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Full Length Article

Organogenesis Induction and Acclimatization of African Blue Lily (*Agapanthus praecox* ssp. *minimus*)

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Abstract

Rapid micropropagation through direct regeneration and organogenesis of *Agapanthus praecox* ssp. *minimus* was established from bulb explants. The effect of various auxins and cytokinins on shoot formation and root induction was studied. The influence of acclimatization treatments such as growth substrates, initial plantlet morphology (prior to acclimatization) and regeneration pathways on growth performance of the plantlets following field transfer was also elucidated. It was found that regeneration of complete plantlets could be achieved after as early as 4 weeks of culture and MS (Murashige and Skoog) media supplemented with 2.0 mg L^{-1} indole-3-butyric acid (IBA) and 2.0 mg L^{-1} 6-furfurylaminopurine (Kinetin) was optimum for shoot formation from bulb explants. Induction of rooting was best achieved on MS media supplemented with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} Kinetin. Callus was readily induced from leaf explants than root explants, although both explant types lacked direct organogenesis potential. Morphology of the plantlets prior to acclimatization and the regeneration pathways (direct and indirect) also affect the growth performance of the plantlets after field transfer. Taller plantlets with more leaves showed the highest increase in height and most number of leaves after 3 months in the field. Plantlets derived from direct regeneration also showed better growth in the field than plantlets derived from somatic embryos. Among different growth substrates, 1:1 ratio of red/black soil resulted in highest percentage of survival rate (96.67%) after field transfer. Morphology (macro and micro) of the *in vitro* plantlets appeared similar (although less developed) to intact plants, indicating no somaclonal variation had occurred. Micropropagation of *A. praecox* ssp. *minimus* via direct regeneration and organogenesis is successful and can be utilized for mass propagation of this species at commercial level.

Keywords: *Agapanthus praecox* ssp. *minimus*; Acclimatization; Growth performance; Micropropagation; Morphology

Introduction

Agapanthus praecox or 'Lily of the Nile' or the 'African Lily' originated from South Africa and was very popular among the native tribes as a traditional remedy to treat prolonged labour (Varga and Veale, 1997). The *Agapanthus* plants had been reported to contain saponins and sapogenins with anti-inflammatory activities. Fungitoxic properties were also observed in *Agapanthus inapertus* (Pretorius *et al*., 2002) and *Agapanthus africanus* (Singh *et al*., 2008; Tegegne *et al*., 2008). Besides, *Agapanthus praecox* was also reported to contain phytoecdysteroids, although the levels were found to be lower than other species within the genus *Agapanthus* (Savchenko *et al*., 1997). Not only that, the violet-blue flower petals of this species was reported to contain valuable anthocyanin pigments (Bloor and Falshaw, 2000; Yaacob *et al*., 2011), which could be manipulated to produce highly commercialized, heat and salt-tolerant organic paint materials (Yaacob *et al*., 2011). The attractive morphological features of *A. praecox* had rendered this species to be popular for landscaping and as an ornamental.

Limited published literatures were found on *in vitro* culture of *Agapanthus* sp., particularly *A. praecox* ssp. *minimus*. Supaibulwatana and Mii (1997) had reported the induction of direct organogenesis from flower buds of *A. africanus* Hoffmanns cultured on MS media through addition of 1.0 mg L^{-1} thidiazuron (TDZ) and naphthalene acetic acid (NAA). Induction of somatic embryogenesis from leaves of intact *A. praecox* ssp. *orientalis* Leighton using 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram) was reported by Suzuki *et al*. (2001; 2002). Regeneration via callus-derived protoplasts of *A. praecox* ssp. *orientalis* had also been achieved (Nakano *et al*., 2003). Titova (2003) mentioned that *A. praecox* is capable of producing dicotyledonous somatic embryos. Wang *et al*. (2012) described events of somatic embryogenesis from caudexes, pedicels and young leaves of *A. praecox* ssp. *orientalis* cultured on MS media supplemented with Picloram and benzyl aminopurine (BAP), while Yaacob *et al*. (2012) successfully obtained embryogenic callus from *A. praecox* ssp. *minimus* cultures fortified with Picloram. Baskaran and Van Staden (2013) induced formation of shoots from shoot tip cultures of *A. praecox* using BAP, indole-3-acetic acid (IAA) and TDZ. In addition to hormone

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combination, different factors such as explant age and type, genotype and culture conditions also affect callus induction and shoot regeneration. Previous publications mostly reported on regeneration via somatic embryogenesis, while direct organogenesis of liliaceous species, particularly *Agapanthus* was rarely reported. The present study reports in detail, the effect of plant growth regulators particularly IBA and Kinetin on induction of shoots and rhizogenesis from leaf, root and bulb explants of this species, which had not been reported before. It was hypothesized that the addition of Kinetin to the culture media will affect root induction and elongation. Taller and leafy plantlets are also expected to exhibit superior growth after acclimatization.

Materials and Methods

Plant Material and Sterilization of Seeds

The seeds of *A. praecox* ssp. *minimus* were collected from Cameron Highlands, Malaysia and cultured on plant growth regulator-free MS (Murashige and Skoog, 1962) media to produce aseptic seedlings of this species. Seeds were sterilized following standard tissue culture protocols (Taha, 1993) but with minor modifications. Seeds were treated with 100%, 70% and 30% (v/v) commercial bleach (chlorox) for 1 min with each concentration. In a laminar flow chamber, the treated seeds were submerged in 70% (v/v) ethanol and rinsed 3 times with sterile distilled water. During the treatment with 100% (v/v) chlorox, 2 drops of Tween-20 were added to reduce surface tensions and facilitate the sterilization process.

The Effect of Various Hormones on *in vitro* Propagation of *Agapanthus praecox* ssp. *minimus*

Various explants (bulb, 0.5 x 0.5 cm leaf and 0.5 cm root) were excised from 1-month-old aseptic seedlings and cultured on MS media supplemented with various plant hormones, such as indole-3-butyric acid (IBA), 6-furfurylaminopurine (Kinetin), naphthalene acetic acid (NAA), benzyl aminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram) at different concentrations to obtain optimum shoot formation and induction of roots. The MS media was added with 3% sucrose and 2 g L^{-1} Gelrite Gellan, pH was adjusted to 5.6±0.1 and media was autoclaved at 120°C for 20 min. The cultures were maintained at 25±1°C with photoperiod of 16 h of light and 8 h of dark. Light intensity was 1000 lux, while relative humidity was 90-100%.

Acclimatization of *In vitro* Grown *Agapanthus praecox* ssp. *minimus*

Complete plantlets were transferred to flower pots containing various growth substrates (black soil, red soil and 1:1 combination of black/red soil) and covered with transparent plastic bags with small air holes to prevent plant dehydration. The plantlets were kept in the culture room at 25±1°C with photoperiod of 16 h of light and 8 h of dark, for 1 month followed by transfer to a greenhouse. The most suitable growth substrate showing the highest percentage of survival rates of this species was determined. The morphology of the *ex vitro* plants were observed and compared with intact plants. The chlorophyll content was also measured and compared. Following successful acclimatization of the *in vitro* plantlets, leaf segments of both *in vitro* and *in vivo* plants were viewed under Scanning electron microscope (SEM, Jeol JSM-6400) to observe any micro-morphological abnormalities and somaclonal variations that might have occurred as a result of tissue culture.

Statistical Analysis

Randomized complete block design (RCBD) with 30 replicates was employed in designing the experiments and assessment of the results. All data were presented as mean ± SE and analyzed using ANOVA and Duncan's multiple range test (DMRT) at $p < 0.05$.

Results

Organogenesis and Micropropagation

Bulb explants of *A. praecox* ssp. *minimus* managed to produce shoots (Fig. 1) after 7 days of culture and form a complete plantlet after 4 weeks (Table 1). In general, the explants responded as early as 1 week when cultured on various culture media. Callogenesis was observed from root and leaf explants with higher concentrations of IBA (1.5-2.0 mg L^{-1}) in the culture media. Callus was also found to be readily induced from leaf explants than root explants, with more than 80% of leaf explants yielded callus when 1.5-2.0 mg L^{-1} IBA was used in combinations with 1.0-2.0 mg L^{-1} Kinetin (Table 1). Addition of Kinetin alone to the culture media produced shoots without roots. However, when cytokinin (1.0 mg L^{-1} Kinetin) was used in combination with 1.0 mg L^{-1} IBA, root formations were improved.

Bulb was observed to be the most responsive explant type and showed the highest organogenesis potential. MS media with 2.0 mg L^{-1} IBA and 2.0 mg L^{-1} Kinetin yielded highest mean number of shoots per explant (4.50 ± 0.38). The regeneration potential of bulb explants was further studied by supplementing the MS media with 2.0 mg L^{-1} of other plant hormones such as NAA, Picloram, 2,4-D, IBA, Kinetin and BAP (Table 2). Similarly, optimum shoot production was achieved when 2.0 mg L^{-1} IBA and 2.0 mg L^{-1} Kinetin were used, while combination of 2.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP yielded the lowest shoot formation with 2.17 ± 0.28 shoots per explant (Table 2).

Influence of auxin and MS media strength on root induction was investigated further (Table 3). As found previously, the addition of Kinetin to the growth media showed better root formation compared to IBA (Table 1).

Table 1: The effects of different concentrations and combinations of IBA and Kinetin on different explants of *Agapanthus praecox* ssp. *minimus* cultured on MS media after 4 weeks of culture

MS media + hormone (mg L^{-1})	Explant type	Observations	Explants with callus (%)	Explants with shoots (%)	No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)
No hormone	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Necrotic	NR	NR	NR	NR
0.5 IBA	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of roots	NR	NR	NR	$3.10 \pm 0.2_{ab}$
1.0 IBA	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of roots	NR	NR	NR	$3.40 \pm 0.2_{ab}$
1.5 IBA	Leaf	Adventitious roots	NR	NR	NR	$2.86 \pm 0.8_{a}$
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of roots	NR	NR	NR	$3.40 \pm 0.2_{ab}$
2.0 IBA	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of roots	NR	NR	NR	$3.47 \pm 0.2_{b}$
0.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots	NR	$90.00 \pm 5.6_{a}$	$2.76 \pm 0.3_{ab}$	NR
1.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots	NR	$90.00 \pm 5.6_{a}$	$3.13 \pm 0.2_{abcde}$	NR
1.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots	NR	$90.00 \pm 5.6_{a}$	$3.60 \pm 0.3_{bcdefgh}$	NR
2.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots	NR	$96.67 \pm 3.3_{a}$	$3.83 \pm 0.3_{cdefgh}$	NR
0.5 IBA + 0.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of roots	NR	$93.33 \pm 3.3_{a}$	$2.90 \pm 0.2_{abc}$	$3.17 \pm 0.2_{ab}$
0.5 IBA + 1.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$100.00 \pm 0.0_{a}$	$3.03 \pm 0.1_{abcde}$	$2.77 \pm 0.1_{a}$
0.5 IBA + 1.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$100.00 \pm 0.0_{a}$	$3.97 \pm 0.2_{defgh}$	$3.07 \pm 0.2_{ab}$
0.5 IBA + 2.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$90.00 \pm 5.6_{a}$	$4.00 \pm 0.3_{efgh}$	$2.87 \pm 0.2_{ab}$
1.0 IBA + 0.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$90.00 \pm 5.6_{a}$	$2.43 \pm 0.2_{a}$	$3.37 \pm 0.2_{ab}$
1.0 IBA + 1.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$90.00 \pm 5.6_{a}$	$3.97 \pm 0.4_{defgh}$	$4.47 \pm 0.3_{c}$
1.0 IBA + 1.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$100.00 \pm 0.0_{a}$	$3.40 \pm 0.2_{bcdefg}$	$3.17 \pm 0.2_{ab}$
1.0 IBA + 2.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Necrotic	NR	NR	NR	NR
	Bulb	Formation of shoots and roots	NR	$93.33 \pm 4.6_{a}$	$3.53 \pm 0.3_{bcdefgh}$	$3.17 \pm 0.2_{ab}$
1.5 IBA + 0.5 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Explant became swollen	NR	NR	NR	NR
	Bulb	Formation of roots and leaves	NR	$96.67 \pm 3.3_{a}$	$3.00 \pm 0.2_{abcd}$	$3.03 \pm 0.2_{ab}$
1.5 IBA + 1.0 Kinetin	Leaf	Necrotic	NR	NR	NR	NR
	Root	Creamy callus formed at the edges	$16.67 \pm 6.9_{a}$	NR	NR	NR
	Bulb	Multiple shoots with formation of roots	NR	$96.67 \pm 3.3_{a}$	$3.87 \pm 0.3_{cdefgh}$	$3.40 \pm 0.2_{ab}$
1.5 IBA + 1.5 Kinetin	Leaf	Creamy white callus	$90.00 \pm .6_{ab}$	NR	NR	NR
	Root	Creamy friable callus	$16.67 \pm 6.9_{a}$	NR	NR	NR
	Bulb	Multiple shoots with formation of roots	NR	$96.67 \pm 3.3_{a}$	$4.17 \pm 0.3_{fgh}$	$3.33 \pm 0.2_{ab}$
1.5 IBA + 2.0 Kinetin	Leaf	Creamy white callus	$100.00 \pm .0_{b}$	NR	NR	NR
	Root	Creamy friable callus	$30.00 \pm 8.5_{a}$	NR	NR	NR
	Bulb	Multiple shoots with formation of roots	NR	$86.67 \pm 6.3_{a}$	$4.23 \pm 0.4_{gh}$	$3.20 \pm 0.2_{ab}$

Table 1: Continued

Table 1: Continued

MS media + hormone (mg L^{-1})	Explant type	Observations	Explants with callus (%)	Explants with shoots (%)	No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)
2.0 IBA + 0.5 Kinetin	Leaf	The edges became swollen	NR	NR	NR	NR
	Root	Creamy white friable callus	$26.67 \pm 8.2_{a}$	NR	NR	NR
	Bulb	Formation of roots and leaves	NR	$100.00 \pm 0.0_{a}$	$3.23 \pm 0.3_{abcdef}$	$3.47 \pm 0.2_{b}$
2.0 IBA + 1.0 Kinetin	Leaf	Creamy white friable callus	$100.00 \pm .0_{b}$	NR	NR	NR
	Root	Creamy white friable callus	$96.67 \pm 3.3_{b}$	NR	NR	NR
	Bulb	Formation of roots and leaves	NR	$96.67 \pm 3.3_{a}$	$4.23 \pm 0.3_{gh}$	$3.37 \pm 0.2_{ab}$
2.0 IBA + 1.5 Kinetin	Leaf	Creamy white friable callus	$96.67 \pm 3.3_{b}$	NR	NR	NR
	Root	Creamy white friable callus	$26.67 \pm 8.2_{a}$	NR	NR	NR
	Bulb	Formation of roots and leaves	NR	$93.33 \pm 4.6_{a}$	$4.33 \pm 0.4_{gh}$	$3.33 \pm 0.2_{ab}$
2.0 IBA + 2.0 Kinetin	Leaf	Creamy white friable callus	$83.33 \pm 6.9_{a}$	NR	NR	NR
	Root	Creamy white friable callus	$100.00 \pm .0_{b}$	NR	NR	NR
	Bulb	Formation of roots and leaves	NR	$90.00 \pm 5.6_{a}$	$4.50 \pm 0.4_{h}$	$3.27 \pm 0.3_{ab}$

Mean values with different letters within a column (between the same explant) are significantly different at $p < 0.05$. IBA = indole-3-butyric acid; Kinetin = 6-furfurylaminopurine; NR = no response

Table 2: The effects of auxins (NAA, Picloram, 2,4-D and IBA) and cytokinins (Kinetin and BAP) on bulb explants cultured on MS media after 4 weeks of culture

MS media + hormone (mg L^{-1})	Observations	Explants with shoots (%)	No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)
2.0 NAA + 2.0 BAP	Formation of roots and leaves	$83.33 \pm 6.92_{a}$	$2.87 \pm 0.32_{a}$	$2.50 \pm 0.27_{ab}$
2.0 NAA + 2.0 Kinetin	Formation of roots and leaves	$73.33 \pm 8.21_{a}$	$2.53 \pm 0.36_{a}$	$2.73 \pm 0.24_{ab}$
2.0 PIC + 2.0 BAP	Formation of roots and leaves	$76.67 \pm 2.85_{a}$	$2.67 \pm 0.38_{a}$	$2.83 \pm 0.25_{ab}$
2.0 PIC + 2.0 Kinetin	Formation of roots and leaves	$83.33 \pm 6.92_{a}$	$2.43 \pm 0.29_{a}$	$2.87 \pm 0.29_{ab}$
2.0 2,4-D + 2.0 BAP	Formation of roots and leaves	$86.67 \pm 6.31_{a}$	$3.03 \pm 0.33_{a}$	$2.60 \pm 0.21_{ab}$
2.0 2,4-D + 2.0 Kinetin	Formation of roots and leaves	$83.33 \pm 6.92_{a}$	$2.43 \pm 0.25_{a}$	$2.40 \pm 0.22_{a}$
2.0 IBA + 2.0 BAP	Formation of roots and leaves	$76.67 \pm 7.85_{a}$	$2.17 \pm 0.28_{a}$	$2.37 \pm 0.19_{a}$
2.0 IBA + 2.0 Kinetin	Formation of roots and leaves	$90.00 \pm 5.57_{a}$	$4.50 \pm 0.38_{b}$	$3.27 \pm 0.25_{b}$

Mean values with different letters within a column (between the same explant) are significantly different at $p < 0.05$. NAA = naphthalene acetic acid; BAP = benzyl aminopurine; Kinetin = 6-furfurylaminopurine; PIC = 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram); 2,4-D = 2,4-dichlorophenoxyacetic acid; IBA = indole-3-butyric acid

Analysis of results showed that all media supplemented with hormones were able to induce rooting, while MS basal media (devoid of hormones) showed no development of roots. MS media fortified with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} Kinetin was the most optimum media for induction of roots from bulb explants (Fig. 2), with mean number of 4.47±0.30 roots per explant (Table 3). The lowest root production was observed when bulb explants were cultured on half strength MS media supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kinetin, with mean number of 1.80±0.32 roots per explant (Table 3).

Acclimatization and Morphology of *Agapanthus praecox* ssp. *minimus* Plantlets

In vitro plantlets obtained from different regeneration pathways such as from direct regeneration and somatic embryogenesis were acclimatized (Fig. 3) as previously described and their growth was monitored and compared to intact plants (control). It was observed that the acclimatized plantlets obtained through direct regeneration showed more number of leaves and were significantly taller than that derived from somatic embryos (Table 4), although inferior than intact plants (control). However, the leaf sizes of both *in vitro* plantlets were not significantly different (Table 4).

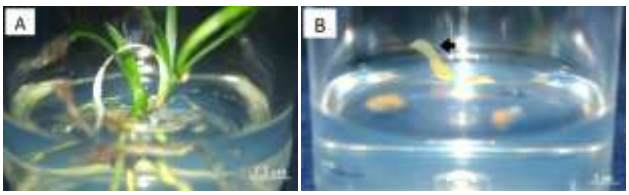

Fig. 1: Development of multiple shoots from bulb explant cultured on MS media supplemented with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} Kinetin (A) and root organogenesis from leaf explant cultured on MS media supplemented with 1.5 mg L^{-1} IBA (B)

Fig. 2: Formation of roots from bulb explant cultured on MS media supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP (A), formation of roots from bulb explant cultured on MS media supplemented with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} Kinetin (B) and formation of roots from bulb explant cultured on MS media supplemented with 2.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP (C)

Table 3: Development of roots from bulb explants of *A. praecox* ssp. *minimus* when cultured in rooting media after 4 weeks

MS Strength (with or without hormones, mg L^{-1})	Explants with roots (%)	No. of roots per explants (mean ± SE)
MS	NR	NR
½ MS	NR	NR
MS + 1.0 IBA + 1.0 Kinetin	$100.00 \pm 0.00_{b}$	$4.47 \pm 0.30_{c}$
MS + 2.0 IBA + 2.0 Kinetin	$100.00 \pm 0.00_{b}$	$3.27 \pm 0.25_{b}$
½ MS + 1.0 IBA + 1.0 Kinetin	$80.00 \pm 7.43_{a}$	$2.50 \pm 0.44_{ab}$
½ MS + 2.0 IBA + 2.0 Kinetin	$80.00 \pm 7.43_{a}$	$2.27 \pm 0.47_{ab}$
MS + 1.0 NAA + 1.0 Kinetin	$76.67 \pm 7.85_{a}$	$2.40 \pm 0.47_{ab}$
MS + 2.0 NAA + 2.0 Kinetin	$100.00 \pm 0.00_{b}$	$2.73 \pm 0.24_{ab}$
½ MS + 1.0 NAA + 1.0 Kinetin	$80.00 \pm 7.43_{a}$	$2.12 \pm 0.36_{ab}$
½ MS + 2.0 NAA + 2.0 Kinetin	$83.33 \pm 6.92_{ab}$	$1.80 \pm 0.32_{a}$

Mean values with different letters within a column are significantly different at $p < 0.05$. IBA = indole-3-butyric acid; Kinetin = 6-furfurylaminopurine; NAA = naphthalene acetic acid; NR = no response

Table 4: Morphology of *in vitro* plantlets after 3 months of acclimatization on peat soil compared to intact plants of similar age

Plantlet category	Plant height (mm)			No. of leaves			Leaf length (mm)		
	Day 0	After 30 days	After 90 days	Day 0	After 30 days	After 90 days	Day 0	After 30 days	After 90 days
Plantlets derived from somatic embryos	$52.10 \pm 2.26_{a}$	$62.10 \pm 1.73_{a}$	$67.10 \pm 1.99_{a}$	$2.50 \pm 0.31_{a}$	$4.20 \pm 0.29_{a}$	$5.70 \pm 0.34_{a}$	$4.40 \pm 0.31_{ab}$	$20.70 \pm 0.75_{a}$	$40.30 \pm 1.18_{a}$
Plantlets derived from direct regeneration	$75.60 \pm 2.01_{b}$	$89.70 \pm 2.50_{b}$	$96.20 \pm 2.34_{b}$	$6.70 \pm 0.42_{b}$	$8.80 \pm 0.49_{b}$	$10.40 \pm 0.50_{b}$	$4.70 \pm 0.37_{b}$	$43.60 \pm 2.86_{b}$	$42.30 \pm 0.87_{a}$
Intact plants (control)	$142.00 \pm 2.61_{c}$	$187.80 \pm 5.20_{c}$	$199.40 \pm 4.53_{c}$	$13.70 \pm 0.79_{c}$	$15.00 \pm 0.75_{c}$	$16.30 \pm 0.83_{c}$	$8.10 \pm 0.39_{c}$	$52.30 \pm 2.12_{c}$	$56.90 \pm 2.16_{b}$

Mean values with different letters within a column are significantly different at $p < 0.05$

Nevertheless, intact plants of similar age (control) showed superior results compared to that of *in vitro* plantlets, in all categories being compared. The prospect of mass propagation of this species through tissue culture had now became more promising as it was shown that the *in vitro* regenerated plantlets can be successfully acclimatized with high survival rates (Table 5), although they exhibit less superior growth performance compared to intact plants (control). However, it was expected that the growth performance of the acclimatized *in vitro* plantlets would be significantly better with time, as plants originated from tissue culture would require some time for hardening process and fully adapt to normal environment, thus becoming more competent.

Furthermore, it was also found that initial plantlet morphology prior to acclimatization played an important role in subsequent growth performance after field transfer. Results indicated that plantlets that were taller on day 0 exhibited the greatest increase in plant height after 3 months. Plantlets with more number of leaves prior to acclimatization also showed the highest number of leaves after 3 months, such as plantlets derived from direct regeneration showed 10.40 leaves compared to plantlets derived from somatic embryos (with only 5.70 leaves) (Table 4).

The suitability of various growth substrates on acclimatization of *A. praecox* ssp. *minimus* plantlets was also compared (Table 5). It was found that 1:1 ratio of black/red soil remained most suitable and resulted in significantly highest survival rates of acclimatized plantlets

Table 5: Survival rates of acclimatized plantlets grown on different types of growth substrates

Type of growth media	Survival rate (%)
Black soil	$86.67 \pm 6.31_{b}$
Red soil	$73.33 \pm 8.21_{a}$
Black soil + red soil (1:1 ratio)	$96.67 \pm 3.33_{c}$

Mean values with different letters within a column are significantly different at $p < 0.05$

(96.67%), followed by black soil (86.67%) and red soil (73.33%). Soil analysis conducted on these growth substrates via X-ray Diffractometer (XRD) revealed that both soil types contain different components or phases, whereby red soil consists of 25 phases with 5 major compounds, while black soil contain only 4 phases with 3 major compounds (Table 6).

Growth performance of the plantlets after field transfer was also evaluated in terms of the chlorophyll content of *in vivo*, *in vitro* and *ex vitro* plants (Fig. 4). It was found that *in vivo* plants showed higher SPAD reading (38.7±5.3 SPAD), compared to *in vitro* (13.3±0.7 SPAD) and *ex vitro* (21.9±3.8 SPAD) plants (Fig. 4). Furthermore, the morphology (macro and micro) of the plantlets was also observed and compared with that of *in vivo* plants, to elucidate any occurrences of morphological abnormalities indicative of somaclonal variations. No distinct morphological irregularities were observed on *in vitro* plantlets, compared to *in vivo* grown plants. However, the size of the leaves (leaf length), plant height

Table 6: Major compounds in red and black soil, identified via X-ray diffractometer (XRD)

Soil type	Reference code	Score	Compound name	Chemical formula
Red soil	98-015-4289	64	Quartz	SiO_2
	98-008-1367	25	Potassium Manganese Oxide Hydrate	$H.8K.27MnO_2$
	98-007-1805	0	Zeolite Rho (Sr-, (NH4)-exchanged, dehydrated)	$H_6.8Al_{12}Cs.N.7O_{96}Si_{36}\ Sr_4$
	98-006-2018	20	Hydrogen	H_2
	98-003-3996	18	Cobalt Dicopper Oxide	$CoCu_2O_3$
Black soil	98-004-3035	37	Cadmium Indium Selenide - Superstructure	$Cd_2In_2Se_5$
	98-064-2216	19	Lithium Oxide	Li_2O
	98-006-5410	16	Titanium (II) Fluoride	F_2Ti

and number of leaves varied depending on the regeneration pathways (Table 4). Nevertheless, no somaclonal variation was observed among the *in vitro* plantlets, for example the leaves looked morphologically similar despite the differences in size (leaf length).

Scanning electron microscopy (SEM) revealed the surface structures of the *in vivo* and *in vitro* samples in detail. It was found that the surface of *in vivo* grown leaf was more rigid and the outline of the epidermal cells (cell walls) looked more defined than leaf of *in vitro* plantlet, as can be seen from the adaxial surfaces of the leaf samples (Fig. 5). *In vivo* grown leaf was also observed to appear very hairy with a lot of hair structures compared to the absence of hair structures on *in vitro* grown leaf (Fig. 5). However, both *in vivo* and *in vitro* grown leaves had similar stoma size (32.86 μm) and shape (anomocytic) (Fig. 5), whereby the guard cells of the stomata were not surrounded by any subsidiary cells. The cross-sections of both *in vivo* and *in vitro* grown *A. praecox* ssp. *minimus* leaf samples were also viewed through SEM, where both *in vivo* and *in vitro* grown leaves had spongy mesophyll that is interspersed with air spaces (Fig. 5).

Discussion

Previously, micropropagation of Liliaceous plant species largely utilized the flowering organs as the explant choice (Dunstan and Short, 1978; Wilmink *et al*., 1995). In the present study, various plant organs such as leaf, root and bulb were used. Results indicated that bulb explant was the most responsive and had higher organogenesis potential than leaf and root explants. Parallel to our finding, bulb has since become the explant choice in tissue culture of many Liliaceous species such as *Lilium philippinensis* (Zamora and Gruezo, 1999), Asiatic hybrid 'Orange Pixie' (Misra and Datta, 2001), *Lilium ledebourii* (Azadi and Khui, 2007) and *Lilium regale* (Saifullah *et al*., 2010).

In the present study, we observed that high concentration of auxin combined with cytokinin yielded callogenesis from leaf and root explants of *A. praecox* ssp. *minimus*. Addition of either auxin or cytokinin alone did not produce callus, but resulted in either formation of roots or shoots. Contrary to our results, a number of studies have shown that auxin such as IBA aided callus induction (Qin *et al.*, 2006; Soomro and Memon, 2007). In the present investigation, no callus induction was observed in cultures supplemented with IBA alone.

Fig. 3: Two-month-old *in vitro* plantlets growing on peat soil and covered with plastic (with small holes) for acclimatization process in the culture room (A) and five-month-old *Agapanthus praecox* plantlet after 90 days of acclimatization (B)

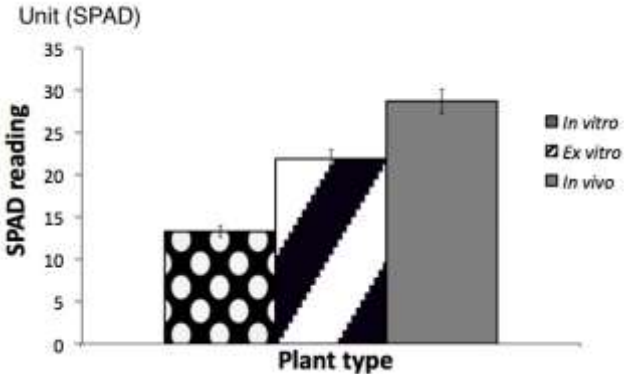

Fig. 4: Comparison of chlorophyll content between the *in vivo*, *in vitro* and *ex vitro* plants

Furthermore, interaction of auxins and cytokinins played a vital role in cell division, growth, development, differentiation and formation of plant organs (Shrivastava and Banerjee, 2008; Purkayastha *et al.*, 2010). It was observed that 1:1 ratio of auxin to cytokinin aided callogenesis from root explants of *A. praecox* ssp. *minimus*, whereby addition of 2.0 mg L^{-1} IBA and 2.0 mg L^{-1} Kinetin yielded 100% callus formation.

Addition of IBA alone resulted in induction of roots from bulb explants of *A. praecox* ssp. *minimus*. Similarly, IBA was found to promote root formation in Chinese jujube, *Ziziphus jujuba* Mill (Zhou and Liu, 2009). In contrast, IBA failed to induce root formation in *L. texensis* (Upadhyaya *et al*., 1992). The present study also investigated

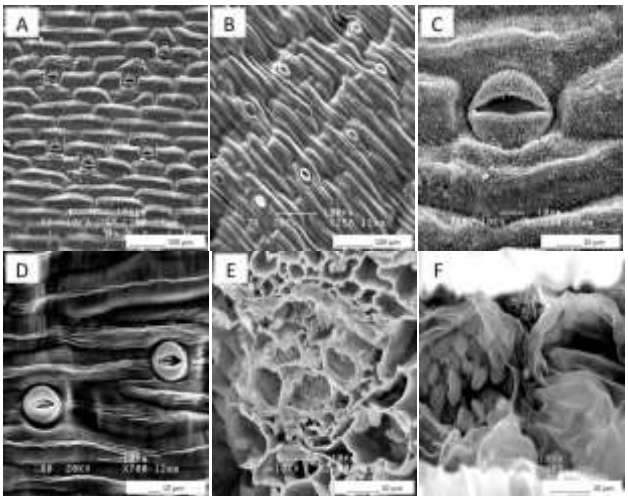

Fig. 5: Scanning electron micrographs (SEM) of *A. praecox* ssp. *minimus* leaf segments; adaxial surface of *in vivo* grown leaf (A), adaxial surface of *in vitro* grown leaf (B), stoma on *in vivo* grown leaf (C), stomata on *in vitro* grown leaf (D), cross-section of *in vivo* grown leaf showing spongy mesophyll (E) and cross-section of *in vitro* grown leaf showing a palisade cell containing chloroplasts (F)

the effects of Kinetin on root induction from *A. praecox* ssp. *minimus* explants. Kinetin was known to yield inhibitory effects when used in tissue culture. For example, Kinetin and IAA retards the growth of lateral roots in *Secale cereale* var. Petkus grown *in vitro* (Yang and Dodson, 1970). Kaminek (1967) also reported complete inhibition of root formation in pea stem sections caused by Kinetin, Ethionine and Chloramphenicol. Moreover, Kinetin also induced cell death in root cortex cells of seedlings of *Vicia faba* ssp. *minor* (Kunikowska *et al*., 2013). However, analysis of results showed that more roots were produced with addition of Kinetin to the media, for example cultures fortified with 1.0 mg L^{-1} IBA and 1.0 mg L^{-1} Kinetin produced the most number of roots after 4 weeks. Similarly, 1.0 mg L^{-1} and 2.0 mg L^{-1} Kinetin was most optimum for rooting and shoot formation of *Matthiola incana* (Hesar *et al*., 2011). Addition of Kinetin to an auxin also promoted callus growth and subsequent indirect rhizogenesis from stem explants of *Dioscoreophyllum cumminsii* (Oselebe and Ene-Obong, 2007). However, Kinetin applied alone will inhibit callus formation and growth of *D. cumminsii* (Oselebe and Ene-Obong, 2007). The present study also report similar findings, where addition of Kinetin alone in *A. praecox* ssp. *minimus* cultures did not result in callus or root formations.

In the present study, it was found that plantlets with more leaves prior to acclimatization showed the most number of leaves after 3 months in the field. However, as reported by Seelye *et al*. (2003) and Huylenbroeck *et al*. (1998), although *in vitro* grown leaves were photosynthetically competent, leaves with higher photosynthetic activity would be formed after acclimatization to replace the *in vitro* grown leaves. These newly-formed leaves, however could still possess less photosynthetic capacity than that of *in vivo* plants (Carvalho *et al*., 2001). It was also found that taller *in vitro A. praecox* ssp. *minimus* plantlets exhibited the greatest increase in plant height after 3 months in the field. Mohammed and Vidaver (1990) also reported similar findings, where it was shown that taller *P. menziesii* plantlets survived better when acclimatized *ex vitro*. *P. menziesii* with an initial shoot height of 41-60 mm showed 53% survival rate, double than that of shorter plantlets.

The present investigation revealed that 1:1 mixture of black/red soil was the most suitable substrate for acclimatization of *in vitro* grown *A. praecox* ssp. *minimus.* The key component in red soil was quartz which consists of a framework of tetrahedra that are bound to other sets of tetrahedra, rendering it to be very large in size and cannot be tightly packed together (Troeh and Thompson, 2005). Quartz contains water molecules or other impurities within the structure and soils containing quartz are also generally acidic (Troeh and Thompson, 2005). This explained the significantly high survival rates of acclimatized plantlets grown on 1:1 combination of red/black soil, as cultivation of *Agapanthus* sp. requires well-drained sandy soil that is slightly acidic (Duncan, 1998). However, different results were reported for other species, for example the African violet, *Saintpaulia ionantha* yielded 95% survival rate when acclimatized on 100% sand compared to only 80%, 70% and 65% survival rates when acclimatized on 1:1 mixture of sand : farmyard manure, 1:1 mixture of sand : coconut husk and 1:1 mixture of sand: ground charcoal, respectively (Khan *et al*., 2007). This shows that acclimatization protocols or treatments such as type of growth substrate should be specifically tailored to meet the needs of different species.

In crux, direct organogenesis was successfully induced from bulb explants of *Agapanthus praecox* ssp. *minimus* through addition of IBA and Kinetin to the growth media. Kinetin was found to promote root growth in *A. praecox* ssp. *minimus* and addition of 1.0 mg L^{-1} of IBA and Kinetin to MS media was optimum for induction of roots. Complete true-to-type plantlets were formed after as early as 4 weeks of culture and were successfully acclimatized in 1:1 mixture of black/red soil with high survival rates (96.67%).

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