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Micropropagation of *Cyrtanthus mackenii Hook. f.*: from tri scales

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Abstract
A procedure for *in vitro* production of bulbs of *Cyrtanthus mackenii* was developed from triscales. The explants were cultured on MS medium supplemented with cytokinins such as BAP (6-Benzyl amino purine), TDZ (Thidiazuron), Zeatin, Kinetin and auxins namely IAA (Indole-3-aceticacid), IBA (Indole-3 butyric acid), NAA (Napthalene acetic acid), individually at different concentrations. A combination of TDZ and NAA in equal concentrations (1mg/l) produced a maximum 14 bulblets. The maximum number of roots (6) root length (4.96cm) was recorded on TDZ (1mg/l) + NAA (1mg/l). Sucrose at various concentrations (30, 60, 90 and 120g/l) was tested to increase the size of bulblets. Increase in the size of bulblets (growth index) was noticed after three months at 60g/l concentration which gave the maximum weight of 4.20gms. Bulblets were then transferred to pots containing a potting mixture of vermiculite: soil (3:1). The plants showed 98% survival. Regenerated plantlets exhibited morphological characters similar to source plant.

Keywords: *C. mackenii*, tri scale explant, growth regulators, sucrose, MS medium

1. Introduction
Cyrtanthus belonging to the family Amaryllidaceae, is well known for its horticultural value, beauty and variety of flower colour and form (Duncan, 1990). *Cyrtanthus mackenii* ("Ifafa Lily") is a well-known ornamental and evergreen species in this genus. The flowers of *Cyrtanthus mackenii* are long and narrow with recurved tips and leaves are narrowly lance – shaped and are sweetly scented. Natural offsets of many bulbous ornamentals are low because they produce few daughter bulbs every year.
Micropropagation is been extensively used for large scale production of elite planting material of desired characteristics. The production of storage organs (corms, tubers or bulbs) during micropropagation can reduce the losses incurred during acclimatization and decrease the time to flower (Ascough *et al*, 2008) [18]. The propagation of *C.mackenii* is mainly by seeds and bulbs, however the seeds may be subject to seasonal availability. *C.mackenii* propagation by bulbs is easy but the multiplication rate is quite low (about five bulblets from one adult bulb per year). *In vitro* micropropagation can offer an alternative way to increase that rate and to obtain aseptic biomass in a shorter period of time. To the best of our knowledge, there are no reports on the tissue culture of *C.mackenii*. The present study was attempted to develop a feasible and reliable method for mass propagation using tri scale explants in *Cyrtanthus mackenii*.

2. Materials and Methods
2.1 Surface sterilization, explants preparation and medium preparation
The bulbs of *C.mackenii* were collected from Government Botanical Garden, Udhagamandalam, Tamil Nadu, India. The root and leaves and the brown coating of the bulbs were removed and washed thoroughly in tap water. Tri scales with basal meristem were used as an explant. The explant was washed several times with tap water along with Teepol for 30 minutes to remove the dirt and debris and treated with a systemic fungicide bavistin (2%) for 30 min and washed thoroughly. An additional treatment of 20 min soaking in antibiotic gentamycin sulphate (0.01%) was essential to eliminate contamination. The explants were then washed and surface sterilized with one or two drops of Tween 80 in ethanol (70%) thoroughly for 20 min and then treated with mercuric chloride (0.02%) and washed repeatedly prior to inoculation.
MS medium was used as the basal medium. The explants were cultured on MS medium with cytokinins such as BAP, Kinetin, Zeatin and TDZ (0.5-3mg/l) and auxins such as NAA, IAA, IBA(0.5-3mg/l) individually. All the cultures were maintained at a temperature of 25 ± 2ºC and at relative humidity of 65-70%. The cultures were kept under white light at intensity of 2000 lux provided from white fluorescent lamps (PHILIPS, INDIA) with 16 hours photoperiodic duration.

2.2 Bulblet multiplication
The explants were cultured in MS basal medium containing various concentrations of TDZ (0.5-3 mg/l), Zeatin (0.5-3mg/l), Kinetin (0.5-3mg/l), NAA (0.5-3mg/l), IAA (0.5-3mg/l) and IBA (0.5-3mg/l) individually and combinations of TDZ (0.5 and 1mg/l) with NAA (0.25, 0.5 and 1mg/l) was also tested for bulblet multiplication. To the above said media 30g/l sucrose was added and solidified with 0.8% agar. The pH was adjusted to 5.8.

2.3 Root formation
The well-developed bulblets were removed from the medium, washed thoroughly to remove the traces of agar and seperated into single bulblet and cultured in MS medium supplemented with Kinetin (0.5-1mg/), NAA (0.5-1mg/l) and IAA (0.5-1mg/l) individually. MS medium containing TDZ (1mg/l) in combination with NAA (0.25mg/l) was also employed to induce rooting.
The fully developed individual bulblets were cultured on MS medium supplemented with sucrose at various concentrations

(30g/l, 60g/l, 90g/l and 120g/l).

2.4 Acclimatization

The regenerated bulblets were washed thoroughly in distilled water to remove the traces of medium and then acclimatized in pots containing a potting mixture of vermiculite: soil (3:1), irrigated with a MS solution and covered with a micro perforated transparent polythene bags and secured with rubber bands to prevent dehydration for a week and then the bags were ventilated by removing the rubber bands and then slowly transferred to garden soil for hardening.

2.5 Statistical Analysis

Each treatment consisted of five replicates and the experiment was repeated thrice. After 2 weeks of culture, data was recorded for shoot initiation and thereafter periodically for shoot development and rooting. Analysis of variance (ANOVA) was performed on all the data to compare concentration effects of growth regulators on multiple shoot regeneration. Means were separated using Duncan's Multiple Range Test (DMRT).

3. Results

The explants did not respond when cultured on MS medium devoid of plant growth regulators (control).Bulblets were formed between the tri scales containing the basal tissue. Single scale and twin scales were not suitable for shoot initiation.

Effect of BAP, Kinetin, Zeatin and TDZ on bulblet formation

Bulblets were initiated on the tri scales on all the media irrespective of the growth regulators tested individually. The effects of BAP, Kinetin, Zeatin and TDZ were studied in the MS media for the number of shoots, shoot length and survival percentage (Table 1). It was found that TDZ was superior over BAP, Kinetin and Zeatin. In the present study TDZ at 1mg/l regenerated highest number of bulblets **(7.63/tri scale),** bulblet diameter (1.90cm) and bulblet length (2.10cm).

3.1 Effect of NAA, IAA and IBA on bulblet formation

The effects of NAA, IAA and IBA were studied in the MS media for the number of shoots, shoot length and survival percentage. NAA failed to induce multiple shoots (Table 1). Maximum bulblet formation was observed in IBA when compared with IAA. IBA (1mg/l) produced 5 bulblets/triscale and IAA (0.5mg/l) produced 3.67 bulblets. Maximum shoot length (9.73cm), bulblet diameter (2.77cm) and bulblet length (3cm) were recorded on IAA (0.5mg/l).

Table 1: Morphometric parameters obtained from tri-scales of *C.mackenii*

Growth regulators	Concentrations (mg/l)	No. of bulblets	Shoot Length (cm)	Bulblet diameter (cm)	Bulblet length (cm)
BAP	0.5	5.67^{a}	7.20^{a}	0.80^{a}	0.7^{a}
	1	4.67^{a}	4.90^{b}	0.40 ^{b}	0.58^{a}
	2	3.00^{b}	2.43^{c}	0.40^{b}	0.60^{a}
	3	2.34^{b}	1.47^{d}	0.33^{b}	1.3^{a}
KN	0.5	4.67^{ab}	4.00^{b}	2.87^{ab}	0.7^{a}
	1	5.67^{a}	5.73^{a}	1.00^{a}	1.16^{a}
	2	5.00^{a}	2.43^{c}	0.43^{b}	0.64^{a}
	3	3.67^{b}	1.27^{d}	0.33^{b}	0.25^{a}
ZEA	0.5	5.67^{a}	4.50^{b}	0.77^{ab}	0.85^{a}
	1	1.00^{c}	5.83^{a}	0.97^{a}	0.94^{a}
	2	2.67^{b}	4.07^{bc}	0.33^{b}	0.45^{a}
	3	2.00^{b}	3.23^{c}	0.70^{ab}	1.26^{a}
TDZ	0.5	2.33^{b}	0.47^{c}	0.43^{b}	0.63^{b}
	1	6.67^{a}	7.63^{a}	1.90 ^{a}	2.10^{a}
	2	2.00^{b}	1.77^{b}	0.60^{b}	0.49^{b}
	3	2.00^{b}	1.76^{b}	0.60^{b}	0.58^{b}
NAA	0.5	1.00^{a}	1.23^{a}	0.47^{a}	0.47^{a}
	1	1.00^{a}	1.27^{a}	0.53 ^{a}	0.54^{a}
	2	1.00^{a}	1.33^{a}	1.60^{a}	0.6^{a}
	3	1.00^{a}	1.23^{a}	0.47^{a}	0.46^{a}
IAA	0.5	3.67^{a}	9.73^{a}	2.77^{a}	3.00^{a}
	1	2.00^{b}	7.57^{b}	2.00^{a}	2.10^{b}
	2	1.67^{b}	7.17^{b}	2.17^{a}	2.13^{b}
	3	1.33^{b}	6.90^{b}	0.80^{b}	1.76^{b}
IBA	0.5	2.33^{b}	5.26^{c}	1.23^{a}	1.5^{a}
	1	5.00^{a}	8.20^{b}	1.47 ^{a}	1.5^{a}
	2	1.00^{b}	9.40^{a}	1.23^{a}	1.56^{a}
	3	1.00^{b}	9.20^{a}	1.27^{a}	1.48^{a}
Hormone (H)$_{6, 56}$		110.48**	329.17**	43.92**	4.07*
Concentration (C)$_{3, 56}$		126.93**	88.35**	15.46**	45.10**
H×C$_{18, 56}$		25.21**	44.03**	6.49**	6.69**

Mean in a column followed by same letter (S) for a hormone are not significantly (P<0.05) different according to Duncan's Multiple Range Test

Combined effect of TDZ and NAA on maximum bulblet formation

The combined effect of TDZ and NAA in the ratio 1:1 produced maximum 14 bulblets with shoot length (7.13cm) and bulblet diameter (2.4cm) and the bulblet length (2.6cm) (Table 2).

Table 2: Effect of growth regulators on the formation of multiple shoots and length

Growth Regulator(Mg/L)		Number Of Bulblets	Shoot Length(Cm)	Bulblet Diameter(Cm)	Bulblet Length(Cm)
TDZ	NAA				
0.5	0.25	2.5^{d}	5.93^{ab}	0.83^{cd}	0.63^{d}
0.5	0.5	3.66^{d}	4.66^{b}	0.50^{d}	1^{cd}
0.5	1	6^{c}	6.13^{ab}	1.16^{bc}	1.26^{c}
1	0.25	10^{b}	5.8^{ab}	1.53^{b}	1.8^{b}
1	0.5	6^{c}	5.1^{b}	0.76^{cd}	0.76^{d}
1	1	14^{a}	7.13^{a}	2.4^{a}	2.6^{a}
F 3,8			1.52ns	32.13**	

3.2 Effect of growth regulators in rooting

Tri scales when cultured on MS medium supplemented with auxins such as IAA and NAA and cytokinin such as Kinetin individually produced shoots and roots in the same medium. Combination of TDZ at 1mg/l and NAA at 0.25mg/l produced 10 bulblets in which the maximum rooting was achieved (Table 3). The maximum number of roots (6.16) and root length (4.96cm) and the maximum growth index as 1.23gms/triscale were formed on TDZ (1mg/l) + NAA (0.25mg/l).

Table 3: Effect of growth regulators on root length and number.

Medium	Concentration(Mg/L)	Number of Bulblets	Number of Roots	Root Length (Cm)	Growth Index (Gm)
Kn	0.5	4.66^{c}	1.34^{c}	0.43^{e}	0.632^{ab}
	1	2.0^{c}	1.34^{c}	0.23^{e}	0.284^{b}
Iaa	0.5	3.67^{a}	5.67^{a}	2.13^{d}	0.728^{ab}
	1	1.67^{c}	1.34^{c}	0.23^{e}	0.213^{b}
Naa	0.5	1.34^{b}	3.85^{b}	3.4^{c}	0.245^{b}
	1	2^{b}	4.23^{b}	4.3^{b}	0.279^{b}
Tdz +Naa	10.025	10^{a}	6.16^{a}	4.96^{a}	1.23^{a}

Mean in a column followed by same letter (S) for a hormone are not significantly ($P<0.05$) different according to Duncan's Multiple Range Test

Bulbing in MS medium with varied sucrose concentrations

The biomass gain of the bulblets when treated with different sucrose concentrations, ranging from 30 to 120g/l, solidified with 8% agar is shown in Table 4.The explants were cultured for 8 weeks under the same light and temperature conditions, being recultured once in the 4thweek. At the end of this period, the increase in the size of bulblets was noticed in 60g/l of sucrose concentration gave the maximum weight of 4.20gms. This increase in carbohydrate supply helps to gain biomass.

Table 4: Effect of sucrose in biomass gain (fresh weight).

Sucrose(Gm/L)	Fresh Weight In Ms Medium(Gm)
30	2.54^{c}
60	4.20^{a}
90	3.36^{b}
120	2.45^{c}
F 3,8	232.81**

3.3 Acclimatization

Tiny bulbs with shoots and roots were selected for acclimatization, once these plants were transferred to soil conditions, they exhibited progressive growth. Survival frequency of the regenerated plantlets under *ex vitro* condition on soil was 98%.The time taken to complete the experiment from culturing to acclimatization was 8 months.

4. Discussion

The present study attempts were made to propagate *Cyrtanthus mackenii* using tri scales along with the basal plate as explants. The *in vitro* micropropagation of *Crinum* 'Ellen Bosanquet' using tri scales as an explant source for the production of bulblets have been reported by Melanie *et al*, (1999) [8]. The use of bulb scales as primary explants for the micropropagation of Narcissus and Lilium species for the production of bulblets has been reported by Tanimato & Matsubara (1995) [6] and Seabrook (1990) [7].

The effects of BAP, Kinetin, Zeatin and TDZ on number of shoots, shoot length and survival percentage were studied. It was found that TDZ was superior over BAP, Kinetin and Zeatin. Cytokinin like compound TDZ has an important regulatory role in plant growth and development (Galston *et al*., 1980 [29] Altman *et al*., 1981 [30] and Yonova *et al*., 1997) [31]. The role of TDZ in shoot initiation in our study was significant. These results coincide with those observed for other bulbous plants, *Lilium* where the role of TDZ in shoot multiplication was well documented (Park *et al*., 1996; Woo *et al*., 2000) [4] and also in *Urginea maritima* where TDZ increased bulblet regeneration (Aasim *et al*., 2008) [1]. Significant variations existed between concentrations(C) 3, 56=126.21 ($P<0.0$), among hormones (H) 6, 56=126.93($P<0.01$), and hormone concentration interaction HxC18, 56=25.21 ($P<0.01$) in number of shoots. Significant

variations existed between concentrations(C) 3,56=88.35 (P<0.01),among hormones(H) 6,56=329.17(P<0.01), and hormone concentration interaction HxC18,56=44.03(P<0.01) in shoot length, concentrations(C) 3,56=15.46(P<0.01),among hormones(H) 6,56=43.92(P<0.01),and hormone concentration interaction HxC 18,56=6.49(P<0.01) in bulblet diameter, concentrations(C) 3,56=45.10 (P<0.01), among hormones(H) 6,56=4.07(P<0.05),and hormone concentration interaction HxC18,56=6.69 (P<0.01) in bulblet length.

In our study NAA individually was less potent in inducing bulblets but in combination with TDZ produced maximum of 14 bulblets and the same effect of the hormone on the growth of bulblets was reported by Chow *et al*. (1992) [19] in *Narcissus bulbocodium* and in *Narcissus tazetta* by Steinitz & Yahel (1982) [9]. It has been reported that auxin is an effective PGR for shoot induction and in combination with a cytokinin, is essential for shoot induction (Paek and Murthy, 2002; Santos *et al*, 2002) [28, 32]. NAA had an inhibitory effect in *Crinum* "Ellen Bosanquet" (Melaine *et al*., 1999) [8]. Low concentrations of TDZ and NAA produced maximum bulblets and the same effect was noticed in bulblet formation in *Tulip* (Alderson *et al*., 1986) [26] and the same effect of hormone on the growth of the bulblets were reported in *Hippeastrum hybridum* (Mii *et al*., 1974) [25] and *Sternbergia clusiana* (Oran & Fattash, 2005) [24].

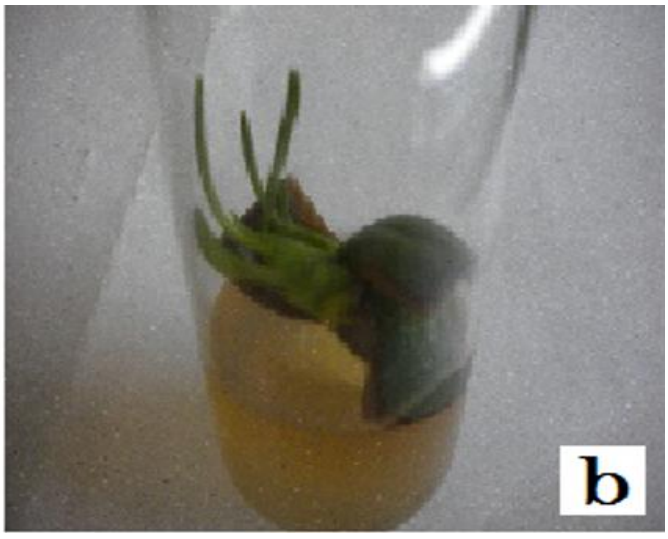
MS medium supplemented with IAA & NAA individually produced rooted shoots, which is in accordance with the root formation in *Watsonia* (Ascough *et al*., 2007) [14]. In *Cyrtanthus clavatus and Cyranthus spiralis* the highest number of roots were observed in bulblets treated with NAA (0.1mg/l) which was reported by Moran *et al*, (2003) [5].

In the present study sucrose at 60g/l enhanced the size of the bulblets and its weight. Similar findings were reported for *Cyrtanthus clavatus* and *C.spiralis* where growth index reveals significant differences (P<0.05) between the bulblets of *C. clavatus* treated with 30g/l of sucrose and those grown with the other sucrose concentrations. The fresh weight of the bulblets ($F_{3, 8} = 232.81$; $p<0.01$) is significant according to Duncans multiple test.

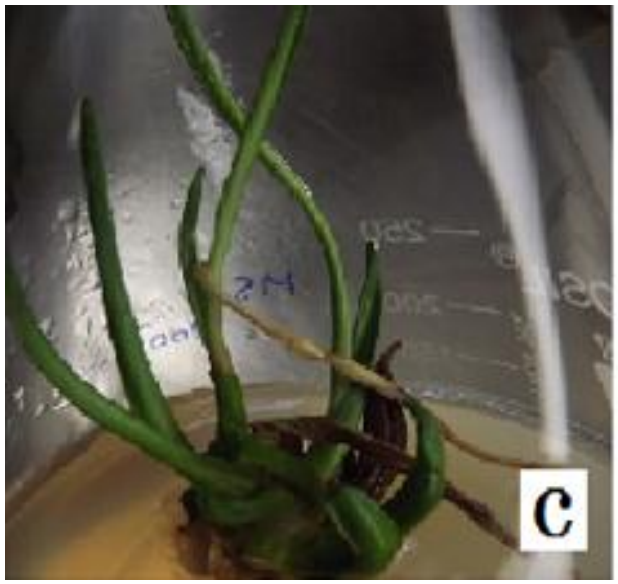
The diameter of the bulbs developed by our method was 2.77cm which was found to be higher than that of *Urginea maritima* (Aasim *et al*., 2008) [1], *Lilium spp*. (Duong *et al*., 2006) [17] and *Albuca spp.* (Ascough & VanStaden 2010) [2]. The study on carbohydrate distribution in bulbs and its ability to initiate adventious bulb shows the co –relation between production of bulbs and levels of carbohydrates (Hanks *et al* 1986) [11].

Bulbous species from warmer areas require warmer culture temperature for growth, higher temperatures stimulate the use of accumulated carbohydrates, due to higher respiration at high temperatures (Fennell 2002) [3], which results in gain in biomass. Sucrose is essential for shoot production (Fennell 2002) [3] and regeneration (De Bruyn *et al.*, 1992) [10]. Bulb formation is stimulated by sucrose in bulbous plants (Chow *et al*., 1992 and De Bruyn & Ferreira, 1992) [19, 10]. *In vitro* oranogenisis and the rate of regeneration are regulated by sucrose (Taeb & Alderson, 1990) [23]. High sucrose levels are required for bulblet growth *in vitro* and low sucrose concentration have been shown to hinder the onset of bulbing (Van aartijk & Van der linde, 1986) [13]. Higher sucrose concentrations resulted in greater multiplication of bulblets (Lian *et al*., 2003). Bulblet formation from shoot clumps was increased by increasing the sucrose concentration in the medium (Chow *et al*., 1992) [19]. Sucrose is the main sugar for storage which occurs in regions which form shoots (Van aartrijk & Blombarn hoorn 1980) [22]. An accumulation of starch in this regions may increase the number of shoots formed and so increase the multiplication rate (Leight, 2009). This implies that carbohydrates improve bulb induction.

a) Habit.



b) Initiation of shoots.



c) Multiple shoot formation.

d) Well developed bulblets with roots.

e) Hardening.

f) Increase in the size of bulblets when treated with 60g/l of sucrose.

Fig 1: Micropropagation of *Cyrtanthus mackenii* from tri scales.

5. Conclusion

The present investigation was attempted to investigate the influence of growth regulators on bulblet formation in *Cytranthus mackenii* attained from tri scales. The number of regenerated shoots was highest on MS medium supplemented with (1 mg/l) TDZ + (1 mg/l) NAA; the number of roots was highest on MS medium containing (1) TDZ + (0.25 mg/l) NAA in combination and individually in (0.5mg/l) IAA. The highest number of bulblets obtained in the present study represents an effective alternative to the conventional method. One bulblet obtained from wild can be dissected into eight sections and if each explant is able to produce a maximum of fourteen bulblets with shoots and roots then this protocol can produce an average of 112 plantlets per bulb. *Cytranthus mackenii* micropropagation presented above is relatively easy and cost effective and many bulblets may be produced using the above protocol.

6. References

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