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Micropropagation of *Crinum* 'Ellen Bosanquet' by tri-scales

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Abstract

Crinum lilies (*Crinum* spp. L.) are tropical and sub-tropical bulbous plants with excellent potential for southern US landscapes. Unfortunately, the more desirable crinum cultivars are slow and expensive to propagate by traditional offsets. The objective of this research was to develop reliable procedures for multiplying *Crinum* 'Ellen Bosanquet' by tissue culture. A sterilization procedure for cleaning explants was developed in which bulb chips containing a basal plate were submersed and agitated in 0.525% hypochlorite for 1 h. For shoot formation, tri-scales (three scales attached to a section of the basal plate) were used as the explant source and grown on MS-based media containing five levels of benzyladenine (BA) (0–22.2 μM) and five levels of naphthaleneacetic acid (NAA) (0–5.3 μM). The greatest shoot formation was obtained from the highest level of BA (22.2 μM) without NAA. In a subsequent experiment, explants were cultured for 4 months on media containing 35.5–88.8 μM BA, and then transferred to hormone-free media for 3 months. BA at 35.5 μM stimulated optimal shoot (8.4) and bulblet (2.8) formation. Plantlets were successfully acclimatized and rooted ex vitro. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Amaryllidaceae; Basal plate; Bulblet; Tissue culture; Micropropagation

Abbreviations: BA, 6-benzyladenine; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; PPF, photosynthetic photon flux

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1. Introduction

Crinum spp. are valuable tropical and subtropical bulbs which are well adapted to the climate of the southern USA. One species, *Crinum americanum* L., is native to southern US. *Crinum lilies* provide a greater diversity of flowering bulbs in a region where few traditional flower bulbs grow. They have little or no chilling requirement, and unlike some traditional Dutch bulbs, they develop their bulbous characteristic as an adaptation to periods of drought rather than cold (De Hertogh and Le Nard, 1993). Besides their beauty, crinum lilies have many highly desirable characteristics for southern US landscapes, such as drought and heat tolerance (Ogden, 1994).

While many improved cultivars of *Crinum* exist, only the less improved cultivars are available in the mainstream nursery industry. Newer, better cultivars, however, are often slow to propagate by offsets (Fig. 1(A)), making them expensive (sometimes as much as US$ 200 per bulb). For this reason, an efficient method of propagating large numbers of these invaluable plants is needed.

One approach is to use tissue culture as a means of propagation. Only limited research has been reported on the tissue culture of crinum species. *Crinum macowanii* has been regenerated in vitro by twin-scaling (Slabbert et al., 1993) and with immature floral stems (Slabbert et al., 1995).

The cultivar 'Ellen Bosanquet' was selected for this research because it is a particularly valuable landscape plant. It has dark pink flowers with flared trumpets and unique undulating foliage. It is hoped that the procedures developed in the course of this research will be applicable to other, rarer *Crinum* spp. and hybrids. The objective of this study was to develop reliable procedures for multiplying crinum lilies by micropropagation. Micropropagation may also be an effective technique for multiplying stock plants of new crinum cultivars, which can then be propagated by slower, conventional offset methods.

2. Materials and methods

2.1. Sterilization

Bulbs used were roughly 8–10 cm in diameter. Several preliminary sterilization experiments were conducted. The most successful sterilization method, which was employed in all subsequent experiments, was as follows: (i) the roots and tunic of the dormant bulbs were removed, and the bulbs soaked in hot (50°C) tap water for 1 h; (ii) bulbs were cut longitudinally into six divisions and further subdivided to yield 12 divisions or chips; (iii) the chips were placed in flasks with a solution of 0.525% sodium hypochlorite containing 0.1% Tween 20 (w/v) and agitated for 1 h; (iv) the chips were rinsed three times with sterilized water under

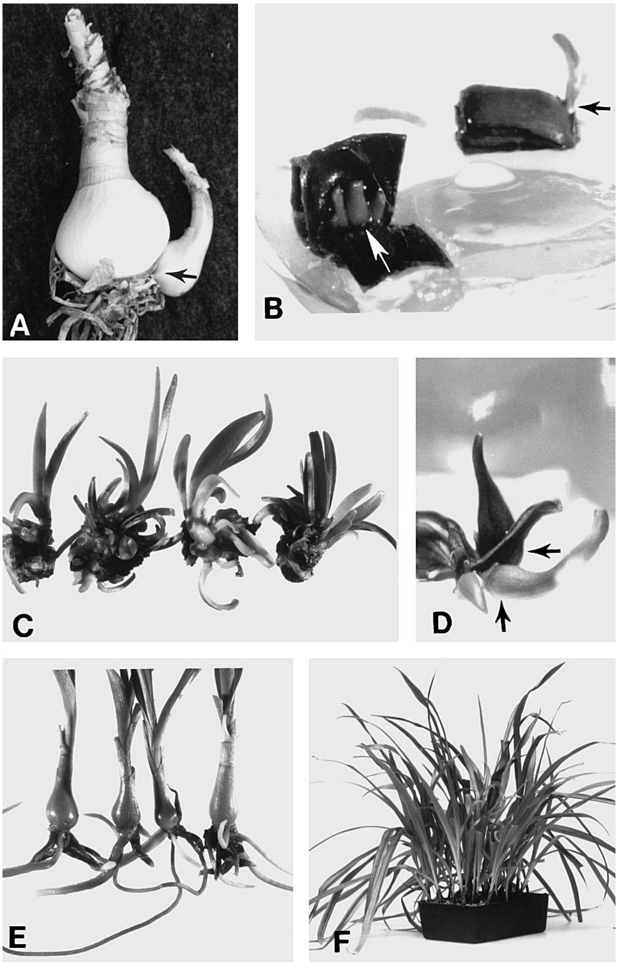

Fig. 1. Micropropagation of *Crinum* sp. 'Ellen Bosanquet' by tri-scales. (A) Traditional offset propagation of *Crinum* with a mother bulb producing a single offset (arrow). (B) Tri-scales with shoots forcing from the basal plate (arrows). (C) Range of shoot formation with tri-scales cultured with 35.5–88.8 μM BA, and then transferred to a hormone-free medium. (D) Shoots swelling at the base to form bulblets (arrows). (E) Shoots with bulblets and roots. (F) Successful acclimatization of a flat of micropropagated *Crinum* sp. 'Ellen Bosanquet'.

a laminar air flow cabinet, the damaged tissue was trimmed, and the chips reduced to tri-scales (three scales attached to a piece of basal plate and usually two to three tri-scales can be derived from one chip); (v) the tri-scales were placed in a solution of 0.263% sodium hypochlorite containing 0.1% Tween 20 (w/v) for 5 min, rinsed once with sterilized water, and placed in culture with the adaxial side down (Fig. 1(B)). Explants were cultured on a hormone-free MS medium (Murashige and Skoog, 1962) containing 3% sucrose, and 7 g l^{-1} agar

(Bacto-Agar, Difco Laboratories, Detroit, MI, USA). Sterilization experiments were arranged in a completely random design (CRD). One explant was grown per jar (200 ml glass baby jars with plastic caps (Magenta Corp., Chicago, IL, USA)), with 10 explants per treatment ($n = 10$). In vitro conditions throughout the research included a 16 h photoperiod at 74 μmol m^{-2} s^{-1} PPF, and a mean temperature of 25 ± 2°C.

2.2. *Influence of BA and NAA on shoot proliferation*

For the first experiment, 0, 4.4, 8.9 and 22.2 μM BA, in combination with 0, 2.7, and 5.3 μM NAA were used in a 4 × 3 factorial experiment in a CRD. The modified MS (Murashige and Skoog, 1962) medium contained 3% sucrose, and 7 g l^{-1} agar. The explants (tri-scales) were grown in 15 jars per treatment, with two explants in each jar ($n = 30$).

2.3. *Influence of darkness on bulb induction*

Explants from the shoot induction proliferation experiment were transferred to a hormone-free MS medium containing 3% sucrose, and 7 g l^{-1} agar. Half of the explants were grown in the light (PPF of 74 μmol m^{-2} s^{-1}), and half in the dark. The mean temperature was 25 ± 2°C ($n = 8$).

2.4. *Influence of explants treated with BA and then subcultured to a hormone-free medium on shoot and bulblet formation*

Tri-scales were cultured for 4 months on a modified MS medium containing 6 g l^{-1} sucrose, and 7 g l^{-1} agar, and either 0, 35.5, 71 or 88.8 μM BA. Explants were then transferred to hormone-free medium for 3 months.

2.5. *Acclimatization*

Bulblets which had leaves at least 5 cm long were removed from their jars, potted individually in 5 cm pots, and grown under intermittent mist for 1 month during late spring (May) in a glasshouse. They were subsequently removed from the mist bench to a shaded section (average maximum PPF of 80 μmol m^{-2} s^{-1}) of the glasshouse for 2 weeks, then exposed to higher light conditions (average maximum PPF of 300 μmol m^{-2} s^{-1}). Greenhouse conditions ranged from 23 ± 2°C to 32 ± 2°C (minimum and maximum temperatures, respectively) and from 65% to 98% relative humidity. Plantlets were irrigated as needed, and fertilized weekly with a 20N-20P-20K soluble fertilizer to supply 200 ppm N.

3. Results

3.1. Sterilization

The sterilization method described in Section 2 with a 1 h exposure to sodium hypochlorite was 90% successful in eliminating any contamination (data not presented), and was used in all subsequent experiments.

3.2. Influence of BA and NAA on shoot induction and proliferation

There were highly significant differences among treatments ($P \leq 0.001$). After 4 months, all BA concentrations (4.4, 8.9, and 22.2 μM), in combination with no NAA, enhanced shoot formation compared to the control (Table 1). The greatest shoot number occurred with 22.2 μM BA and no NAA, although this treatment was not statistically different from 4.4 μM BA and no NAA. NAA had an inhibitory effect. Of the eight treatments containing NAA, all yielded significantly fewer shoots than the control. Many of the explants grown on media containing NAA became enlarged and swollen. No organs or calli arose, but the original tissue expanded and appeared hypertrophic. Eventually, these explants collapsed. The few shoots which formed from tri-scales in the NAA media were abnormal in appearance, whereas shoots from the treatments with BA and no NAA were usually slender and tubular.

Table 1
Effect of BA and NAA on shoot proliferation of tri-scales of *Crinum* sp. 'Ellen Bosanquet'

BA (μM)	NAA (μM)	Shoot No. per explant[a,b]
0.0	0.0	0.8 ± 0.2
0.0	2.7	0.3 ± 0.0
0.0	5.3	0.0 ± 0.0
4.4	0.0	1.4 ± 0.3
4.4	2.7	0.3 ± 0.1
4.4	5.3	0.2 ± 0.1
8.9	0.0	1.2 ± 0.2
8.9	2.7	0.3 ± 0.2
8.9	5.3	0.3 ± 0.2
22.2	0.0	2.1 ± 0.6
22.2	2.7	0.4 ± 0.1
22.2	5.3	0.5 ± 0.2

[a] Data represents a mean of 30 tri-scales ± SE.
[b] Data analyzed with ANOVA using GLM procedure (SAS Institute, Cary, NC, USA); $P \leq 0.0001$.

3.3. Influence of darkness on bulb induction

Tiny bulbs are bulblets which are thickened at the base, containing multiple scales and a basal plate (Fig. 1(D) and (E)). In vitro produced shoots are narrow and initially do not contain scales or a basal plate (Fig. 1(C)). Tissue culture or micropropagation, traditionally, involves four stages: (i) sterilization; (ii) shoot induction and proliferation; (iii) root induction; and (iv) acclimatization. However, as bulb crops have such a unique structure, the procedures for culturing these plants differ slightly. A bulb induction stage is necessary, while a rooting stage is not needed. In this experiment, explants from the shoot induction proliferation experiment were transferred to a hormone-free MS medium. Half of the explants were grown under light (PPF of 74 μmol m^{-2} s^{-1}), and half in the dark, for 5 months. The purpose of the dark treatment was to simulate etiolated underground conditions in which bulbs normally form. Neither darkness nor residual hormone effect in the tissue of previously cultured plants enhanced bulblet formation compared to controls (data not presented). While some bulblet formation was observed, there was no correlation to light or dark treatments.

3.4. Influence of explants treated with BA and then subcultured in a hormone-free media on shoot and bulblet formation

Tri-scales were initially cultured for 4 months on a modified MS medium either at 0, 35.5, 71 or 88.8 μM BA. Explants were then transferred to hormone-free media for 3 months. BA at 35.5 μM stimulated optimal shoot and bulblet formation, particularly when explants were transferred to hormone-free media during the final 3 months of culture (Table 2; Fig. 1(C)–(E)).

Table 2
Shoot and bulb formation of *Crinum* sp. 'Ellen Bosanquet' tri-scales cultured with BA and then subcultured to a hormone-free medium[a]

BA (μM)	Shoot No.	Shoot height (cm)	Subcultured shoot No.	Subcultured shoot height (cm)	Shoot weight/ bulbs
0.0	2.8 ± 0.9	13.7 ± 1.3	–	–	–
35.5	8.3 ± 0.9	2.4 ± 0.4	8.4 ± 0.9	3.5 ± 0.4	2.8 ± 0.3
71.0	9.3 ± 1.2	1.2 ± 0.1	7.5 ± 0.8	1.4 ± 0.2	1.4 ± 0.2
88.8	8.4 ± 1.0	1.1 ± 0.1	8.3 ± 1.2	1.5 ± 0.2	1.8 ± 0.3
Significance[b]	0.0002	0.0001	NS	0.0001	0.0100

[a] Data represents a mean of 25 tri-scales ± SE.
[b] Significance level for ANOVA using GLM procedure (SAS Institute, Cary, NC, USA).

3.5. Acclimatization

Plantlets were acclimatized successfully and rooted ex vitro (Fig. 1(F)). There was a 100% survival rate for transplanted bulblets, and none became dormant. After 3 months, the bulblets were approximately 1.47 cm in diameter at the largest point, with an average plant height of 27.0 cm (measured from the base of the bulb neck to the tip of the tallest leaf).

4. Discussion

This research demonstrates that *Crinum* sp. 'Ellen Bosanquet' can be micropropagated successfully by tri-scales. Optimal regeneration occurred with BA in the absence of NAA. Slabbert et al. (1993) used up to 20 mg l^{-1} (88.8 μM) BA plus ancymidol on *C. macowanii* to improve bulblet initiation. With the micropropagation of *Crinum* sp. 'Ellen Bosanquet', we observed that culturing explants in 35.5 μM BA, and then transferring them to a hormone-free media was more beneficial than using 88 μM BA and then transferring. While Chow et al. (1992) reported that BA actually inhibited bulblets on *Narcissus*, researchers working with selected amaryllis species reported that BA increased shoots and bulblets (Custers and Bergervoet, 1992), which agrees with our results. Slabbert et al. (1993) reported that optimal bulblet formation of *C. macowanii* occurred with 10–30 g l^{-1} of sucrose. We found that 18 g l^{-1} sucrose depressed shoot and bulb growth, and that 6 g l^{-1} was more beneficial for *Crinum* sp. 'Ellen Bosanquet' (data not presented).

Slabbert et al. (1995) had success using scapes (immature floral stems) of *C. macowanii* as explants, and O'Rourke et al. (1991) derived plantlets from the ovary and pedicel tissue of *Hippeastrum*. Although there are certain problems associated with using scapes (such as the limitation of explant sources since some crinum lilies bloom only once a year), this method of micropropagation might prove valuable with other *Crinum* cultivars.

However, in our case, tri-scales of *Crinum* sp. 'Ellen Bosanquet' have excellent regeneration capability. The success of ex vitro establishment clearly indicates that micropropagation is an effective technique for crinum lily propagation. Micropropagation may also be an effective technique for multiplying stock plants of new cultivars, which can then be propagated by slower, conventional offset methods. It is hoped that procedures developed in the course of this research will also be applicable to other, rarer *Crinum* spp. and cultivars.

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