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Micropropagation and *in vitro* short-term storage of *Globba sherwoodiana* W.J. Kress & V. Gowda

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Abstract

Globba sherwoodiana is an important cut-flower plant in Thailand which is still cultivated using conventional vegetative propagation. *In vitro* culture is a useful technique for increasing the efficiency of plant production and breeding programs. This research studied suitable culture media for *G. sherwoodiana* micropropagation and *in vitro* storage with genetic fidelity analysis. The sterile shoots were cultured on semi-solid Murashige and Skoog (MS) medium supplemented with 30 different treatments of 6-benzylaminopurine (BA) or 1-naphthaleneacetic acid (NAA). The results showed that explants cultured on MS medium supplemented with a concentration of BA higher than 2 mg/L could induce significantly higher numbers of new shoots, but they were hyperhydric. The explants cultured on MS medium supplemented with a concentration of NAA lower than 1.5 mg/L could induce significantly higher numbers of normal roots. The most suitable medium for shoot proliferation was MS medium supplemented with 1 mg/L BA, while MS medium supplemented with a concentration of NAA lower than 1 mg/L was suitable for root induction. For short-term storage culture, the single shoot explants were cultured on medium of semi-solid MS, ½ MS or ¼ MS supplemented with 10 g/L, 20 g/L, 30 g/L, 40 g/L or 50 g/L sucrose for 60 d, 120 d and 180 d. After storage, the stored explants were recovered by transferring to MS medium and suitable slow growth media were evaluated based on the growth rate, percentage of plantlet survival and new shoot proliferation after recovery. No polymorphic band profiles were found in the clones analyzed using inter simple sequence repeats at the end of each storage period.

Introduction

Globba sherwoodiana W.J. Kress & V. Gowda is a tropical rhizomatous geophyte and belongs to the Zingiberaceae. The genus *Globba* has approximately 100 species that are distributed broadly around South East Asia, especially in Thailand, Myanmar, Indonesia, Malaysia and Laos (William et al., 2004; Larsen and Larsen, 2006). Thailand exports some *Globba* species as commercial ornamental bulbs in global ornamental plant markets. *G. winitii* C.H. Wright, *G. rosea* Gagnep., *G. sherwoodiana* W.J. Kress & V. Gowda and *G. schomburgkii* Hook.f. are *Globba* species commonly sold as cut flowers and in pot plants. *G. sherwoodiana* was originally known as *G. magnifica* (William et al., 2004). It was found in Myanmar and reported in 2012 as a new species in the genus *Globba* Section *Globba* with a combination of white, sharply reflexed and imbricate inflorescence bracts and glabrous bright-green leaves (Gowda et al., 2012). *G. sherwoodiana* has been available through the ornamental plant market since 1990 as *G. magnifica* 'White Dragon' (Paz et al., 2004), and is the main cut-flower *Globba* species in

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Thailand (Boonkorkaew et al., 2010). Generally, *Globba* spp. can be propagated from underground rhizomes, bulbils and seeds (Pimmuen et al., 2014). However, conventional propagation is slow, producing about 10–12 plants per rhizome annually under field conditions (Kumsiri, 2002). New shoots that have divided from old clumps might contain soilborne diseases or internal diseases from mother plants. Therefore, the number of new rhizomes is not sufficient to support the high market demand. *In vitro* propagation is suitable for producing high numbers of disease-free plants and there have been reports regarding the proper medium formulas for *Globba* micropropagation, such as Gamborg B5 medium (Gamborg et al., 1968) containing 20 % sucrose supplemented with 3.0 mg/L 6-benzylaminopurine (BA) for *G. brachyanthera* K.Schum. (Kho et al., 2010); and Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA) and 3 mg/L BA for *G. marantina* L. (Pimmuen et al., 2014). This literature revealed that each *Globba* species has its own suitable medium formula for propagation. Moreover, maintenance of parental plants and hybrids is an important procedure in a plant breeding program. Global warming and rainfall deficiency can cause serious problems for many kinds of plant cultivation (Meteorological Department, 2019). The global warming and rainfall deficiency are also major problems for *ex situ Globba* germplasm management and plant characteristic evaluation. Therefore, *in vitro* storage might be a useful method to protect from plant pathogen impacts or improper cultivating environments. The slow growth technique is one *in vitro* conservation method that reduces the growth rate while also providing improved viability and expanding the intervals between subcultures and consequently, promoting the miniaturization of cultured plants by reducing the strength of the medium nutrients, using plant growth retardants or by controlling osmotic pressure (Ashmore, 1997). For some plant species belonging to the ginger family, *in vitro* conservation has been developed using a slow growth storage technique. Dekkers et al., (1991) successfully stored *Zingiber officinale* Rosc. by controlling the sugar concentration and storage temperature. Parida et al. (2016) reported an *in vitro* conservation protocol for *G. marantina* L. using combinations of 3 mg/L kinetin and 0.5 mg/L NAA supplemented with 10 g/L sucrose and 10 g/L mannitol for retarding the growth rate of the cultured plants. Some other plant species have been successfully raised using minimal growth *in vitro* culture by controlling growth factors such as plant growth regulators (PGR), sugar supplementation and temperature (Bonnier and Van Tuyl, 1997; Martin et al., 2003). Some reports have confirmed the true-to-type of Zingiberaceae plantlet production with PGR addition in culture media such as *Zingiber moran* and *Z. zerumbet* (Das et al., 2013), *Curcuma caesia* Roxb. (Ghosh et al., 2013) and *Kaempferia angustifolia* Roscoe (Haque et al., 2018). However, the application of PGRs during *in vitro* culture for a long period may cause plant malformation growth or genetic changes (Reid et al., 1995). Thus, it is necessary to evaluate the genetic consistency of micropropagated plants using cytological or molecular markers to ensure the quality of plants (Mallon et al., 2010). There are various techniques to determine and confirm anomalies, such as cytological and molecular markers. However, inter simple sequence repeats (ISSR) markers have been widely tested (Bornet et al., 2001) to elucidate genetic uniformity in micropropagated plantlets such as *Saccharum officinarum* (Hsie et al., 2015), *Arracacia xanthorrhiza* Bancroft (Vitamvas et al., 2019) and *Guadua magna* Londoño & Filg. (Nogueira et al., 2019). Therefore, the current study aimed to develop a suitable culture medium for *in vitro* propagation and to use different strengths of MS medium supplemented with different sucrose concentrations to minimize the growth rate of *G. sherwoodiana* for short-term storage and to verify the genetic uniformity of the stored raised plants using ISSR.

Materials and Methods

Plant materials and surface sterilization

Globba sherwoodiana samples were collected from Maejo University, Chiang Mai, Thailand and maintained in a greenhouse at Kasetsart University, Bangkok, Thailand. Sprouting buds (0.5–1.0 cm^3) were excised from rhizomes and washed with liquid detergent and kept under running tap water for 2 hr. The excised buds were separated from the outer scales and the buds were soaked in 70% ethanol for 30 s and then immersed in 20% Clorox containing two drops of Tween-20 for 30 min with continuous shaking. The explants were rinsed three times with sterile distilled water. Apical shoots were excised from sterile explants and cultured on semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 20 g/L sucrose, 2.5 g/L Kelcogel® at pH 5.8. The culture was maintained at 25 ± 2ºC under a cool daylight lamp (Philips TLD 36W/865) at about 30 µmol/m^2/s for a daily photoperiod of 16 hr.

Effect of 6-benzylaminopurine and 1-naphthaleneacetic acid concentrations on explant growth

After surface sterilization, the *in vitro* shoots (1 cm in height) of *G. sherwoodiana* were separated from shoot clumps and cultured on semi-solid MS medium supplemented with BA (0 mg/L, 1 mg/L, 2 mg/L, 3 mg/L, 4 mg/L or 5 mg/L) or NAA (0 mg/L, 0.5 mg/L, 1 mg/L, 1.5 mg/L or 2 mg/L) or 20 combinations of BA and NAA, 20 g/L sucrose and 2.5 g/L Kelcogel® at pH 5.8. The shoots were cultured in '4 ounce' bottles (three shoots per bottle) which contained 20 mL of each medium treatment under cool daylight at about 30 µmol/m^2/s for a daily photoperiod of 16 hr at 25 ± 1ºC. The explants were twice subcultured without trimming every 30 d. The means were recorded of new shoot number, plant height, root number and root length. The experimental design used was a completely randomized design (CRD) with two replications (60 explants/replication).

Effect of medium salt strength and sucrose concentration on growth of short-term storage explants

A factorial experiment using a CRD with two replications (60 explants for each replication) was conducted to study the effect of three levels of salt strength and five sucrose concentrations in

the medium. Single shoots (1 cm) were isolated from the proliferating cultures and transferred to '4 ounce' bottles containing 20 ml of semi-solid MS, half strength MS (½ MS) or quarter strength MS (¼ MS) medium (five shoots per bottle) supplemented with 10 g/L, 20 g/L, 30 g/L, 40 g/L or 50 g/L sucrose and 2.5 g/L Kelcogel® at pH 5.8. The explants were cultured without subculturing for 60 d, 120 d or 180 d. Following storage, the final shoot length was recorded. After each separate storage period, small shoots were excised from the stored shoot clumps and transferred to semi-solid MS medium with 20 g/L sucrose and cultured for 30 d. Evaluation was based on the percentage survival (the number of shoots which visually did not exhibit complete necrosis after storage and were able to convert per one repetition) and the proliferation rate (number of shoots per explant).

Assessment of stored explant genetic stability using inter simple sequence repeats analysis

After short-term storage, the plantlets raised from the stored explants for each treatment were randomly chosen for ISSR analysis. Young leaves of plantlets (150 mg) were frozen in liquid nitrogen. DNA isolation was performed using the modified method of Doyle and Doyle (1987) based on cetyl trimethylammonium bromide (CTAB) extraction buffer. Plants without storage treatment were used as a control. The reaction mixture for polymerase chain reaction (PCR) (25 µL) contained: 1 × optimized DyNAzyme buffer (1.5 mM $MgCl_2$), 200 µM each dNTP, 0.5 µM primer, 50 mg DNA and 1 unit of DyNAzyme II DNA polymerase (Fermentas; Canada). Analysis was performed using eight ISSR primers (Table 1). The PCR reactions were carried out in a thermocycler (Bio Rad MyCycler; USA) with an initial denaturation of the DNA at 94°C for 2 min and then 35 cycles of amplification consisting of denaturation at 94°C for 30 s, primer annealing at melting temperature (Tm) calculated for each primer for 30 s and primer extension at 72°C for 2 min. The final extension was performed at 72°C for 7 min. The PCR products were electrophoresed in 1% agarose gel using 1× tris borate EDTA (TBE) running buffer and RedSafe™ nucleic acid staining solution. The size of amplicons was visualized under ultraviolet light and estimated using a DNA ladder (100–5,000 bp). Only clear and reproducible bands were considered in the analysis.

Table 1 List of eight ISSR primers used for testing genetic fidelity among *in vitro* raised plantlets from stored explants

Primer	Sequence
UBC813	5' - CTCTCTCTCTCTCTCTT - 3'
UBC817	5' -CACACACACACACACAA- 3'
UBC818	5' -CACACACACACACACAG- 3'
UBC857	5' - ACACACACACACACACYG - 3'
UBC860	5' - TGTGTGTGTGTGTGTGRA - 3'
UBC868	5' - GAAGAAGAAGAAGAAGAA - 3'
UBC892	5' - TAGATCTGATATCTGAATTCCC - 3'
UBC895	5' - AGAGTTGGTAGCTCTTGATC - 3'

Statistical analysis

The data were analyzed using analyses of variance based on the SPSS® software (version 16.0; SPSS Inc.; Chicago, IL, USA). Duncan's multiple range test was used to test for significant differences among treatment means at $p < 0.05$.

Results and Discussion

Effect of 6-benzylaminopurine and 1-naphthaleneacetic acid concentration on explant growth

The explants were cultured on semi-solid MS medium supplemented with different concentrations of BA and NAA for 60 d. The results showed that explants cultured on the MS medium supplemented with 5 mg/L BA produced the highest mean (±SD) number of new shoots (4.23 ± 1.02 shoots per explant), which was significantly higher than for the other treatments (Table 2). Almost all clumps of new shoots derived from the MS medium supplemented with 5 mg/L BA were a small size and exhibited hyperhydricity, which is a physiological malformation resulting in excessive hydration, yellowing, swelling, glassiness and leaf curling, which directly affect propagation (Kevers et al., 2004). In the current study, the low-quality shoots due to hyperhydricity were in shoot clumps raised from explants cultured on MS medium supplemented with 2 mg/L BA and higher concentrations. Kadota and Niimi (2003) reported that hyperhydricity was positively related to cytokinin concentration, which may explain the hyperhydricity resulting from using a high concentration of cytokinin in the culture medium. However, it is unlikely that the tissue PGR level alone directly affects hyperhydricity; Ivanova and Van Staden (2011) reported that ventilation of culture vessels and using the proper gelling agent can relieve hyperhydricity. The plant heights of explants cultured on MS medium supplemented with BA at a concentration higher than 2 mg/L or a concentration of NAA higher than 1.5 mg/L or combinations between concentrations of BA higher than 2 mg/L together with concentration of NAA higher than 1 mg/L were significantly lower than plant height cultured on PGR-free MS medium. Explants cultured on the MS medium supplemented with 2 mg/L NAA could induce the significantly highest number of roots. The root numbers of explants cultured on the MS medium supplemented with BA or combinations between higher concentrations of BA and NAA were significantly lower than for the PGR-free MS medium or for a lower concentration of BA in the combination medium samples. Explants cultured on MS medium samples supplemented with 0.5 mg/L or 1 mg/L NAA or with a combination between 1 mg/L or 2 mg/L BA and 0.5 mg/L NAA had significantly longer roots than for other treatments. The current results indicated that the optimum concentration of NAA to promote root induction in the range 0.5–1 mg/L, which was in agreement with the report by Kho et al. (2010) who used 0.1–0.5 mg/L NAA in their culture media for root induction of *G. brachyanthera* K. Schum. and similar to the results of Pimmuen et al. (2014) for root induction of *G. marantina* L. However, explants cultured on MS medium supplemented with 2 mg/L NAA

or 5 mg/L BA or combinations between BA and concentrations of NAA higher than 2 mg/L had callus formation at the pseudostem base with fewer and undersized roots produced. Nguyen et al. (2005) and Yusuf et al. (2011) also reported that MS medium supplemented with 2.0 mg/L NAA could enhance root induction in *Curcuma zedoaria* and *Boesenbergia rotunda*, respectively. Auxin and cytokinin seem to be necessary for plant cell division. Auxins are involved in cell division, cell elongation, vascular tissue differentiation, rhizogenesis and root formation, embryogenesis and inhibition of axillary shoot growth (Chawla, 2002; George et al., 2008; Park et al., 2010). Nevertheless, a high concentration of auxin or cytokinin might cause callus formation from explants of *in vitro* cultured ginger as reported by Musfir et al., (2019) and Miri (2020). In the current research, explants cultured on MS medium supplemented with 5 mg/L BA could induce the significantly highest number of new shoots, but almost all the new shoot clumps were of low quality and exhibited hyperhydricity. Concentrations of NAA higher than 2 mg/L caused abnormal root induction. Thus, the suitable balance for *G. sherwoodiana* multiplication should be MS medium supplemented with 1 mg/L BA. MS medium supplemented with a concentration of NAA lower than 1 mg/L could induce high numbers of normal roots and so was the appropriate medium for root induction of *in vitro* shoots.

Effect of medium salt strength and sucrose concentration on growth of short-term storage explants

The plant height was recorded after storing explants cultured on semi-solid MS, ½ MS or ¼ MS medium supplemented with different sucrose concentrations for 60, 120 or 180 d . The shoot heights of explants cultured on MS, ½ MS, or ¼ MS medium supplemented with 20 g/L or 30 g/L sucrose for 60, 120 or 180 d were significantly higher than for the other treatments (Table 3). Almost all shoots cultured on MS, ½ MS or ¼ MS medium supplemented with 10 g/L or 50 g/L sucrose for any of the storage periods had significantly lower plant heights than for the other treatments. A high or low concentration of sucrose can reduce the growth rate due to osmotic stress (Grout, 1991; Withers, 1991). As the sucrose level in the medium increases,

Table 2 Effects of concentration and combination of BA and NAA in semi-solid Murashige and Skoog medium on growth of *Globba sherwoodiana* after culturing for 60 d

Concentration of plant growth regulator (mg/L)		Number of shoots	Plant height (cm)	Number of roots	Root length (cm)
BA	NAA				
0	0	2.62 ± 0.32 ^{n}	2.76 ± 0.29 ^{a}	22.04 ± 3.79 ^{ef}	1.58 ± 0.19 ^{de}
1.0	0	3.46 ± 0.34 ^{c}	2.77 ± 0.20 ^{a}	20.37 ± 5.34 ^{fg}	1.54 ± 0.35 ^{efg}
2.0	0	3.48 ± 0.35 ^{c}	2.42 ± 0.33 ^{defg}	18.37 ± 4.99 ^{ghi}	1.31 ± 0.42 ^{klm}
3.0	0	3.86 ± 0.65 ^{b}	1.95 ± 0.25 ^{i}	17.96 ± 4.64 ^{hi}	1.35 ± 0.29 ^{jklm}
4.0	0	3.80 ± 0.77 ^{b}	1.61 ± 0.19 ^{l}	15.59 ± 4.71 ^{jkl}	1.26 ± 0.28 ^{m}
5.0	0	4.23 ± 1.02 ^{a}	1.09 ± 0.23 ^{n}	12.76 ± 3.38 ^{m}	0.94 ± 0.37 ^{o}
0	0.5	2.68 ± 0.27 ^{lmn}	2.79 ± 0.26 ^{a}	22.94 ± 3.89 ^{de}	2.41 ± 0.33 ^{a}
0	1.0	2.65 ± 0.30 ^{nm}	2.69 ± 0.32 ^{ab}	24.73 ± 2.89 ^{cd}	2.55 ± 0.24 ^{a}
0	1.5	2.69 ± 0.25 ^{lmn}	2.41 ± 0.50 ^{efg}	27.13 ± 4.29 ^{b}	1.51 ± 0.44 ^{efgh}
0	2.0	2.68 ± 0.27 ^{lmn}	2.57 ± 0.46 ^{bcd}	30.03 ± 5.92 ^{a}	0.67 ± 0.16 ^{p}
1.0	0.5	3.04 ± 0.49 ^{fghi}	2.47 ± 0.47 ^{cdef}	17.90 ± 4.49 ^{hi}	2.53 ± 0.32 ^{a}
	1.0	3.37 ± 0.44 ^{cd}	2.61 ± 0.33 ^{bc}	26.52 ± 4.23 ^{bc}	1.76 ± 0.33 ^{c}
	1.5	3.08 ± 0.45 ^{fgh}	2.45 ± 0.45 ^{def}	23.06 ± 7.20 ^{de}	1.56 ± 0.19 ^{def}
	2.0	2.90 ± 0.39 ^{hijk}	2.54 ± 0.38 ^{cde}	23.97 ± 5.91 ^{d}	1.93 ± 0.28 ^{b}
2.0	0.5	3.13 ± 0.45 ^{efg}	2.34 ± 0.28 ^{fg}	18.40 ± 6.57 ^{ghi}	2.56 ± 0.28 ^{a}
	1.0	3.23 ± 0.55 ^{def}	2.44 ± 0.33 ^{def}	14.76 ± 4.60 ^{lm}	1.51 ± 0.17 ^{efgh}
	1.5	2.91 ± 0.38 ^{hijk}	2.35 ± 0.27 ^{fg}	18.80 ± 4.82 ^{ghi}	1.66 ± 0.35 ^{cd}
	2.0	2.85 ± 0.34 ^{jkl}	2.28 ± 0.51 ^{g}	19.45 ± 6.38 ^{fgh}	1.02 ± 0.34 ^{n}
3.0	0.5	3.21 ± 0.45 ^{def}	2.09 ± 0.38 ^{h}	15.28 ± 3.81 ^{kl}	1.49 ± 0.21 ^{efghi}
	1.0	3.15 ± 0.44 ^{efg}	1.98 ± 0.28 ^{hi}	16.96 ± 6.66 ^{ijk}	1.45 ± 0.23 ^{fghij}
	1.5	2.83 ± 0.36 ^{jklm}	1.88 ± 0.31 ^{ij}	19.70 ± 6.02 ^{fgh}	1.48 ± 0.19 ^{efghi}
	2.0	2.86 ± 0.34 ^{ijkl}	1.78 ± 0.35 ^{jk}	21.40 ± 7.26 ^{ef}	1.34 ± 0.29 ^{jklm}
4.0	0.5	3.17 ± 0.43 ^{ef}	1.73 ± 0.43 ^{kl}	17.85 ± 4.20 ^{hi}	1.67 ± 0.34 ^{cd}
	1.0	3.30 ± 0.50 ^{cde}	1.72 ± 0.48 ^{kl}	17.29 ± 4.58 ^{ij}	1.37 ± 0.46 ^{ijklm}
	1.5	2.78 ± 0.28 ^{klmn}	1.69 ± 0.39 ^{l}	14.19 ± 3.20 ^{lm}	1.29 ± 0.27 ^{ghijk}
	2.0	2.82 ± 0.36 ^{jklm}	1.65 ± 0.34 ^{kl}	16.78 ± 6.05 ^{ijk}	1.03 ± 0.43 ^{n}
5.0	0.5	3.21 ± 0.48 ^{def}	1.26 ± 0.46 ^{m}	13.93 ± 5.10 ^{lm}	1.35 ± 0.44 ^{jklm}
	1.0	3.20 ± 0.46 ^{def}	1.12 ± 0.39 ^{n}	12.86 ± 4.23 ^{m}	1.40 ± 0.21 ^{hijkl}
	1.5	3.05 ± 0.58 ^{fghi}	1.12 ± 0.41 ^{n}	13.25 ± 4.35 ^{m}	1.30 ± 0.29 ^{klm}
	2.0	2.97 ± 0.44 ^{ghij}	1.09 ± 0.30 ^{n}	10.88 ± 3.07 ^{n}	0.96 ± 0.38 ^{o}

BA = 6-benzylaminopurine (BA); NAA = 1-naphthaleneacetic acid.

Mean ± SD values in same column with different lowercase superscripts are significantly ($p < 0.05$) different.

the water content in the plant cell and the cell volume decrease. The decreasing cell volume would result in lower turgor pressure in the cells, and loss of turgor would result in growth reduction (Lazar et al., 2003). On the other hand, a decrease in the sucrose concentration in the medium enhanced the reduction in growth and the photosynthetic ability of plantlets (Desjardins et al., 1995). However, some shoots cultured on MS, ½ MS or ¼ MS medium supplemented with 10 g/L sucrose for longer than 120 d had yellow leaf symptoms on the leaf margin and the whole leaf had turned a light brown color (Fig. 1). Sucrose is considered the most suitable energy source for plant *in vitro* propagation (Yun-peng et al., 2012.) as it comprises the major proportion of most tissue culture media compared to the other constituents (Shibli et al., 2006). Sucrose in culture medium may assist in water conservation and in maintaining the osmotic potential of cells (Hazarika, 2003) and is related to the stomatal density and the photosynthetic pigment content (Mohamed and Alsadon, 2010; Iarema et al., 2012). Hazarika et al. (2000) reported that the starch and chlorophyll contents decreased when the sugar concentration in culture media was reduced. Similarly, Serret et al. (1997) reported that culturing shoot explants on a low sucrose concentration medium caused yellow leaf symptoms in *Gardenia jasmoides* plantlets. Furthermore, cultured explants were normally subcultured every 30–45 d to maintain nutrition levels, pH, osmoticum and necessary chemicals in the culture medium. Therefore, a deficiency of a nutrient and the carbon source in the culture medium might cause yellow leaf symptoms in plantlets cultured longer than 120 d without being subcultured in this experiment. Recent studies also showed that the *in vitro* culture conditions for short and medium-term conservation can increase the oxidative stress, senescence and the accumulation of ethylene in culture vessels (Thakur et al., 2015; El-Dawayati et al., 2018).

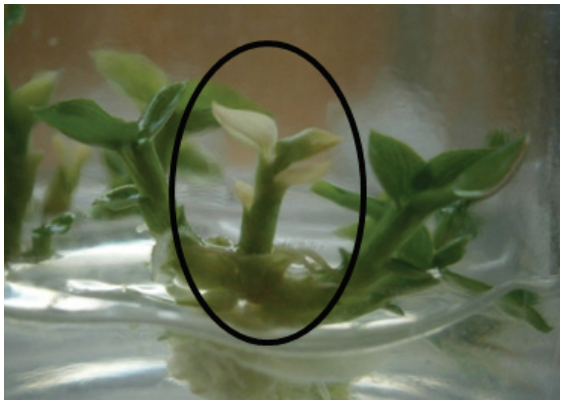

Fig. 1 Yellow leaf symptom of plantlets cultured on Murashige and Skoog (MS), ½ MS or ¼ MS supplemented with 10 g/L sucrose for longer than 120 d

Table 3 Effect of sucrose concentration in semi-solid Murashige and Skoog (MS), ½ MS or ¼ MS medium on plant height of *Globba sherwoodiana* after culturing for 60 d, 120 d and 180 d

Medium	Sucrose concentration (g/L)	Plant height (cm)		
		60 d	120 d	180 d
MS	10	1.26 ± 0.74 ^{ef}	3.00 ± 0.54 ^{d}	4.27 ± 1.05 ^{d}
	20	2.98 ± 0.51 ^{a}	4.43 ± 0.95 ^{a}	7.22 ± 1.01 ^{a}
	30	3.04 ± 0.49 ^{a}	4.33 ± 0.85 ^{a}	7.04 ± 0.81 ^{a}
	40	1.67 ± 1.10 ^{cde}	3.45 ± 0.34 ^{bc}	7.02 ± 0.72 ^{a}
	50	1.43 ± 0.94 ^{def}	3.10 ± 0.36 ^{cd}	6.42 ± 0.52 ^{b}
½ MS	10	1.33 ± 0.86 ^{def}	3.06 ± 0.46 ^{cd}	4.19 ± 1.00 ^{d}
	20	3.02 ± 0.47 ^{a}	4.28 ± 1.03 ^{a}	7.21 ± 0.73 ^{a}
	30	2.26 ± 1.28 ^{b}	4.19 ± 1.00 ^{a}	7.09 ± 0.91 ^{a}
	40	1.78 ± 0.76 ^{cd}	3.66 ± 0.44 ^{b}	6.32 ± 1.22 ^{b}
	50	1.76 ± 1.11 ^{cd}	3.25 ± 0.46 ^{cd}	5.07 ± 0.95 ^{c}
¼ MS	10	1.03 ± 0.57 ^{f}	3.05 ± 0.41 ^{d}	4.15 ± 1.00 ^{d}
	20	3.05 ± 0.54 ^{a}	4.18 ± 1.03 ^{a}	7.23 ± 0.82 ^{a}
	30	2.99 ± 0.48 ^{a}	4.15 ± 1.00 ^{a}	7.21 ± 0.95 ^{a}
	40	1.88 ± 0.31 ^{bc}	2.63 ± 0.38 ^{e}	5.20 ± 0.82 ^{c}
	50	1.41 ± 1.06 ^{def}	2.59 ± 0.29 ^{e}	5.17 ± 1.16 ^{c}
Analysis of variance				
Medium		*	*	*
Sucrose concentration		*	*	*
Medium × Sucrose concentration		**	***	*

Mean ± SD values in same column with different lowercase superscripts are significantly ($p < 0.05$) different.

*, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

After storing explants by culturing on semi-solid MS, ½ MS or ¼ MS medium supplemented with different sucrose concentrations for 60, 120 or 180 d, the explants were transferred to culture on MS medium supplemented with 20 g/L sucrose for 30 d. The survival percentage decreased as the storage period increased (Table 4). The survival percentage was significantly higher in the first 60 d of storage than for the two longer storage periods. The percentages of survival plantlets cultured on MS, ½ MS or ¼ MS medium supplemented with 20 g/L or 30 g/L sucrose were significantly higher than for the other treatments. The most common carbohydrate source used is sucrose, at a concentration of 3 % as recommended by Murashige and Skoog (1962). The exogenous sucrose supply may increase the endogenous content of carbohydrate stocks such as starch, sucrose, fructose and glucose in the micropropagated plants. In addition, it may favor acclimatization and accelerate physiological adaptations (Jo et al., 2009). Similar results have been reported for explants cultured on medium supplemented with 20 g/L or 30 g/L sucrose with *Alocasia amazonica* (Jo et al., 2009) and *Zingiber officinale* (Sathyagowri et al., 2011). The percentage of survival plantlets stored longer than 120 d reduced to 43–83 % and decreased to 26–61 % after storage for 180 d (Table 4). In spite of having the significantly highest survival by plantlets cultured on MS medium supplemented with 20 g/L sucrose after storage for 180 d, the plantlets in some storage bottles had reached the lid of the bottle and the contamination rate rose with the extension of storage duration. Since the objective of this experiment was to retard the growth of plantlets, the plant growth rate and percentage of survival had to be considered. The growth rate of explants stored for 60, 120 or 180 d with post-storage recovery by transferring to MS medium supplemented with 20 g/L sucrose for 30 d, showed that the explants cultured on MS medium supplemented with 40 g/L sucrose for 60 d, MS medium supplemented with 50 g/L sucrose for 120 d and MS medium supplemented with 10 g/L sucrose for 180 d all had significantly lower growth rates than normal plantlets, while their percentages of explant survival after recovery were higher than for the other treatments (Tables 3 and 4).

After storage for 60, 120 or 180 d, treatments of explants cultured on MS and ½ MS medium supplemented with 20 g/L or 30 g/L sucrose were transferred to culture on MS medium supplemented with 20 g/L sucrose for 30 d. The numbers of new shoot proliferation were significantly higher than for the other storage treatments (Table 5). Explants cultured on ¼ MS medium supplemented with 50 g/L sucrose had a significantly lower number of induced new shoots than the other treatments for each storage period after recovery. The MS medium has some mineral compounds related to endogenous cytokinin biosynthesis. An increase in the salt strength of the medium might induce expression of genes responsible for the biosynthesis of cytokinins, resulting in the accumulation of these hormones in plants (Takei et al., 2004; Wang et al., 2004). Cytokinins are primarily responsible for breaking apical dominance and consequent lateral shoot induction; breaking apical dominance is fundamental in the first cell division (Pasternak et al., 2000).

Table 4 Percentage of survival shoots of *Globba sherwoodiana* after *in vitro* storage for 60 d, 120 d or 180 d and transferring to semi-solid Murashige and Skoog (MS) medium for 30 d

Medium	Sucrose concentration (g/L)	Percentage survival after storage period (%)		
		60 d	120 d	180 d
MS	10	90.0 ± 3.78 ^{e}	65.0 ± 6.55 ^{de}	39.4 ± 6.78 ^{c}
	20	100.0 ± 0.00 ^{a}	83.4 ± 5.71 ^{a}	61.9 ± 3.72 ^{a}
	30	100.0 ± 0.00 ^{a}	81.9 ± 4.58 ^{a}	50.6 ± 4.17 ^{b}
	40	97.5 ± 2.67 ^{a}	74.4 ± 4.17 ^{bc}	45.0 ± 5.35 ^{b}
	50	90.0 ± 5.35 ^{bc}	69.4 ± 4.17 ^{cd}	38.8 ± 6.41 ^{c}
½ MS	10	72.5 ± 5.98 ^{bc}	61.9 ± 5.94 ^{ef}	28.8 ± 6.41 ^{de}
	20	100.0 ± 0.00 ^{a}	80.0 ± 5.35 ^{a}	45.6 ± 4.17 ^{b}
	30	98.5 ± 2.27 ^{a}	79.4 ± 4.96 ^{ab}	45.6 ± 7.29 ^{b}
	40	92.5 ± 2.67 ^{bc}	67.5 ± 4.63 ^{de}	33.8 ± 5.18 ^{cd}
	50	91.9 ± 2.59 ^{bc}	56.9 ± 5.30 ^{f}	29.4 ± 4.17 ^{de}
¼ MS	10	79.4 ± 3.20 ^{d}	43.1 ± 5.30 ^{hi}	27.5 ± 5.35 ^{e}
	20	92.5 ± 2.67 ^{bc}	48.8 ± 4.43 ^{g}	37.5 ± 5.35 ^{c}
	30	93.1 ± 2.59 ^{bc}	48.1 ± 2.59 ^{gh}	34.4 ± 5.63 ^{cd}
	40	86.9 ± 4.58 ^{c}	43.8 ± 4.43 ^{ghi}	30.0 ± 8.02 ^{de}
	50	75.0 ± 4.63 ^{e}	43.1 ± 6.23 ^{i}	26.3 ± 4.43 ^{e}
Analysis of variance				
Medium		*	*	*
Sucrose concentration		*	*	*
Medium × Sucrose concentration		*	*	*

Mean ± SD) values in same column with different lowercase superscripts are significantly ($p < 0.05$) different.

* indicates significant at $p < 0.05$.

Based on the analysis of variance, the MS salt strength had a significant interaction with the sucrose concentration regarding plant height, percentage of explant survival and new shoot proliferation after recovery. The MS salt strength seemed to be the important factor for promoting growth rate of *Globba* explants. The trends for explant height, percentage of plantlet survival and new shoot proliferation were higher when the concentration of MS salt strength increased. The optimum concentration of sucrose in the culture medium for growth acceleration was in the range 20–40 g/L, whereas lower (10 g/L) and higher (50 g/L) sucrose concentrations retarded the growth of the *Globba* explants cultured at all three concentration levels of MS salt strength.

According to Tables 3, 4 and 5, the suitable slow growth medium that could retard growth and produce a high percentage of plantlet survival after recovery should be MS medium supplemented with 40 g/L sucrose with storage for 60 d, MS medium supplemented with 50 g/L sucrose with storage for 120 d and MS medium supplemented with 10 g/L sucrose with storage for 180 d. Interestingly, shoots cultured on MS medium supplemented with 10 g/L sucrose for 180 d had a significantly lower growth rate with a high percentage of explant survival after recovery. The slow growth of plantlets cultured on the low sucrose concentration medium might have been caused by the osmotic stress that resisted metabolic activities. However, culturing medium without sucrose or with only a low sucrose concentration could increase the photosynthetic capacity of plantlets (Kozai, 2010). Thus, shoots cultured on MS medium supplemented with 10 g/L sucrose for 180 d had a slow growth rate with a high percentage of plantlet survival after recovery and a high level of new shoot proliferation. Through the modification of the medium composition by adjusting the sugar content, minerals, growth regulators and osmoticum, inhibition of cell division can be achieved, which significantly limits growth and shoot development (Shibli et al. 2006; Lambardi and Ozudogru 2013). Some studies have reported successfully using modified MS medium together with sucrose concentration adjustment with Fraser photinia (Akdemir et al., 2010) and *Chilotanum* potato (Muñoz et al., 2019). For *in vitro* culturing of *Globba* species, MS medium supplemented with 2% or 3 % sucrose is generally used (Kho et al., 2010; Pimmuen et al., 2014). Since commercial *Globba* species are propagated by rhizome division, a microrhizome induction technique is necessary. Sharma and Singh (1995) reported that using MS medium supplemented with 8 mg/L BAP could produce microrhizomes. Bhat et al. (1994) and Zheng et al. (2008) indicated that rhizome formation *in vitro* is affected by many factors, including photoperiod, mineral nutrition, culture method and carbon source. Stimulation of tuber, bulb and rhizome formation by increasing the sucrose concentration in the medium up to 6% or 8% has been reported in ginger (Rout et al., 2001), *Ipsea malabarica* (Martin, 2003) and calla lily (Ebrahim, 2004). Sucrose may be essential as an osmoticum and energy source, while at higher concentrations it might also have a role as a signal for microrhizome formation (Jo et al., 2009). The current study used the high sucrose concentration medium as osmoticum to retard the growth

Table 5 Effect of sucrose concentration in semi-solid Murashige and Skoog (MS), ½ MS or ¼ MS medium on new shoot numbers of *Globba sherwoodiana* after *in vitro* storage for 60 d, 120 d or 180 d and transferring to semi-solid MS medium for 30 d

Medium	Sucrose concentration (g/L)	Number of new shoots		
		60 d	120 d	180 d
MS	10	1.82 ± 0.25 ^{de}	1.76 ± 0.30 ^{c}	1.82 ± 0.31 ^{b}
	20	2.72 ± 0.32 ^{a}	2.66 ± 0.24 ^{a}	2.16 ± 0.34 ^{a}
	30	2.60 ± 0.20 ^{a}	2.62 ± 0.31 ^{a}	2.19 ± 0.47 ^{a}
	40	1.74 ± 0.78 ^{def}	1.68 ± 0.21 ^{cd}	1.86 ± 0.25 ^{b}
	50	1.55 ± 0.84 ^{defg}	1.58 ± 0.26 ^{de}	1.06 ± 0.27 ^{d}
½ MS	10	1.83 ± 0.50 ^{d}	1.81 ± 0.38 ^{c}	1.32 ± 0.26 ^{c}
	20	2.65 ± 0.28 ^{a}	2.64 ± 0.29 ^{a}	2.16 ± 0.47 ^{a}
	30	2.52 ± 0.54 ^{ab}	2.55 ± 0.26 ^{a}	2.19 ± 0.29 ^{a}
	40	1.54 ± 0.37 ^{efg}	1.50 ± 0.19 ^{ef}	1.14 ± 0.38 ^{d}
	50	1.40 ± 0.45 ^{g}	1.40 ± 0.30 ^{f}	0.99 ± 0.20 ^{d}
¼ MS	10	1.50 ± 0.36 ^{fg}	1.52 ± 0.35 ^{def}	1.00 ± 0.22 ^{d}
	20	2.27 ± 0.46 ^{bc}	2.21 ± 0.26 ^{b}	1.80 ± 0.37 ^{b}
	30	2.10 ± 0.50 ^{c}	2.11 ± 0.37 ^{b}	1.75 ± 0.36 ^{b}
	40	1.58 ± 0.28 ^{defg}	1.53 ± 0.36 ^{def}	1.07 ± 0.38 ^{d}
	50	1.11 ± 0.70 ^{h}	1.12 ± 0.22 ^{g}	0.78 ± 0.26 ^{e}
Analysis of variance				*
Medium		*	*	
Sucrose concentration		*	*	*
Medium × Sucrose concentration		*	*	*

Mean ± SD values in same column with different lowercase superscripts are significantly ($p < 0.05$) different.

* indicates significant at $p < 0.05$.

of the stored explants. Microrhizomes were not present during *in vitro* culture until 180 d, perhaps because the sucrose concentration and the nutrients in the culture medium were decreased by the metabolic activities of the explants during the long period of culturing without any subculturing.

Assessment of stored explant genetic stability using inter simple sequence repeats analysis

The genetic fidelity of plantlets raised during the recovering under each treatment of explant storage were confirmed using eight ISSR primers. All primers amplified the genomic fragments and provided reproducible bands. In total, 40 consistently amplified DNA fragments were considered. The 142 amplified band fragments were produced by the eight ISSR primers and 40 loci were observed. Fragment sizes were in the range 150–1,400 bp. The number of observed loci per primer was in the range 3–7 bands with an average of 5 (Table 6). The produced band profiles were monomorphic and there was no variation among all regenerated plantlets under each storage treatment.

Somaclonal variation, a common phenomenon in plant cell cultures, includes all types of variations among plants or cells and derives from all kinds of tissue culture (Skirvin et al., 1993). Usually, variability occurs spontaneously and can be a result of temporary changes or permanent genetic changes in cells or tissue during *in vitro* culture. Temporary changes result from epigenetic or physiological effects and are nonheritable and reversible (Kaeppler et al., 2000). Numerous factors have been reported to cause somaclonal variation, such as the genetics of the explant source, initiation of tissue culture plants from organized meristematic growth, and the type and concentration of plant growth regulators (Yaacob et al. 2013). Plant growth regulators and osmotic pressure are reported as types of abiotic stress that can induce somaclonal variation (Lestari, 2006). However, some studies have reported no genetic variations on raised plantlets after storage on slow growth medium such as for *Lavandula officinalis* Chaix. (Archana et al., 2014) and date palm (El-Bahr et al., 2016). With *Globba* species, there have been some reports confirming the stability of genetic fidelity of micropropagation or conservation using different PGR doses (Kho et al., 2010; Parida et al., 2016). Nevertheless, there have been no reports on *in vitro* storage of *Globba* spp. using a combination of concentration level adjustment of the medium salt strength and of sucrose during the *in vitro* storage. The genetic fidelity results from the current research indicate that plantlets regenerated from short term storage explants cultured on MS, ½ MS, or ¼ MS medium supplemented with 10 g/L, 20 g/L, 30 g/L, 40 g/L or 50 g/L sucrose for 180 d retained their genetic stability. The results obtained from this research are useful practically in increasing the efficiency of a *Globba* breeding program because parental germplasm and hybrid populations can be stored in a culture room with high genetic stability and require a reduced frequency of subculturing while waiting for suitable planting conditions in field trials.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 6 Number of amplified bands, number of loci bands generated by eight inter simple sequence repeat primers using genomic DNA of plantlets raised after storage of explants for 180 d

Primer	Number of loci	Number of polymorphic loci	Range of amplification (bp)
UBC813	5	0	350–1,200
UBC817	6	0	300–1,100
UBC818	3	0	200–1,000
UBC857	7	0	250–1,400
UBC860	4	0	200–1,250
UBC868	6	0	300–1,300
UBC892	5	0	150–1,100
UBC895	4	0	250–1,300
Total	40		

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