. Significant differences among the treatments were determined by performing multiple comparison tests using Duncan's multiple range test with $P < 0.05$ defined as significant (SAS 2003).

RESULTS AND DISCUSSION

Effect of NaOCl treatment and culture medium on seed germination

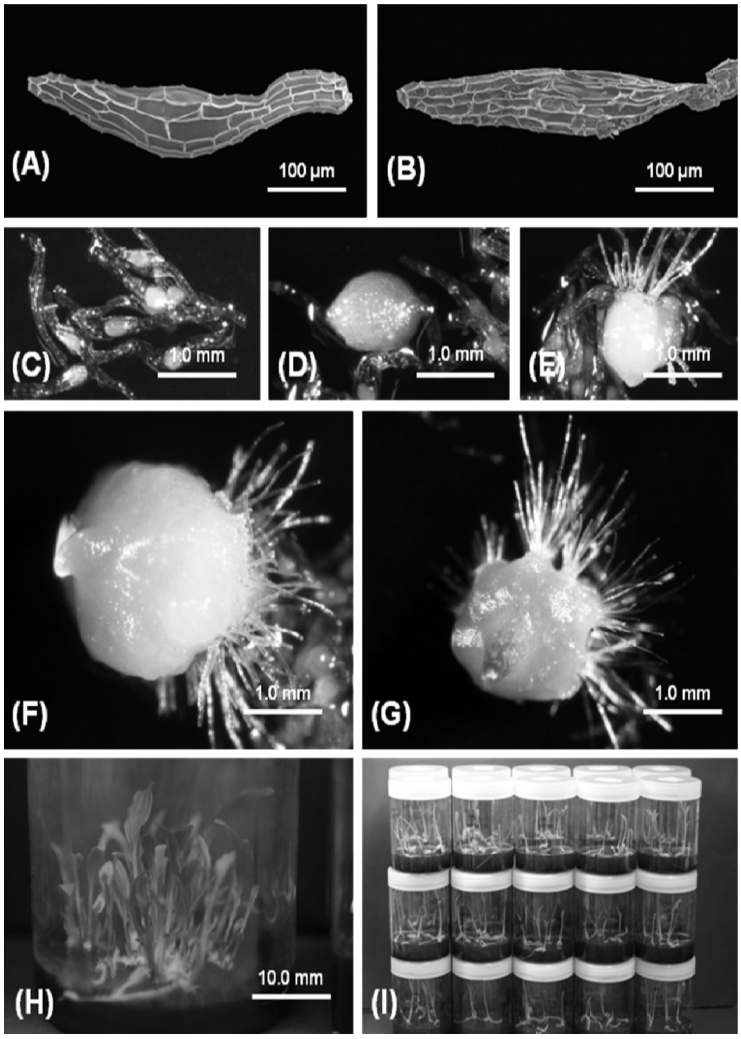

Fig. 1. Developmental stages of asymbiotically cultured *Calanthe discolor* seeds. (A) Surface of seed coat without NaOCl treatment. (B) Surface of seed coats after 1% NaOCl treatment for 30 min by SEM. (C) Hyaline embryo, seed coat intact. (D) Embryos swollen after 4 weeks of culture. (E) Swelled embryos present rhizoids after 6 weeks of culture. (F) Appearance of protomeristem and rhizoid elongation after 7 weeks culture. (G) Appearance of chlorophyllous protomeristem after 10 weeks of culture. (H) Shoot formation *via* the shoot axis were cultured on POM medium containing 1.0 mg/L GA_3 after 10 weeks of culture. (I) Well-developed plantlets were removed from POM medium without 1.0 mg/L GA_3.

The effect of NaOCl treatment on seed germination was examined. After 30 min of 1% NaOCl treatment, the seed coat was bleached completely and the zygotic embryo inside the seed coat could be clearly seen (Fig. 1C). Embryo swelling was first scored 4 weeks after sowing (Fig. 1D). After NaOCl treatment of the seeds, embryo swelling was frequent after 8 weeks of culture. The highest embryo swelling in zygotic embryos was noticed when seeds were treated for 30 min with 1% NaOCl in POM medium (Table 1). POM medium (88.2%) induced significantly greater embryo swelling than SGM (55.4%) and MS (66.7%) media. When immature seeds of *C. discolor* were cultured on POM, SGM, and MS media without 1% NaOCl treatment 11.4%, 8.2%, and 4.1% swelling was observed 2 weeks of culture (Table 1).

Table 1

Effect of 1% NaOCl treatment and culture media on embryo swelling and protocorm formation from *Calanthe discolor* seeds after 10 weeks of culture on medium supplemented with 20 g/L sucrose and 3.0 g/L gelrite

Time [min]	Swelled embryo formation [%]			Protocorm formation [%]		
	POM	SGM	MS	POM	SGM	MS
0	11.4 ± 2.1*d	8.2 ± 1.1e	4.1 ± 2.3f	0d	0d	0d
30	88.2 ± 3.1a	55.4 ± 6.1c	66.7 ± 7.2b	82.3 ± 6.2a	44.1 ± 3.8b	14.8 ± 5.2c

*Data are the means ± SD, of three experiments. Different alphabetical letters are significantly different according to Duncan's multiple range test at $P < 0.05$. SGM, POM, and MS indicate seed germination medium, phytomax orchid maintenance medium, and Murashige and Skoog medium, respectively

Table 2

Effect of light source (LED) on chlorophyll content of *Calanthe discolor* after 12 weeks of culture on POM medium supplemented with sucrose (20 g/L) and gelrite (3.0 g/L)

Light sources	Chlorophyll contents			
	Chl a	Chl b	Total Chlorophyll contents	Chla ratio b
FL	3.21±0.01	0.17±0.01	3.37±0.01	18.52±0.19
RL	4.32±0.01	0.23±0.01	4.55±0.01	18.87±0.11
BL	0.08±0.01	0.02±0.01	0.11±0.01	5.23±0.94

*Data are the means ± SD, of three experiments. Different alphabetical letters are significantly different according to Duncan's multiple range test at $P < 0.05$

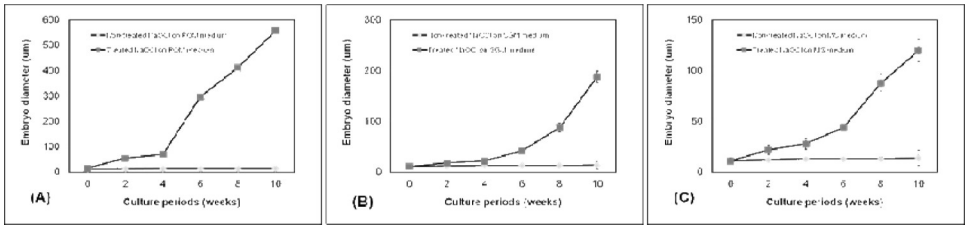

Fig. 2. Effects of culture medium and 1% NaOCl treatment on embryo diameter of Calanthe discolor after 0, 2, 4, 6, 8, and 10 weeks in vitro culture. (A) Effect of treatment with or without 1% NaOCl on seeds in POM medium. (B) SGM medium. (C) MS medium. Data are the mean ± SD of three experiments

After 8 weeks in POM medium without NaOCl, the protocorm formation was very low compared to that in seeds treated with NaOCl (Table 1). In contrast, immature seeds treated with NaOCl began to germinate within 8 weeks (Fig. 1E). Immature seeds with NaOCl treatment cultured in POM medium showed a greatly enhanced frequency of protocorm formation. The maximum protocorm formation was recorded for seeds treated with 1% NaOCl for 30 min and cultured on POM medium (82.3%), followed by SGM (44.1%) and MS (14.8%) medium, whereas the three media without NaOCl treatment (0%) showed the least protocorm formation. Morphological development of *C. discolor* from seed to protocorms was observed (Fig. 1E-G). The maximum embryo diameter was recorded for seeds treated with or without 1% NaOCl and cultured on POM medium (Fig. 2A), followed by SGM (Fig. 2B) medium, and MS medium (Fig. 2C). Within 10 weeks of culture on POM medium supplemented with GA_3 1.0 mg × L^{-1}, the plantlets attained a height of 4-6 cm with converting shoot primordia and 1-2 roots (Fig. 1H). The well-rooted plantlets (approximately 6-7 cm long with 3-4 roots) were removed from the POM medium without GA_3 (Fig. 1I). SEM analysis revealed that NaOCl treatment resulted in the small perforation of seed coats (Fig. 1B) but intact in NaOCl non-treatment (Fig. 1A), indicating that NaOCl treatment strongly affect the rigidity of seed coat by decomposition of cell wall materials. Seed germination of some orchid species is typically very low or nonexistent in ex vitro and in vitro conditions (Ault and Blackmon 1987; Anderson 1996). Terrestrial orchids have more stringent requirements for germination, but little information is available regarding the specific requirements for terrestrial orchids (Fast 1982). NaOCl is a disinfecting agent that is widely used for seed surface sterilization (Bewley and Black 1994), and it is also known to favor seed germination or to overcome seed dormancy in *Cypripedium reginae*, *C. parviflorum*, and *Platanthera grandiflora* (Vujanovic *et al.* 2000). Yildiz *et al.* (2002) reported that treatment with NaOCl for 20 minutes enhanced the germination of *Linum usitatissimum* seeds. The promotion of germination by NaOCl is thought to be due to scarification of the seed coat, which allows more water and oxygen absorption, or from the enhancement of oxidative respiration by the extra supply of oxygen arising from decomposition of NaOCl (Vujanovic *et al.* 2000). In addition, the

effectiveness of disinfection agents such as NaOCl and $Ca(OCl)_2$ for stimulation of germination of orchid seeds has been reported in *Cypripedium acaule* and *Dendrobium* species (St-Arnaud *et al.* 1992; Malmgren 1996), but the underlying mechanism has not yet been reported. The possible mechanisms of action underlying the induction of seed germination or disruption of dormancy by NaOCl have been considered to be partial degradation of the seed coat and/or the solubilization and oxidation of growth inhibitors. Harvais (1982) considered the stimulating effect of surface sterilization with NaOCl to be a physiological effect of the endogenous inhibitor abscisic acid (ABA) being washed away from the seed.

Effect of LED light on seedling growth and chlorophyll measurement

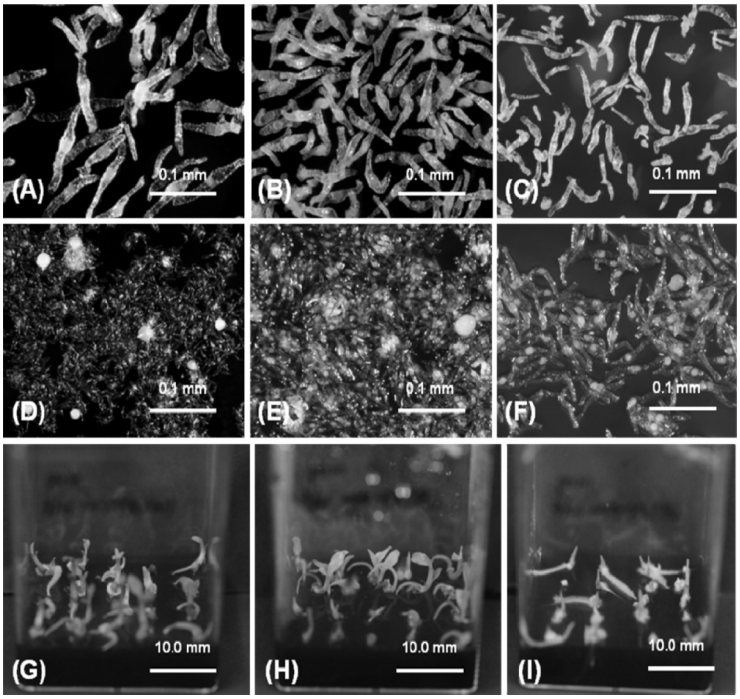

Fig. 3. *Calanthe discolor* seeds asymbiotically cultured in various media without plant growth regulator treatment. (A) Seeds cultured without NaOCl treatment for 12 weeks in POM medium. (B) In SGM medium. (C) In MS medium. (D) With NaOCl treatment for 12 weeks in POM medium. (E) In SGM medium. (F) In MS medium. (G) Plantlet growth on FL after 12 weeks of culture. (H) Plantlet growth on RL after 12 weeks of culture. (I) Plantlet growth on BL after 12 weeks of culture.

Shoot length at 12 weeks after sowing was 2.74 cm, 3.11 cm, and 1.39 cm under fluorescent light (FL), red LED –light (RL), and blue LED –light (BL), respectively, in the POM medium (Fig. 3, Table 2). The root length and fresh weight measurements were all significantly greater for seedlings cultured under

RL with a wavelength of 625 nm (Fig. 4). The highest total chlorophyll content was observed in seedlings cultured under RL (4.55 mg/g), followed by FL (3.37 mg/g), and BL (0.1 mg/g) (Table 2). The induction of protocorm growth using LEDs has also been previously reported in *Calanthe satsuma* (Fukai *et al.* 1997). The inhibition of seed germination by lighting has been reported for many terrestrial orchid species, such as *Calanthe tricarinata* (Godo *et al.* 2010) and *Habenaria macroceratitis* (Stewart and Kane 2006). LED lighting systems also regulate in vitro seedling growth. In *Bletilla ochracea,* a maximum seedling growth of 55% was obtained using various LED-lights (Godo *et al.* 2011).

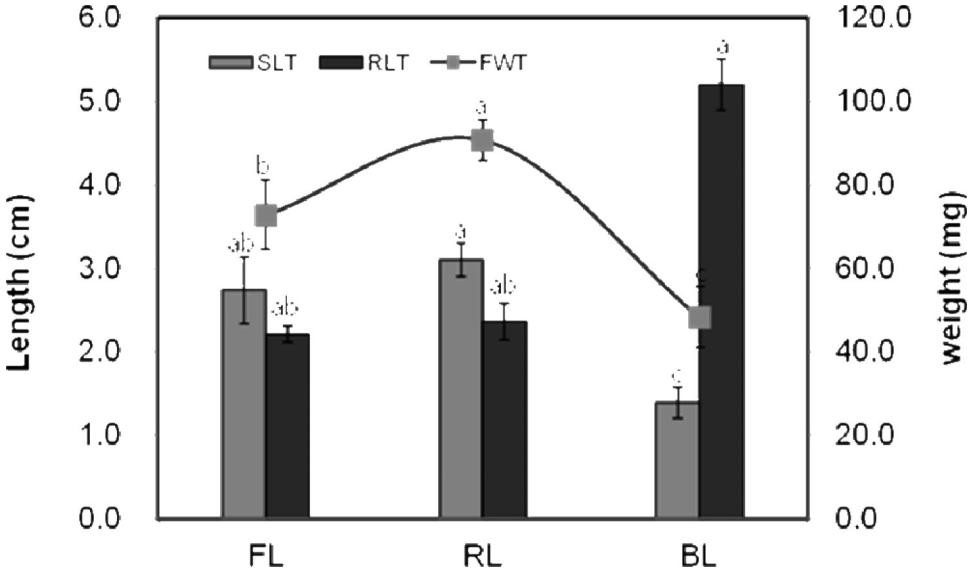

Fig. 4. Effect of light source (fluorescent light and red or blue LED-light) on seedling growth (shoot and root growth, fresh weight measurement) of *Calanthe discolor* after 12 weeks of culture on POM medium supplemented with sucrose (20 g/L) and gelrite (3.0 g/L). SLT, RLT, and FWT indicate shoot length, root length, and fresh weight, respectively. Data are the mean ± SD of three experiments. Different alphabetical letters are significantly different according to Duncan's multiple range test with significant defined as $P < 0.05$

This study demonstrated an efficient micropropagation system for *C. discolor* using seeds and axenic seedling-derived rhizome for explants culture. Asymbiotic seed germination and cloning through rhizomes provided a large number of vigorous seedlings and rhizome-derived clones for ornamental exploitation and eco-restoration of the ensuing rhizomes as the storage organ for propagation. Association with a symbiotic fungus, at least during transplantation from *in vitro* culture, may be an option to enhance growth and to establish species in the field. However, the protocol described in the present study could be useful for commercial nurseries that conduct mass propagation and ex situ conservation of *C. discolor*.

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