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In vitro Regeneration and Acclimatization Protocols of Selected Ornamental Plants (*Agapanthus praecox*, *Justicia betonica* and *Celosia cristata*)

(Protokol Regenerasi Secara *In vitro* dan Aklimatasi Tumbuhan Hiasan Terpilih (*Agapanthus praecox*, *Justicia betonica* dan *Celosia cristata*))

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ABSTRACT

This paper discussed on the effectiveness of BAP and NAA growth hormones on establishment of plant regeneration for selected ornamentals; Agapanthus praecox, Justicia betonica *and* Celosia cristata. *Various explants (leaf, stem, shoot tip and bulb) derived from one-month-old aseptic seedlings of* A. praecox *and* C. cristata*, as well as explants from intact plants of* J. betonica *were utilized to achieve complete plant regeneration of these species. MS medium supplemented with various hormones, with an emphasis on BAP and NAA was tested to obtain direct and indirect regeneration. Both* A. praecox *(bulbs) and* C. cristata *(shoots) formed complete plantlets on MS added with 0.5-2.0 mg/L BAP and NAA, while direct regeneration was achieved for* J. betonica *on MS media containing BAP. Several methods were attempted to acclimatize the regenerants, with* A. praecox *gave the highest percentage of survival rates (96.67%), followed by* J. betonica *(80.00%) and* C. cristata *(75.00%).*

Keywords: Acclimatization; Agapanthus praecox*;* Celosia cristata*;* Justicia betonica*; plant growth regulators*

ABSTRAK

Kertas ini menerangkan keberkesanan hormon pertumbuhan BAP dan NAA terhadap regenerasi tumbuhan hiasan terpilih; Agapanthus praecox, Justicia betonica *dan* Celosia cristata. *Pelbagai eksplan (daun, batang, hujung pucuk dan ubi) yang berasal daripada anak benih aseptik* A. praecox *dan* C. cristata *yang berusia satu bulan, serta eksplan daripada tumbuhan utuh* J. betonica *telah digunakan untuk menperoleh regenerasi lengkap spesies ini. Media MS yang dibekalkan dengan pelbagai hormon, dengan penekanan kepada BAP dan NAA telah diuji untuk mendapatkan regenerasi secara langsung atau tidak langsung. Kedua-dua* A. praecox *(ubi) dan* C. cristata *(pucuk) membentuk anak pokok lengkap di dalam media MS yang ditambah dengan 0.5-2.0 mg/L BAP dan NAA, manakala regenerasi secara langsung telah dicapai oleh* J. betonica *di dalam media MS yang mengandungi BAP. Beberapa kaedah telah dicuba dalam pengikliman anak pokok, dengan* A. praecox *memberikan peratusan kadar kelangsungan hidup tertinggi (96.67%), diikuti oleh* J. betonica *(80.00%) dan* C. cristata *(75.00%).*

Kata kunci: Agapanthus praecox*;* Celosia cristata; *hormon pertumbuhan;* Justicia betonica*; pengikliman*

INTRODUCTION

Plant propagation through *in vitro* methods is mainly aimed to produce plants of superior qualities, free from microorganisms and with high multiplication rates. A number of reports dealing with this aspect of tissue culture has been published for many species such as *Asplenium nidus* (Higuchi & Amaki 1989), *Petunia hybrida* (Dimasi-Theriou 1993), *Ananas comosus* (Debergh & Be 2006; Hamad & Taha 2008), *Lilium regale* (Saifullah et al. 2010) and *Platycerium coronarium* (Taha et al. 2011). Another important point to consider when dealing with *in vitro* work is the changes that occur when cells are transferred from *in vivo* environment to *in vitro* environment. The changes in cellular parameters such as ploidy level, Mitotic Index (MI), mean cell and nuclear areas can be associated with regeneration potentials (Taha & Francis 1990). Reports on association of somaclonal variations and *in vitro* flowering as a result of tissue culture with cytogenetic changes has been published for several species including *Trifolium alexandrium* (Masoud & Hamta 2008) and *Celosia cristata* (Taha & Wafa 2012). For example, different cellular characteristics were detected in embryogenic and non-embryogenic callus tissues of sugar beet (Moghaddam & Taha 2005).

Since the introduction of tissue culture technique, it has contributed significantly in various fields, such as agriculture, forestry, horticulture and medicinal. Successful plant regeneration from small pieces of plant tissues and organs, either through micropropagation or somatic embryogenesis indicates that almost any plant can respond to tissue culture/*in vitro* protocols, as exemplified by many species such as *Gerbera jamesonii* (Hasbullah et al. 2011), cauliflower (Siong et al. 2011) as well as fern species (Aspiras 2010; Taha et al. 2011).

However, the prospect of *in vitro* plant regeneration will not be fully utilized if the procedure for acclimatization is neglected.

Among the popular ornamental plants, establishment of high frequency of regeneration of those species was widely reported, while ornamental plants such as *Agapanthus praecox*, *Justicia betonica* and *C. cristata* may not yet gain popularity and commercial values equivalent to roses, carnation and orchids. However, exploitation and manipulation of these species promise great potentials. *A. praecox* plants for instance, are used by native South African tribes to treat pregnancy-related illness and prolonged labor (Varga & Veale 1997) and *A. praecox* was reported to contain anthocyanin pigments with potential as commercial organic colourant (Yaacob et al. 2011). On the other hand, *Justicia betonica* has for a long time been used for slope planting, as well as to treat amenorrhoea, rheumatism and scaly skin (Burkill 1985) while *C. cristata* is a popular plant for landscaping and as roadside plants. This paper compared micropopagation of three selected ornamental species (*A. praecox*, *J. betonica* and *C. cristata*) as possible commercializable roadside or ornamental plants. The species showing faster and more efficient growth *in vitro* with high viability after field transfer was identified.

Protocols for acclimatization of these three species has been successfully developed by adapting the field transfer method as reported for *Solanum melongena* (Taha & Tijan 2002), but with several minor modifications. The present work not only described the establishment of *in vitro* regeneration of the three species from various explants, but also successful acclimatization methods and suitable substrates for field transfer of these species.

Materials and Methods

Explant preparation and culture conditions

Seeds of *C. cristata* were obtained from nurseries around Kuala Lumpur, Malaysia and those of *A. praecox* ssp. *minimus* collected from Cameron Highlands, Malaysia were sterilized in the usual manner and standard tissue culture protocols were followed (Mohamed & Taha 2011; Taha 1997). One-month-old aseptic seedlings were used as explant sources for *in vitro* culture of these species, while explants for *J. betonica* were obtained from intact plants. Leaves (0.5 × 0.5 cm), stems (0.5 cm), shoot tips (1 cm) and young bulbs were used to initiate cultures of these species. MS medium supplemented with various concentrations and combinations of BAP and NAA was used, with 30 replicates in each experiment. Cultures were maintained at 25±1ºC with 16 h light and 8 h dark.

Acclimatization of regenerants

The regenerants were acclimatized following methods described by Taha and Tijan (2002). Briefly, the regenerated plantlets were transferred onto enclosed containers with small air holes containing various types of garden soil and maintained in the culture room for one month. Then, the plantlets were transferred to the green house for one month and subsequently to the field with no shades to ensure the plantlets can successfully adapt to the natural environment. The growth performance of the *ex vitro* plantlets in the natural environment was monitored. The morphological features of the acclimatized regenerants were also compared to detect any irregularities and somaclonal variations that might occur due to tissue culture stress. The shape of flowers, leaves, plant height and root morphology of each acclimatized regenerants (subject to their species) were also compared.

Statistical analysis

Data analysis and assessment of results were conducted based on randomized complete block design (RCBD) with 30 replicates. Statistical variance test (ANOVA) and Duncan's multiple range test (DMRT) with least significant differences at 5% level were employed.

Results and Discussion

Both *A. praecox* and *C. cristata* were observed to respond readily to plant growth hormones, whereby it was found that both species started to exhibit callus growth after one to two weeks of culture (Tables 1 and 2). Both leaf and root explants of *A. praecox* could produce friable creamy callus on MS medium supplemented with equal concentrations of BAP and NAA (0.5-2.0 mg/L), while bulb explants exhibited direct regeneration and form a complete plantlet after two to four weeks (Table 1). However, *J. betonica* was the most difficult to regenerate *in vitro*, where it was observed that direct regeneration of this species occurred as early as 5 weeks and high auxin concentrations (1.0 to 2.0 mg/L NAA) were found to inhibit callus growth in all explant types (Table 3).

The production of coloured callus was only obtained from *C. cristata*, whereby it was observed that leaf, stem and root explants of this species could produce red and orange callus when cultured on MS medium containing only BAP (Table 2). In agreement with the findings by Taha and Wafa (2012), purplish red callus was also obtained from leaf, stem and root explants of *C. cristata* cultured on media containing 0.5-2.0 mg/L BAP and 0.5 mg/L NAA (Table 2). In the present work, direct regeneration of both *J. betonica* and *C. cristata* was obtained from *in vitro* cultures of shoot explants, while direct regeneration of *A. praecox* was obtained through *in vitro* cultures of bulb explants. The responses of various explants to different concentrations of hormones are documented in Tables 1-3.

Generally, formation of multiple shoots in *C. cristata* was observed after as early as twenty days in culture on MS fortified with BAP and NAA (Table 2), but not on MS added with other hormones e.g. Zeatin, IAA and Kinetin (data not shown). Regeneration of plantlets with abnormal stems was also observed in *C. cristata* when grown on

TABLE 1. The effects of NAA and BAP at different concentrations on various explants of *A. praecox* after 4 months, cultured at 25 +1°C under 16 h light and 8 h dark

Hormone concentration (mg/L)		Explant type	Callus (%)	Colour of callus	Direct regeneration (%)	Multiple shoot (%)	Observations
NAA	BAP						
-	-	Leaf	NR	-	NR	NR	Necrosis
		Root	NR	-	NR	NR	Necrosis
		Bulb	NR	-	NR	NR	Necrosis
0.5	0.5	Leaf	NR	-	NR	NR	Cream callus
		Root	93.3 ± 1.3[a]	C/W	NR	NR	Cream callus
		Bulb	NR	-	95.3 ± 3.3[b]	NR	Direct regeneration after 3 weeks
1.0	1.0	Leaf	100.0 ± 0.0[b]	C/W	NR	NR	Cream callus
		Root	100.0 ± 0.0[b]	C/W	NR	NR	Cream callus
		Bulb	NR	-	100.0 ± 0.0[c]	NR	Direct regeneration after 3 weeks
1.5	1.5	Leaf	96.7 ± 1.3[a]	C/W	NR	NR	Cream callus
		Root	93.3 ± 3.7[a]	C/W	NR	NR	Cream callus
		Bulb	NR	-	100.0 ± 0.0[c]	NR	Direct regeneration after 2 weeks
2.0	2.0	Leaf	100.0 ± 0.0[b]	C/W	NR	NR	Cream callus
		Root	100.0 ± 0.0[b]	C/W	NR	NR	Cream callus
		Bulb	NR	-	83.3 ± 1.2[a]	NR	Direct regeneration after 2 weeks

Mean values within a column (between the same explant) are significantly different at $p \leq 0.05$
Percentage (%): number of explants producing callus, direct regeneration or multiple shoots
Colour of callus: (C) cream, (W) white

MS supplemented with only BAP (0.5-2.0 mg/L), parallel to the findings by Taha and Wafa (2012). Moreover, formation of multiple shoots in *A. praecox* only occurred on MS fortified with 1.5 mg/L IBA and 1.0-2.0 mg/L Kinetin (data not shown), however combinations of BAP and NAA resulted in regeneration of complete plantlets as early as 2 to 4 weeks of culture (Table 1). The formation of multiple shoots in *J. botanica* only occurred in MS fortified with combinations of high concentrations of auxin with low concentrations of cytokinin (1.5 mg/L NAA and 0.5 and 1.5 mg/L BAP as well as in MS fortified with 2.0 mg/L NAA and 0.5 mg/L BAP).

All *in vitro* grown *A. praecox, C. cristata* and *J. betonica* plantlets were acclimatized based on the methods described by Taha and Tijan (2002). The plantlets were transferred to several growth substrates such as black (peat) soil, red soil or sand and the best growth substrate for each species was identified (Table 4). It was very important to note that the acclimatization methods following *in vitro* cultures were essential to maximize the quantity or amount of survived plantlets, which is an important aspect for the purpose of commercialization. Tissue culture techniques promised a higher yield and rapid reproduction of clonal plants (with superior qualities), thus optimum survival rates of the plantlets following field transfer is important to maximize profit and minimize wastage. For example, *in vitro* grown *C. cristata* plantlets failed to survive following their transfer to soil, even in culture room conditions (at a temperature of 23-26°C). However, a significant increase of survival rates were recorded when the acclimatized *C. cristata* plantlets were covered in clear plastic bags (with holes) for 2 weeks in the culture room, prior to field transfer to the greenhouse.

This indicates that *in vitro* plantlets must be kept in a 'familiar' environment (covered) before being completely transferred to normal environmental conditions to minimize the physiological stress, therefore ensuring a better survival. Other than that, it is also very important to note that each species may require a different growth substrate for a better survival. For example, *A. praecox* plantlets were found to survive better in 1:1 ratio of black soil: red soil than in only black or red soil (data not shown). The acclimatization methods and suitable growth substrates for *A. praecox, C. cristata* and *J. betonica* are depicted in Table 4. The morphology of the *ex vitro* plantlets were compared and it was found that no morphological abnormalities were observed. For example, the shape of leaf and root of acclimatized *A. praecox* plantlets appeared similar to *A. praecox* plants grown *in vivo*. Flowers of *C. cristata* were also observed to be morphologically similar to flowers of *in vivo* grown *C. cristata*, despite the small size. The formation of callus to complete *in vitro* regeneration and acclimatization of *A. praecox*, *C. cristata* and *J. betonica* plantlets is shown in Figures 1-3.

Among the hormones usually used for culturing explants, BAP and NAA seems to have desirable effects on many plant species. In fact, the effects of BAP and NAA on tissue culture of various species have been widely reported. For instance, Mukhri and Yamaguchi (1986) reported that BAP had positive effects on formation of

TABLE 2. The effects of NAA and BAP at different concentrations on various explants of *C. cristata* after 4 weeks, cultured at 25 +1°C under 16 h light and 8 h dark

[Hormone] (mg/L)		Explant type	Callus (%)	Colour of callus	DR (%)	MS (%)	Explant type	Callus (%)	Colour of callus	DR (%)	MS (%)
NAA	BAP										
-	-	Stem	NR	-	NR	NR	Root	NR	-	NR	NR
		Leaf	NR	-	NR	NR	Shoot	NR	-	15.7 ± 3.5^{a}	75.3 ± 4.9^{hi}
0.5	-	Stem	40.0 ± 0.6^{c}	C	NR	NR	Root	NR	-	NR	NR
		Leaf	30.7 ± 2.3^{b}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	90.3 ± 1.4^{j}
1.0	-	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	9.7 ± 1.5^{ab}	C	NR	NR
		Leaf	70.0 ± 2.9^{f}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	50.0 ± 2.8^{f}
1.5	-	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	52.3 ± 6.5^{ef}	C	NR	NR
		Leaf	80.0 ± 0.6^{g}	C	NR	NR	Shoot	NR	-	90.6 ± 2.6^{cd}	50.0 ± 2.9^{f}
2.0	-	Stem	90.0 ± 2.9^{h}	W/C	NR	NR	Root	86.0 ± 2.1^{g}	C	NR	NR
		Leaf	55.0 ± 2.9^{de}	C	NR	NR	Shoot	NR	-	85.3 ± 3.2^{c}	15.2 ± 0.7^{ab}
-	0.5	Stem	100.0 ± 0.0^{i}	O	NR	NR	Root	55.3 ± 0.9^{f}	W	NR	NR
		Leaf	90.0 ± 2.9^{h}	W	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	90.3 ± 1.5^{j}
-	1.0	Stem	60.0 ± 2.9^{e}	O	NR	NR	Root	81.0 ± 4.9^{g}	W/C	NR	NR
		Leaf	40.0 ± 2.3^{c}	W	NR	NR	Shoot	NR	-	89.8 ± 3.9^{cd}	39.7 ± 2.0^{e}
-	1.5	Stem	30.0 ± 1.2^{b}	R/O	NR	NR	Root	15.3 ± 4.9^{ab}	W	NR	NR
		Leaf	15.0 ± 0.6^{a}	W	NR	NR	Shoot	NR	-	14.8 ± 2.2^{a}	70.0 ± 1.2^{h}
-	2.0	Stem	25.0 ± 0.6^{b}	R	NR	NR	Root	5.2 ± 1.3^{a}	C	NR	NR
		Leaf	10.3 ± 0.9^{a}	W	NR	NR	Shoot	NR	-	90.3 ± 1.5^{cd}	79.7 ± 2.6^{i}
0.5	0.5	Stem	70.0 ± 2.9^{f}	C	NR	NR	Root	19.8 ± 0.7^{bc}	C	NR	NR
		Leaf	40.0 ± 2.9^{c}	O	NR	NR	Shoot	NR	-	90.5 ± 5.5^{cd}	100.0 ± 0.0^{k}
0.5	1.0	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	19.8 ± 2.7^{bc}	C	NR	NR
		Leaf	80.0 ± 0.6^{g}	Y	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	59.7 ± 2.6^{g}
0.5	1.5	Stem	90.0 ± 2.8^{h}	C	NR	NR	Root	49.7 ± 0.9^{ef}	C	NR	NR
		Leaf	100.0 ± 0.0^{i}	R	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	100.0 ± 0.0^{k}
0.5	2.0	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	100.0 ± 0.0^{h}	C	NR	NR
		Leaf	100.0 ± 0.0^{i}	R	NR	NR	Shoot	NR	-	90.3 ± 1.5^{cd}	60.0 ± 2.9^{g}
1.0	0.5	Stem	80.0 ± 1.7^{g}	C	NR	NR	Root	15.0 ± 2.9^{ab}	C	NR	NR
		Leaf	90.0 ± 1.2^{h}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	9.8 ± 3.0^{a}
1.0	1.0	Stem	10.0 ± 2.9^{a}	C	NR	NR	Root	20.7 ± 5.2^{bc}	C	NR	NR
		Leaf	15.3 ± 0.9^{a}	C	NR	NR	Shoot	NR	-	9.7 ± 2.6^{a}	49.8 ± 1.6^{f}
1.0	1.5	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	29.7 ± 3.2^{cd}	C	NR	NR
		Leaf	59.3 ± 2.3^{e}	C	NR	NR	Shoot	NR	-	49.3 ± 3.5^{b}	40.3 ± 1.5^{e}
1.0	2.0	Stem	50.0 ± 1.2^{d}	C	NR	NR	Root	39.7 ± 2.6^{de}	C	NR	NR
		Leaf	49.7 ± 0.9^{d}	C	NR	NR	Shoot	NR	-	95.0 ± 1.2^{de}	20.3 ± 0.9^{bc}
1.5	0.5	Stem	50.0 ± 2.9^{d}	C	NR	NR	Root	29.7 ± 1.5^{cd}	C	NR	NR
		Leaf	30.0 ± 0.6^{b}	C	NR	NR	Shoot	NR	-	90.6 ± 2.0^{cd}	25.2 ± 1.9^{cd}
1.5	1.0	Stem	70.0 ± 0.6^{f}	C	NR	NR	Root	40.3 ± 11.3^{de}	C	NR	NR
		Leaf	50.7 ± 2.3^{d}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	30.0 ± 1.2^{d}
1.5	1.5	Stem	91.0 ± 0.5^{h}	C	NR	NR	Root	80.3 ± 8.9^{g}	C	NR	NR
		Leaf	59.7 ± 0.9^{e}	C	NR	NR	Shoot	NR	-	95.0 ± 2.9^{de}	39.7 ± 2.6^{e}
1.5	2.0	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	100.0 ± 0.0^{h}	C	NR	NR
		Leaf	100.0 ± 0.0^{i}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	10.0 ± 1.2^{a}
2.0	0.5	Stem	30.0 ± 0.6^{b}	C	NR	NR	Root	20.3 ± 1.5^{bc}	C	NR	NR
		Leaf	10.0 ± 0.6^{a}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	14.8 ± 1.6^{ab}
2.0	1.0	Stem	40.0 ± 1.7^{c}	C	NR	NR	Root	20.3 ± 2.0^{bc}	C	NR	NR
		Leaf	10.3 ± 1.5^{a}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	40.3 ± 2.0^{e}
2.0	1.5	Stem	100.0 ± 0.0^{i}	C	NR	NR	Root	75.2 ± 0.7^{g}	C	NR	NR
		Leaf	94.7 ± 2.6^{hi}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	10.0 ± 1.5^{a}
2.0	2.0	Stem	70.0 ± 5.0^{f}	C	NR	NR	Root	NR	-	NR	NR
		Leaf	60.3 ± 2.0^{e}	C	NR	NR	Shoot	NR	-	100.0 ± 0.0^{e}	10.2 ± 1.6^{a}

Mean values with different letters within a column (between the same explant) are significantly different at $p \leq 0.05$
Percentage (%): number of explants producing callus, direct regeneration or multiple shoots
DR: Direct regeneration, MS: Multiple shoots, NR: No response
Colour of callus: (C) cream, (W) white, (O) orange, (R) red, (Y) yellow

TABLE 3. The effects of NAA and BAP at different concentrations on various explants of *J. betonica* after 15 weeks, cultured at 25 +1°C under 16 h light and 8 h dark

[Hormone] (mg/L)		Explant type	Callus (%)	Colour of callus	DR (%)	MS (%)	Explant type	Callus (%)	Colour of callus	DR (%)	MS (%)
NAA	BAP										
-	-	Leaf	15.0 ± 1.0a	W	20.0 ± 5.0c	NR	Stem	50.0 ± 0.5e	W	50.0 ± 0.4i	NR
							Shoot	14.6 ± 0.4c	W	21.3 ± 0.1f	NR
0.5	-	Leaf	NR	-	20.0 ± 2.8c	NR	Stem	47.3 ± 0.4d	W	56.4 ± 1.2j	NR
							Shoot	NR	-	NR	NR
1.0	-	Leaf	NR	-	35.0 ± 2.0e	NR	Stem	24 .1 ± 0.6b	W	NR	NR
							Shoot	NR	-	NR	NR
1.5	-	Leaf	NR	-	50.0 ± 0.5i	NR	Stem	33.3 ± 1.5c	W	NR	NR
							Shoot	NR	-	16.7 ± 1.5e	NR
2.0	-	Leaf	NR	-	70.0 ± 1.0j	NR	Stem	NR	-	NR	NR
							Shoot	NR	-	NR	NR
-	0.5	Leaf	40.1 ± 2.5d	W	NR	NR	Stem	NR	-	14.1 ± 2.1a	NR
							Shoot	7.1 ± 1.0b	W	13.5 ± 0.2d	NR
-	1.0	Leaf	NR	-	1.5 ± 0.6a	NR	Stem	NR	-	20.0 ± 0.1c	NR
							Shoot	NR	-	4.6 ± 0.4b	NR
-	1.5	Leaf	86.2 ± 1.5e	W	NR	NR	Stem	NR	-	27.0 ± 1.1e	NR
							Shoot	0.5 ± 0.0a	W	83.4 ± 9.7j	NR
-	2.0	Leaf	90.3 ± 0.7f	W	NR	NR	Stem	NR	-	40.2 ± 0.8g	NR
							Shoot	NR	-	45.2 ± 8.1i	NR
0.5	0.5	Leaf	36.5 ± 0.6c	W	42.6 ± 7.5h	NR	Stem	21.4 ± 0.1a	W	43.3 ± 0.1h	NR
							Shoot	NR	-	NR	NR
0.5	1.0	Leaf	NR	-	36.5 ± 0.7f	NR	Stem	NR	-	40.0 ± 0.2g	NR
							Shoot	23.5 ± 4.7d	W	25.0 ± 1.5g	NR
0.5	1.5	Leaf	NR	-	40.5 ± 0.9g	NR	Stem	NR	-	24.4 ± 0.3d	NR
							Shoot	NR	-	2.5 ± 0.3a	NR
0.5	2.0	Leaf	NR	-	41.6 ± 0.5gh	NR	Stem	NR	-	16.7 ± 1.7b	NR
							Shoot	NR	-	10.15 ± 1.2c	NR
1.0	0.5	Leaf	18.9 ± 0.7b	W	70.0 ± 1.3j	NR	Stem	NR	-	NR	NR
							Shoot	NR	-	NR	NR
1.0	1.0	Leaf	NR	-	23.3 ± 0.5d	NR	Stem	NR	-	35.6 ± 1.0f	NR
							Shoot	NR	-	15.5 ± 0.5e	NR
1.0	1.5	Leaf	NR	-	NR	NR	Stem	NR	-	NR	NR
							Shoot	NR	-	12.5 ± 0.1d	NR
1.0	2.0	Leaf	NR	-	NR	NR	Stem	NR	-	NR	NR
							Shoot	NR	-	NR	NR
1.5	0.5	Leaf	NR	-	36.5 ± 0.4f	NR	Stem	NR	-	55.6 ± 2.6j	NR
							Shoot	NR	-	NR	25.0 ± 2.6
1.5	1.0	Leaf	NR	-	NR	NR	Stem	NR	-	14.4 ± 0.4a	NR
							Shoot	NR	-	32.2 ± 0.3h	NR
1.5	1.5	Leaf	NR	-	NR	NR	Stem	NR	-	27.7 ± 0.7e	NR
							Shoot	NR	-	NR	41.2 ± 0.2
1.5	2.0	Leaf	NR	-	NR	NR	Stem	NR	-	NR	NR
							Shoot	NR	-	NR	NR
2.0	0.5	Leaf	NR	-	16.2 ± 1.1b	NR	Stem	NR	-	40.1 ± 1.4g	NR
							Shoot	NR	-	NR	14.7 ± 0.5
2.0	1.0	Leaf	NR	-	NR	NR	Stem	NR	-	36.6 ± 0.8f	NR
							Shoot	NR	-	NR	NR
2.0	1.5	Leaf	NR	-	NR	NR	Stem	NR	-	41.2 ± 0.9g	NR
							Shoot	NR	-	NR	NR
2.0	2.0	Leaf	NR	-	NR	NR	Stem	NR	-	25.2 ± 0.7d	NR
							Shoot	NR	-	NR	NR

Mean values with different letters within a column (between the same explant) are significantly different at $p \leq 0.05$
Percentage (%): number of explants producing callus, direct regeneration or multiple shoots
DR: Direct regeneration, MS: Multiple shoots, NR: No response
Colour of callus: (W) white

TABLE 4. Acclimatization methods, suitable substrates and survival rates of *A. praecox*, *C. cristata* and *J. betonica* after 5 months being transferred to soil

Plant	Acclimatization methods	Suitable substrate	Survival rate (%)
A. praecox	Wash all adhering media, transfer to growth substrate, cover with plastic bags (with holes) and keep in the culture room for 1 month, transfer to greenhouse	Black soil : red soil (1:1)	96.67
C. cristata	Wash all adhering media, transfer to growth substrate, cover with plastic bags (with holes) and keep in the culture room for 2 weeks, transfer to greenhouse	Black soil : sand (3:1)	75.00
J. betonica	Wash all adhering media, transfer to growth substrate, cover with plastic bags (with holes) and keep in the culture room for 2 weeks, transfer to greenhouse	Black soil	80.00

Percentage (%): number of *ex vitro* plantlets survived after acclimatization

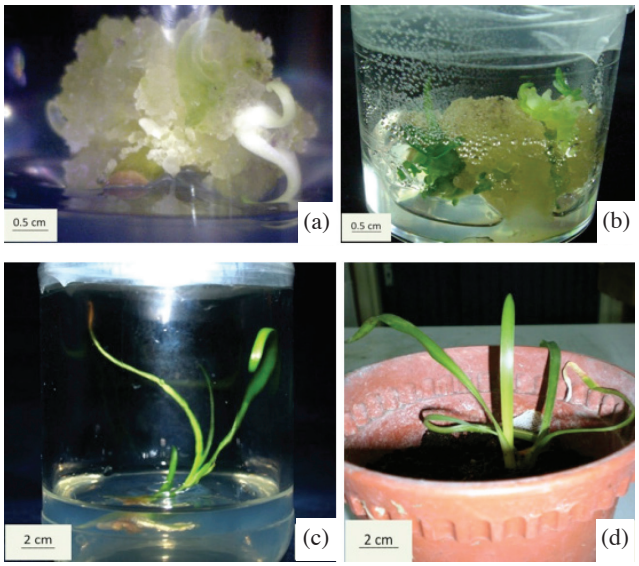

FIGURE 1. (a) Callus growth from leaf explant of *A. praecox*, (b) formation of multiple shoots in *A. praecox*, (c) regeneration of complete *A. praecox* plantlet and (d) successfully acclimatized 2-month-old *A. praecox* plantlet

roots and shoots in tissue culture of *Curcuma domestica*, while combinations of BAP and NAA resulted in formation of auxiliary shoots from callus of this species. Al-Saif et al. (2011) also reported positive results on shoot growth and proliferation of pineapple using combinations of NAA and BAP. Furthermore, equal concentrations of BAP and NAA at 2.0 mg/L was found to be effective for multiple shoots formation and subsequent plant regeneration from 4-month-old stems of *Lawsonia inermis* syn. *L. alba* or henna plant (Rahiman & Taha 2011). In the present study, *J. betonica* explants also showed positive responses when equal concentration of BAP and NAA was used in the range of 0.5-2.0 mg/L (Table 3).

In the present study, we proposed combinations of BAP and NAA as a universal combo, fundamental in tissue culture of many species. The tissue culture techniques and acclimatization protocols described in this paper can be used as a model for tissue culture and field transfer of other species. For instance, *Celosia cristata* has been used as model crop for low cost media options for *in vitro* culture (Daud et al. 2011), signifying the suitability of this species as a model for other species. We therefore proposed a universal combo of growth hormones (combinations of BAP and NAA) as a model for tissue culture of many plants. Acclimatization of *in vitro* regenerated plantlets is of utmost importance to fully utilize the *in vitro* products for commercialization. The *in vitro* plantlets are acclimatized in the culture room within enclosed plastic bags (with small holes) prior to field transfer, to ensure optimum survival of the tissue culture plants.

CONCLUSION

The combinations of BAP and NAA were found to be of substantial importance in tissue culture of various ornamental species, such as *A. praecox* ssp. *minimus*, *C. cristata* and *J. betonica*. Based on the findings depicted in

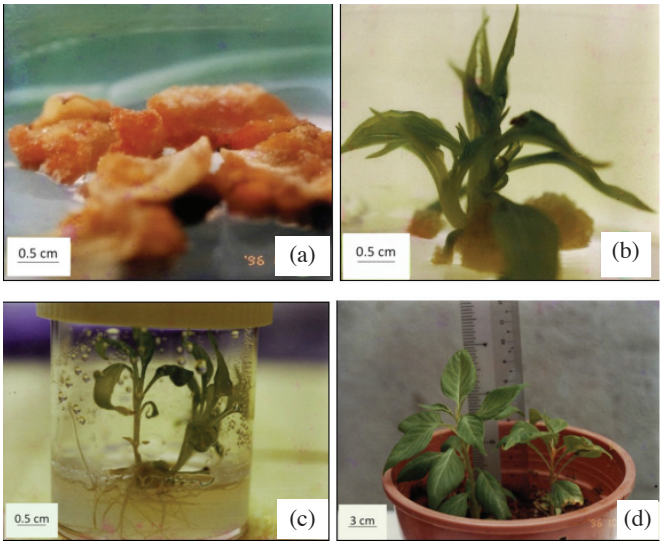

FIGURE 2. (a) Orange callus from leaf explant of *C. cristata*, (b) formation of multiple shoots in *C. cristata*, (c) regeneration of complete *C. cristata* plantlet and (d) successfully acclimatized 2-month-old *C. cristata* plantlet

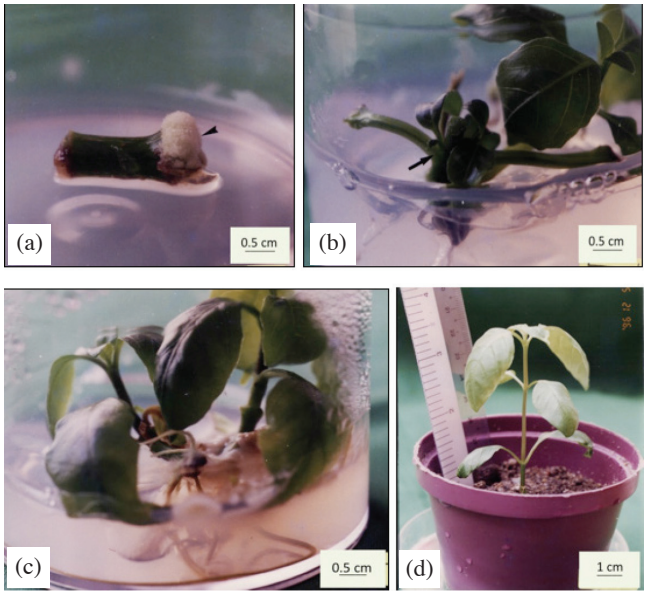

FIGURE 3. (a) Orange callus from leaf explant of *J. betonica*, (b) formation of multiple shoots in *J. betonica*, (c) regeneration of complete *J. betonica* plantlet and (d) successfully acclimatized 2-month-old *J. betonica* plantlet

the present study, it was proposed that BAP and NAA can be employed in *in vitro* culture of other ornamental species, whereby BAP and NAA at equal concentration can yield production of callus with embryogenic potential while the use of BAP and NAA at other concentrations can result in direct regeneration and rhizogenesis. The importance of acclimatization and a good field transfer method was also described in the present study. All *in vitro* regenerants must be acclimatized on suitable growth substrates and maintained in the culture room to assist in early adaptation to the natural environment and ensure high survival rates. Further transfer of the *ex vitro* plantlets to the green house can be pursued after the plantlets has shown early signs of successful *ex vitro* transfer (increase in plant girth).

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