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Somatic embryogenesis and plant regeneration from bulb, leaf and root explants of African blue lily (*Agapanthus praecox* ssp. *minimus*)

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Abstract

The African blue lily (*Agapanthus praecox* ssp. *minimus*) is a valuable plant, reported to contain medicinal compounds such as saponins, sapogenins and phytoecdysteroids, besides gaining popularity as an ornamental and landscape species. This paper reports on high efficiency and rapid *in vitro* propagation of *Agapanthus praecox* ssp. *minimus* via somatic embryogenesis from tissues derived from sterile root, leaf and bulb explants obtained from one-month-old aseptic seedlings of this species. Detailed observations on developmental stages of somatic embryos (from globular to coleoptilar) of this monocotyledonous species were also reported in the present investigation. Friable callus which gave rise to high plant regeneration rate (95%) was lucratively produced within 1-2 months after the explants were cultured on Murashige and Skoog (MS) medium supplemented with picloram, 2,4-D, TDZ and combinations of NAA and BAP (0.5 mg/L to 2.0 mg/L). Subsequently, the proliferated calli were transferred onto the same media compositions at 2 weeks interval to encourage extensive production of somatic embryos, followed by transferring to plant growth regulator-free MS medium for complete plant regeneration. Analysis of results showed that explant types highly influenced the degree of response to hormone treatments, whereby root explants were the most responsive. Picloram (a systemic herbicide) was the most effective in inducing somatic embryogenesis from leaf explants, while 2,4-D had excellent influence on root and bulb explants. Further development of somatic embryos to complete plantlets was achieved on MS basal medium. Regenerated plantlets were then hardened and acclimatized on black (peat) soil with 86.67 ± 6.31% survival rate. Scanning electron microscopic studies on leaf tissues showed no morphological variations between the *in vivo* and *in vitro* grown plants, hence mass propagation through tissue culture for true-to-type production of this species is feasible.

Keywords: *Agapanthus praecox* ssp. *minimus*; somatic embryos; *in vitro* regeneration; scanning electron microscopy; acclimatization.

Abbreviations: BAP - 6-benzyl aminopurine; 2,4-D - 2,4- dichlorophenoxyacetic acid; NAA - α-naphthalene acetic acid; PIC – picloram; TDZ – thiadiazuron.

Introduction

Originating from South Africa, the *Agapanthus* species, especially the violet/blue blooming flowers of *Agapanthus praecox* ssp. *minimus* have been increasingly popular around the world for ornamental and landscaping purposes. *A. praecox*, or the 'African lily' or the 'lily of the Nile' (Suzuki et al., 2001; Mor et al., 1984) have long been used by the native South African for medicinal purposes, especially to treat prolonged labour (Varga and Veale, 1997), and has been dubbed as the plant of fertility and pregnancy. The plants within the genus *Apaganthus* were also found to possess antifungal properties (Pretorius et al., 2002; Singh et al., 2008; Tegegne et al., 2008) as well as phytoecdysteroids, although lesser in *A. praecox* compared to other species within the genus (Savchenko et al., 1997). Furthermore, *A. praecox* flowers were also found to contain anthocyanin, with potentials to be utilized as natural colourant (Bloor and Falshaw, 2000; Yaacob et al., 2011). Somatic embryogenesis offers many advantages as compared to conventional breeding, especially in terms of mass propagation of selected genotypes through the use of bioreactors. Furthermore, somatic embryos can be utilized for production of synthetic seeds, whereby the somatic embryos were encapsulated and germinated as natural seeds (Norgaard et al., 1993). Somatic embryogenesis also allows higher yield of plants to be produced in shorter time and may be cryopreserved for future uses (Norgaard et al., 1993). In addition, somatic embryogenesis also serves as a very good model to study embryo development in plants, especially in terms of the biochemical and morphological changes that may have occurred due to the changes in gene expression patterns resulting from somatic embryogenesis process (Santos et al., 2005). Somatic embryogenesis is also a very beneficial tool for rapid production of clonal elite cultivars, where an embryo or a plant can be derived from a single somatic cell (Vicient and Martinez, 1998). Somatic embryogenesis had been reported in *Picea abies* (Hakman and Von Arnold, 1985; Chalupa, 1985) as well as in various conifers and cycads (Jain et al., 1995). On the other hand, the use of bioreactors for mass propagation through somatic embryogenesis had been successful, as reported for *Coffea canephora* (Robusta) and *Coffea arabica* (Ducos et al., 2007). Li et al. (1998) had also achieved somatic embryogenesis in *Theobroma cacao* grown on DKW (Driver

and Kuniyuki, 1984) media supplemented with 9 μM 2,4-D, 22.7 nM TDZ, 2% sucrose and 0.2% phytagel. However, very few published work were found for tissue culture of *Agapanthus* sp., especially on *Agapanthus praecox* ssp. *minimus*. It had been reported that direct organogenesis was readily induced from flower buds of *Agapanthus africanus* Hoffmanns cultured on MS media fortified with 1 mg/L combinations of BAP, Kinetin or TDZ with NAA, 2,4-D or picloram (Supaibulwatana and Mii, 1997). Combinations of TDZ and NAA (both at 1 mg/L) were reported to yield the highest percentage (62%) of direct shoots induction from *A. africanus* flower buds (Supaibulwatana and Mii, 1997). They also stated that TDZ was more effective than Kinetin and BAP in shoot induction, while NAA was more efficient compared to 2,4-D and picloram, when used in combination with other cytokinins (Supaibulwatana and Mii, 1997). Suzuki et al. (2001; 2002) on the other hand reported somatic embryogenesis from leaves of intact *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton when cultures were supplemented with picloram. The objectives of the present work are to establish an efficient *in vitro* propagation method for *Agapanthus praecox* ssp. *minimus*, as an alternative to the conventional approach via the use of bulbs. In the current work, through *in vitro* manipulation of plant hormones, regeneration via somatic embryogenesis could be achieved by using various explants such as leaves, roots and bulbs. A variety of callus inducing hormones were utilized, either applied singly (2,4-D, picloram and TDZ) or in combinations (NAA and BAP). A total of 21 hormone combinations were tested. All stages of somatic embryogenesis were identified and complete plantlets were formed and finally successfully acclimatized. Comparison of morphological characteristics between *in vivo* and *in vitro* grown plants was also made. Although production of embryogenic callus from intact *A. praecox* ssp. *orientalis* (Leighton) Leighton leaves had been previously reported (Suzuki et al., 2001; Suzuki et al., 2002), successful efficient whole plant regeneration via somatic embryogenesis from roots and young bulbs had never been reported before. Besides, Suzuki et al. (2001; 2002) only reported on the use of picloram in producing the somatic embryos, but in the present study, a variety of other hormones such as NAA, BAP, 2,4-D and TDZ were also investigated.

Results

Induction of embryogenic callus

Sterile leaf, root and bulb explants of *Agapanthus praecox* ssp. *minimus* were cultured on MS (Murashige and Skoog, 1962) media supplemented with different concentrations and combinations of α-naphthalene acetic acid (NAA), 6-benzyl aminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), picloram (PIC) and thiadiazuron (TDZ). In the present study, 2,4-D, PIC and TDZ singly applied were used to study somatic embryogenesis of *A. praecox ssp. minimus* since they were found to be able to induce formation of callus in this species. On the other hand, combinations of NAA and BAP were used when single applications of either NAA or BAP failed to induce callus in some cases. Formation of either roots or shoots was observed when only NAA or BAP was applied. Also, equal concentrations of NAA and BAP were used throughout the experiment, with inference that combinations of hormones at equal concentrations would yield somatic embryos, as reported in previous study on *Lawsonia inermis* syn. *L. alba* (Rahiman and Taha, 2011). Creamy friable callus was obtained when root and bulb explants were cultured on MS media supplemented with 0.5-2.0 mg/L 2,4-D (Tables 1 and 2) and also when root and leaf explants were cultured on MS media supplemented with combinations of 0.5-2.0 mg/L NAA and BAP (Tables 1 and 3). In contrary, yellow callus was obtained when root and leaf explants were cultured on MS media supplemented with PIC, as well as from leaf explants cultured on MS media containing high concentrations (1.0 – 2.0 mg/L) of 2,4-D (Tables 1 and 3). Yellowish green callus was produced from root explants cultured on MS fortified with TDZ (Table 1). All somatic embryos were transferred onto MS basal medium for further development of shoots and roots. The resulting plantlets were then subsequently transferred to soil and acclimatized in the green house for further growth and development. Most hormone treatments used in this study managed to induce the production of embryogenic callus from leaf explants of *A. praecox ssp. minimus* except for TDZ treatments, where no callus formation was obtained, even at higher concentrations (Table 3). Similar observations were recorded for root explants, whereby it was found that most hormone treatments had yielded somatic embryogenesis from root explants except PIC (Table 1). It was also found that combinations of NAA and BAP resulted in the formation of embryogenic callus from both root and leaf explants (for most concentrations tested), except for leaf explants cultured on MS fortified with 0.5 mg/L NAA and BAP (Tables 1 and 3). Furthermore, it was also observed that 2,4-D was the most competent hormone for somatic embryogenesis in *A. praecox ssp. Minimus*, yielding somatic embryos from all the three explant types (Tables 1-3) except from leaf explants cultured on MS media fortified with 0.5 mg/L 2,4-D (Table 3). In contrary, TDZ was found to be the least effective in induction of somatic embryogenesis in *A. praecox ssp. minimus*, with only root cultures yielding embryogenic callus (Table 1). The most suitable hormone to induce the production of somatic embryos for leaf explants was 2 mg/L picloram, with the highest percentage of callus weight (64%), though only a mediocre 60% of the explants managed to produce embryogenic callus (Table 3). However, 2 mg/L 2,4-D was found to be the most suitable hormone to induce somatic embryogenesis from both root and bulb explants, with percentage of callus weight of 39.53% from 100% of root explants forming callus (Table1) and an astonishingly high percentage of callus weight (92.30%) from 90% of bulb explants which produced callus (Table 2). Among all the three explants used in this study, root explants were found to be the most responsive, while bulbs were the least responsive in inducing somatic embryogenesis. This is clearly depicted in Fig 1, where 52.38% from a total of 21 hormone combinations including cultures maintained in the dark (data not shown), that were used in this study managed to produce somatic embryos in root explants, followed by 47.62% for the leaf explants and 19.05% for the bulb explants. Hence, this indicated that roots were the most responsive explants for induction of somatic embryogenesis in this species. Somatic embryogenesis observed from the monocotyledonous *A. praecox ssp. minimus* showed that cells had undergone periclinal and anticlinal divisions, yielding the development of globular somatic embryos, which subsequently elongated

Table 1. Callus induction from root explants of *A. praecox ssp. minimus* cultured on MS medium supplemented with various hormones after 4 months of culture.

[PIC] mg/L	[NAA] mg/L	[BAP] mg/L	[2,4-D] mg/L	[TDZ] mg/L	Explant with callus (%)	Callus growth (weight, %)	Observations
0.5					96.67	$13.95^{b} \pm 0.6$	Yellow compact callus (non-embryogenic)
1.0					100.00	$20.93^{d} \pm 0.4$	Yellow compact callus (non-embryogenic)
1.5					96.67	$32.56^{f} \pm 0.9$	Yellow compact callus (non-embryogenic)
2.0					100.00	$37.21^{g} \pm 0.5$	Yellow compact callus (non-embryogenic)
	0.5	0.5			93.33	$6.98^{a} \pm 0.3$	Creamy white friable callus (embryogenic)
	1.0	1.0			100.00	$16.28^{c} \pm 0.4$	Creamy white friable callus (embryogenic)
	1.5	1.5			93.33	$20.93^{d} \pm 0.2$	Creamy white friable callus (embryogenic)
	2.0	2.0			100.00	$27.91^{e} \pm 0.3$	Creamy white friable callus (embryogenic)
			0.5		93.33	$16.28^{c} \pm 0.5$	Creamy friable callus (embryogenic)
			1.0		100.00	$20.93^{d} \pm 0.5$	Creamy friable callus (embryogenic)
			1.5		96.67	$30.23^{f} \pm 0.3$	Creamy friable callus (embryogenic)
			2.0		100.00	$39.53^{g} \pm 0.7$	Creamy friable callus (embryogenic)
				0.5	-	-	No formation of callus
				1.0	83.33	$32.81^{f} \pm 0.5$	Yellowish green compact callus (embryogenic)
				1.5	96.67	$33.73^{f} \pm 0.7$	Yellowish green compact callus (embryogenic)
				2.0	86.67	$33.51^{f} \pm 1.2$	Yellowish green compact callus (embryogenic)

*means with different letters in the same column differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test.

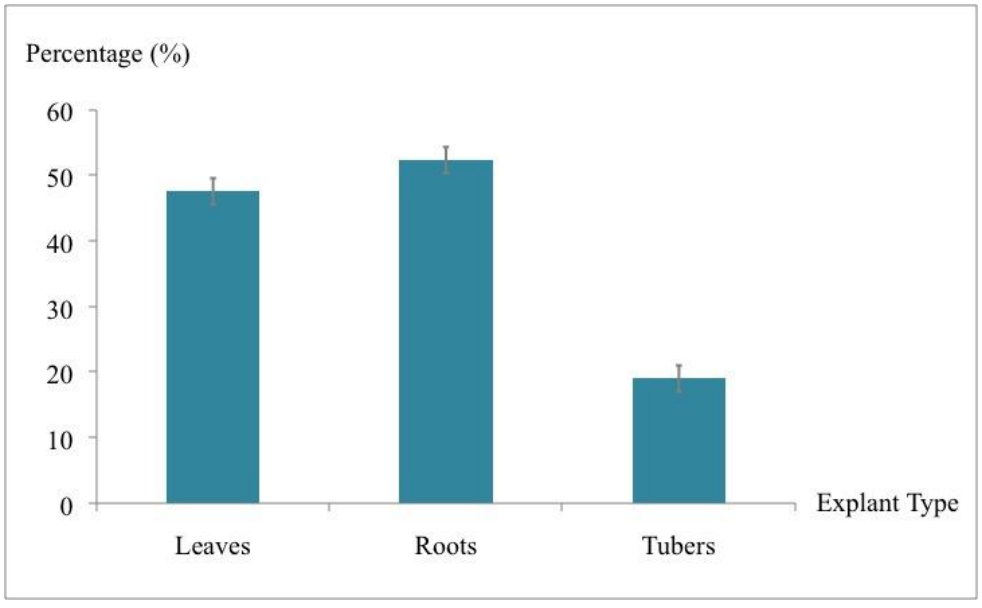

Fig 1. Responses (depicted in terms of percentage, %) of different types of explants to different hormone treatments.

and undergone scutellar and coleoptilar stages (Fig 2). The development of microshoots of *A. praecox ssp. minimus* is summarized in Fig 3, while Fig 4 showed the complete and successfully acclimatized *A. praecox ssp. minimus* plantlet.

Morphology of somatic embryo-derived in vitro plantlets versus in vivo grown plants

Visual observations on morphology of both *in vivo* and somatic embryo-derived *in vitro* grown plants revealed no distinct abnormalities. The *in vivo* plants were observed to be taller and have bigger leaf diameter than *in vitro* plantlets (data not shown). The shapes and structures of leaves between both *in vivo* and *in vitro* plants were similar, whereby the leaves were long, narrow and leathery. Furthermore, the root morphology of both *in vivo* and *in vitro* plants was also similar, with roots that were thick and hairy. However, the roots of *in vivo* plants were creamy white, while the roots of *in vitro* plantlets looked slightly greenish due to accumulation of chlorophyll in the root tissues during incubation in the culture room. The growth of *in vitro* grown plantlets were subjected to many limiting factors such as the size of tissue culture vessels as well as controlled supply of nutrients and growth hormones, resulting in smaller plantlets with under-developed morphological characters. Scanning electron microscopy (SEM) showed that *in vivo* grown *A. praecox ssp. minimus* leaf had more stomata than leaf of somatic embryo-derived *in vitro* grown *A. praecox ssp. minimus* (Fig 5a and 5b). Morphologically, as observed via SEM, the surface of *in vivo* leaf was more rigid compared to the soft structure of the *in vitro* grown *A. praecox ssp. minimus* leaf. These can be observed in Fig 5a and 5b, where the surface of *in vitro* grown *A. praecox ssp. minimus* leaf appeared to be undulating and not smooth. In addition, the surface of the *in vivo* leaf was hairy (Fig 5c), while the *in vitro* leaf had no hair structures (Fig 5d). However, the size of the stoma of both *in vivo* and *in vitro* grown *A. praecox ssp. minimus* leaves was similar (32.86 cm). Successful acclimatization of somatic embryo-derived plantlets of this species was further attested by the SEM studies, which showed that no morphological differences or abnormalities had occurred in the *in vitro* grown plantlets. However, hardening of the *in vitro* grown plantlets would develop with time, following *ex vitro* acclimatization.

Discussion

Previously, micropopagation of Liliaceous plant species had been widely produced via utilization of the flowering organs, resulting in direct shoot formation (Wilmink et al., 1995) and indirect plant regeneration via somatic embryogenesis of the flowering organs (Dunstan and Short, 1978). The present study however depicts the use of other possible explants as the sources of *in vitro* culture for this species, which may become useful as alternatives or supplementary to the inflorescence parts that may not be available at all times. The present study also adheres to the findings of Supaibulwatana and Mii (1997) and Suzuki et al. (2001; 2002), where picloram had been found to be the most suitable hormone in inducing somatic embryogenesis from leaves of *Agapanthus* sp. However, the present study also revealed that successful induction of somatic embryogenesis from *A. praecox ssp. minimus* leaves also occurred through additions of 2,4-D and combinations of NAA and BAP. Besides, the present study also indicated successful production of somatic embryos from other explants such as *A. praecox ssp. minimus* roots and young bulbs. The root explants were found to be the most responsive and the easiest to culture *in vitro*, with successful somatic embryogenesis induction in 52.38% of all the hormones tested including cultures maintained in the dark (data not shown). The current study therefore provides a new insight into achieving efficient regeneration systems for micropropagation of *Agapanthus praecox* ssp. minimus, where more explant choices can be utilized in mass propagation of *Agapanthus praecox in vitro*. Rapid and more profitable production of this species can now be achieved by utilizing all these available possible explants. However, further researches are in progress to reduce culture time and hence large scale *in vitro* production of this species. Picloram was found to be very effective in inducing callus growth from leaf and root explants of this species, on top of able to influence the formation of somatic embryos from friable callus derived from *in vitro* cultures of *A. praecox ssp. minimus* leaves. The effectiveness of picloram in inducing callus growth was also reported by Sener et al. (2008), where it was shown that Linsmaier and Skoog (1965) media supplemented with 7.5 mg/L picloram gave the highest callus induction rate (31.3%) in *in vitro* cultures of immature inflorescence of spring barley cultivars (*Hordeum vulgare* L.). Conger et al. (1982) also reported similar findings, where picloram was found to be effective in inducing callus in gramineae species such as orchardgrass (*Dactylis glomerata* L.), tall fescue (Festuca arundinacea Schreb.) and annual ryegrass (*Lolium multiflorum Lam.*). In addition, picloram had also been found to be very efficient for callus induction in other species such as potato, Solanum tuberosum L. (Hagen et al., 1990), wheat, *Triticum aestivum* L. (Mendoza and Kaeppler, 2002), wetland monocot cattail, *T. latifolia* and *J. accuminatus* (Rogers et al., 1998; Sarma and Rogers, 1998). Furthermore, embryogenic callus was readily induced from all explants (root, leaf and bulb) cultured on MS media fortified with 2,4-D, except in leaf cultures fortified with 0.5 mg/L 2,4-D, suggesting that 2,4-D is a universally competent hormone for somatic embryogenesis of *A. praecox ssp. minimus*. Poon et al. (2011) also reported direct somatic embryogenesis from hypocotyl explants of cauliflower (*Brassica oleracea* var. botrytis) cultured on MS with 2,4-D. 2,4-D had also been shown to be very effective in inducing callus growth in cotton (Kolganova et al., 1992). However, the effectiveness of 2,4-D on callus induction is subject to plant species, whereby it had been reported that dicamba and picloram were especially superior than 2,4-D for callus induction in tall fescue plant, *Festuca arundinacea* (Conger et al., 1982). Both Rogers et al. (1998) and Sarma and Rogers (1998) also reported callus growth in *T. latifolia* and *J. accuminatus* via the use of hormones 2,4-D and NAA, although the use of picloram had been shown to be the most

Table 2. Callus induction from bulb explants of *A. praecox ssp. minimus* cultured on MS medium supplemented with various hormones after 4 months of culture.

[PIC] mg/L	[NAA] mg/L	[BAP] mg/L	[2,4-D] mg/L	[TDZ] mg/L	Explant with callus (%)	Callus growth (weight, %)	Observations
0.5					-	-	No formation of callus
1.0					-	-	No formation of callus
1.5					-	-	No formation of callus
2.0					-	-	No formation of callus
	0.5	0.5			-	-	No formation of callus
	1.0	1.0			-	-	No formation of callus
	1.5	1.5			-	-	No formation of callus
	2.0	2.0			-	-	No formation of callus
			0.5		83.33	$15.38^{a} \pm 0.4$	Creamy white friable callus (embryogenic)
			1.0		100	$46.15^{b} \pm 0.3$	Creamy white friable callus (embryogenic)
			1.5		100	$69.23^{c} \pm 0.6$	Creamy white friable callus (embryogenic)
			2.0		90.00	$92.30^{d} \pm 0.3$	Creamy white friable callus (embryogenic)
				0.5	-	-	No formation of callus
				1.0	-	-	No formation of callus
				1.5	-	-	No formation of callus
				2.0	-	-	No formation of callus

*means with different letters in the same column differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test.

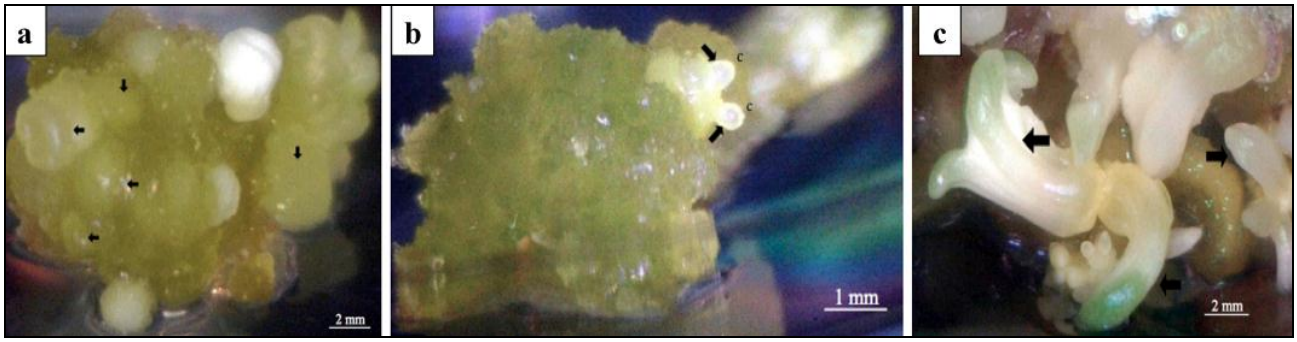

Fig 2. Stages of somatic embryogenesis in *Agapanthus praecox* ssp. *minimus*: (a) Globular-shaped phase somatic embryo produced from leaf explant cultured on MS media supplemented with 2 mg/L PIC. (b) Somatic embryo produced from leaf explant cultured on MS media supplemented with 2 mg/L PIC, at the scutellar stage showing the coleoptile (c). (c) Somatic embryo produced from bulb explant cultured on MS media supplemented with 1 mg/L 2,4-D, at the coleoptilar stage. Arrows show different stages of somatic embryogenesis.

effective. Apart from that, we also found that high concentrations of NAA and BAP greatly influenced the formation of embryogenic callus from *A. praecox ssp. minimus* leaf and root explants. NAA and BAP in combinations had also been reported to yield positive results in tissue culture of many species, such as in promoting shoot growth and proliferation of pineapple (Al-Saif et al., 2011) as well as formation of multiple shoots and subsequent plantlet regeneration from stems of *Lawsonia inermis* syn. *L. alba* or henna plant (Rahiman and Taha 2011). Besides, higher concentrations of NAA and BAP were also found to enhance the percentage of callus weight from *in vitro* cultures of *A. praecox ssp. minimus* leaf and root explants, suggesting a direct correlation exists between NAA and BAP concentrations with callus formation. Similar observations were obtained by Devendra et al. (2011), where the frequency of callus formation from nodal explants of *Eclipta alba* L. Hassk increased with increasing concentrations of NAA. The competence of NAA, BAP and 2,4-D in inducing somatic embryogenesis had also been previously reported in *Brassica* species, where 3 mg/L BAP, 2 mg/L NAA and 2 mg/L 2,4-D were found to be the most efficient hormones to induce somatic embryogenesis from hypocotyl explants of *Brassica napus* L. ssp. 'Talayeh' (Zeylani et al., 2010). However, contrasting results were reported for *Citrus macroptera* Mont.var. anammensis, whereby nucellus tissues grown on MS media supplemented with 2,4-D and Kinetin managed to produce callus but failed to induce somatic embryogenesis (Miah et al., 2002). In *Rosa hybrida* cvs. Carefree Beauty, the use of 2,4-D only managed to induce callus growth, but other hormones such as TDZ, BAP and GA3 were needed for production of somatic embryos (Li et al., 2002).

Materials and methods

Plant material

Seeds of *Agapanthus praecox* ssp. minimus collected from Cameron Highlands, Malaysia were surface sterilized

Table 3. Callus induction from leaf explants of *A. praecox ssp. minimus* cultured on MS medium supplemented with various hormones after 4 months of culture.

[PIC] mg/L	[NAA] mg/L	[BAP] mg/L	[2,4-D] mg/L	[TDZ] mg/L	Explant with callus (%)	Callus growth (weight, %)	Observations
0.5					83.33	$8.00^{b} \pm 0.3$	Yellow friable callus (embryogenic)
1.0					100.00	$20.00^{d} \pm 0.2$	Yellow friable callus (embryogenic)
1.5					83.33	$40.00^{f} \pm 0.7$	Yellow friable callus (embryogenic)
2.0					60.00	$64.00^{h} \pm 0.2$	Yellow friable callus (embryogenic)
	0.5	0.5			-	-	No formation of callus
	1.0	1.0			100.00	$16.00^{c} \pm 0.6$	Creamy white friable callus (embryogenic)
	1.5	1.5			96.67	$32.00^{e} \pm 0.2$	Creamy white friable callus (embryogenic)
	2.0	2.0			100.00	$48.00^{g} \pm 1.1$	Creamy white friable callus (embryogenic)
			0.5		-	-	No formation of callus
			1.0		100.00	$4.00^{a} \pm 0.1$	Yellow friable callus (embryogenic)
			1.5		71.43	$16.00^{cd} \pm 0.6$	Yellow friable callus (embryogenic)
			2.0		100.00	$44.00^{g} \pm 0.5$	Yellow friable callus (embryogenic)
				0.5	-	-	No formation of callus
				1.0	-	-	No formation of callus
				1.5	-	-	No formation of callus
				2.0	-	-	No formation of callus

*means with different letters in the same column differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test.

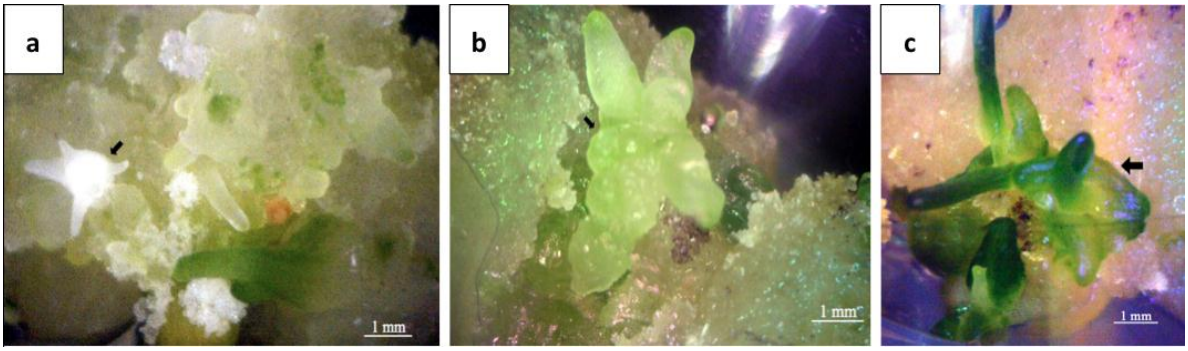

Fig 3. Formation of microshoots from somatic embryo of *Agapanthus praecox* ssp. *minimus*, from root explant cultured on MS media supplemented with 1 mg/L NAA + 1 mg/L BAP: (a) Shoot organogenesis started to occur on the creamy white friable callus after 3 months of culture. (b) Further development of adventitious microshoots after 1 week. (c) Further development and maturation of adventitious microshoots after 2-3 weeks. Arrows show development of the microshoots.

following standard tissue culture protocols (Taha, 1993) but with minor modifications. The seeds were treated with 100%, 70% and 30% (v/v) commercial bleach (Chlorox) for 1 minute at each concentration, followed by submersion in 70% (v/v) ethanol and finally by 3 times rinsing with sterile distilled water. Two drops of Tween-20 were also added during the treatment with 100% (v/v) Chlorox to facilitate the sterilization process and reduce surface tensions. The seeds were cultured on MS (Murashige and Skoog, 1962) media without hormones, supplemented with 30 g/L sucrose, pH 5.6 ± 0.1, solidified with 2 g/L gelrite and maintained in the culture room at 25 ± 1 ºC with 16 hours light and 8 hours dark for 4 weeks. Explants were subcultured into small-sized sterile jam jars after they had grown to approximately 4 cm in height. Different parts (root, young bulb and leaf explants) of 4-week-old explants were used for callus induction and production of embryogenic tissues.

Embryogenic callus induction

The leaf and root explants were cut into small pieces (2 cm in length) and young bulbs that were free from leaves and roots were used to produce embryogenic callus. In this study, different concentrations of picloram, PIC (0.5, 1.0, 1.5, 2.0 mg/L), 2,4- dichlorophenoxyacetic acid, 2,4-D (0.5, 1.0, 1.5, 2.0 mg/L), thiadiazuron, TDZ (0.5, 1.0, 1.5, 2.0 mg/L) as well as equal concentrations of alpha-naphthalene acetic acid, NAA and 6-benzyl aminopurine, BAP (0.5, 1.0, 1.5, 2.0 mg/L) were used to initiate and investigate the formation of somatic embryogenesis in *A. praecox ssp. minimus*. The

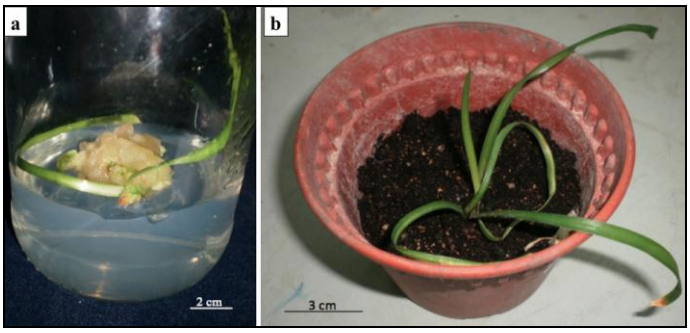

Fig 4. Regeneration and acclimatization of somatic embryo-derived complete plantlet of *Agapanthus praecox* ssp. *minimus*: (a) Regeneration of complete plantlet from somatic embryo derived from bulb explant cultured on MS medium supplemented with 1 mg/L 2,4-D. (b) Successfully acclimatized plantlet (3-month-old).

explants (root, leaf and bulb) were cultured on MS media supplemented with the designated hormone combinations with 30 g/L sucrose, pH 5.6 ± 0.1, solidified with 2 g/L gelrite and incubated in the culture room at 25 ± 1 ºC with 16 hours light and 8 hours dark for 4 months.

Regeneration and acclimatization of complete plantlets

Matured somatic embryos and microshoots of *A. praecox ssp. minimus* were transferred to plant growth regulator-free MS medium to induce production of more shoots and roots and subsequently to form complete plantlets. The MS basal medium was added with 30 g/L sucrose, pH 5.6 ± 0.1 and solidified with 2 g/L gelrite. The cultures were maintained in the culture room at 25 ± 1 ºC with 16 hours light and 8 hours dark. All the resulting plantlets from indirect regeneration via somatic embryogenesis of *A. praecox ssp. minimus* were acclimatized for further growth and development. The plantlets were transferred to black soil in vases covered with plastic bags and acclimatized in the culture room for one month. Subsequently, the plantlets were transferred into a green house, and their ability to fully adapt to the natural environment was monitored.

Morphology of in vivo and in vitro grown plants

The morphological characteristics of both *in vivo* and *in vitro* grown *A. praecox ssp. minimus* were observed and comparisons were made to elucidate any morphological abnormalities that might have occurred as a result of tissue culture protocols. The leaf shape, leaf structure, mean plant height and mean leaf diameter of the acclimatized *in vitro* grown plantlets were measured and compared to *in vivo* plants of similar age (3-month-old). Root morphology (thickness, colour and structure) of *in vivo* and *in vitro* grown plantlets were also compared. Leaf segments of *in vivo* and *in vitro* grown *A. praecox ssp. minimus* were also examined under scanning electron microscope (SEM) (Jeol JSM-6400) to distinguish any morphological differences between the *in vivo* and *in vitro* leaf tissues and to detect any occurrences of somaclonal variation.

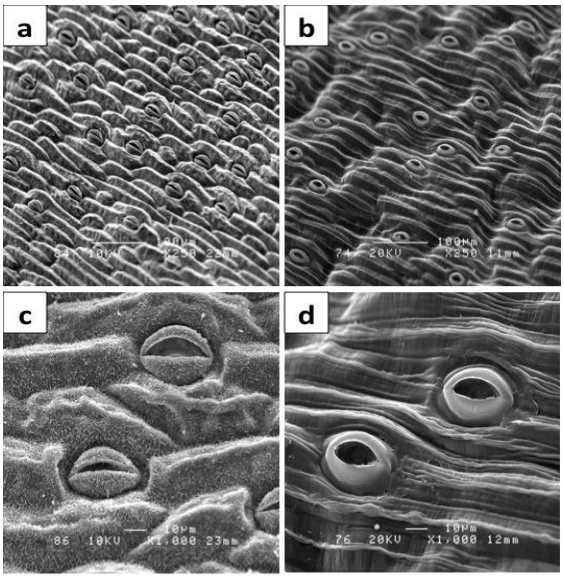

Fig 5. SEM of *in vivo* and *in vitro* grown *A. praecox ssp. minimus* leaves, showing no morphogenic variations had occured. (a-b) Abaxial surface of *in vivo* leaf showing a rigid epidermal surface and numerous stomata, compared to an uneven and 'softer' epidermal surface (abaxial) of *in vitro* leaf, also with less stomata than *in vivo* leaf. (**c-d**) Stoma of *in vivo* and *in vitro* leaf showing similar shape and size, as well as the lack of hair structures on *in vitro* leaf compared *to in vivo* leaf.

Statistical analysis

Different concentrations of hormones were assessed using complete block design with 30 replicates, which is standard in tissue culture research to decrease error and enhance accuracy. The results were analyzed by using statistical variance test (ANOVA) and compared with least significant differences (LSD) at 5% level, to discern the effects and differences between the hormone treatments.

Conclusion

Callus initiation followed by somatic embryogenesis was successfully obtained on MS supplemented with different

combinations and concentrations of hormones (picloram, 2,4-D, TDZ, NAA and BAP). It has been shown that explant types largely influenced the response to hormone treatments, for example 2 mg/L picloram was found to be the best hormone to induce somatic embryogenesis in *A. praecox ssp. minimus* leaf explants, while 2 mg/L 2,4-D was the most suitable for somatic embryogenesis induction in root and bulb explants of *A. praecox ssp. minimus*. It was also found that root explants responded more readily to hormone treatments for induction of somatic embryogenesis, followed by leaves and lastly the bulbs. *In vitro* regeneration and acclimatization were also successfully obtained and the regenerants maintained the true-to-type morphological characteristics of the plant, except smaller in size with under-developed structures.

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