

***In-vitro* Propagation of *Crossandra infundibuliformis* var. *Danica* Through Shoot Tip and Callus Culture**

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ABSTRACT. *Crossandra* (*Crossandra infundibuliformis* var. *Danica*) is one of Sri Lanka's flowering potted plants, recently introduced to the international floriculture market. The challenge today is to develop this plant for its ornamental characters, while maintaining its demand for many more years. *In-vitro* culture has often been acknowledged as one of the most promising avenues in plant improvement. This requires the availability of reliable protocols for micropropagation. Therefore, efforts were made to develop efficient *in-vitro* protocols for shoot tip and callus cultures. Tender leaves and immature stem explants were cultured in modified MS media containing two sucrose levels (2% and 3%) with different combinations of 2-4, D and kinetin for callus initiation. The highest callus formation (100%) was observed in immature stems in the medium supplemented with 2% sucrose, 1 mg/l 2-4, D and 1 mg/l kinetin after 4 weeks of culture in the dark. The best callus proliferation (90%) was observed in the medium supplemented with 2% sucrose, 4 mg/l 2-4, D and 5 mg/l kinetin after 8 weeks of culture in the dark. Shoot tips were cultured on half MS medium supplemented with different combinations of BAP and IAA. BAP alone (3 mg/l) produced the best results in terms of % survival and average number of shoots per culture. In the multiplication stage, selected significant level of BAP for shoot establishment was further elaborated without IAA. The highest shoot multiplication and growth was observed in the medium supplemented with 1.5 mg/l BAP. The regenerated shoots were elongated, and rooted on hormone free MS medium. Addition of activated charcoal (0.2%) to the above medium improved rooting and 95% plant survival was achieved during acclimatization of the rooted plantlets.

INTRODUCTION

Ornamental crops include many varieties of a wide range of plants, which, in some countries make a substantial contribution to the value of the horticultural products of those countries. For modern and industrialised horticulture, there is always a demand and necessity for new varieties. The plant *Crossandra* var. *Danica* is a beautiful flowering shrub that has recently been introduced to the international floriculture market. It is a natural chimera (spontaneous mutation) of one tropical flowering shrub known as *Crossandra infundibuliformis* L. which belongs to the genus *Crossandra* in the Family *Acanthaceae*.

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The challenge today is to develop this plant for its ornamental values, while maintaining its other unique and existing ornamental characters such as glossy and green ovate leaves, long lasting tubular flowers which bloom on top of green flowering spikes suited for export market. To fulfil this target, genetic variability within this crop must be increased using plant-breeding methods (Longman, 1985).

The combination of *in-vitro* culture techniques with induced mutagenesis is a fast, rapid and efficient method to obtain new cultivars from well-adapted genotypes. The paraxial techniques of plant regeneration and transgenesis in combination with induced mutation can be used for the improvement of vegetatively propagated plants, particularly ornamentals and fruit trees (Datta, 1997).

In-vitro techniques allow induction of mutations in large numbers of propagules in a small space and several cycles of sub culture can be carried out in a short duration. Following the treatment with physical or chemical mutagens, the resulting chimeras can be separated into solid mutant sectors rapidly, and the population can be advanced to produce sufficiently large numbers for selection. In addition, there is no loss of the mutants, because micropropagules are sub cultured under insect and disease free conditions. Cultures of apical meristems, axillary buds, microcuttings and microplants, regenerative and embryogenic calli and cell suspensions, allow mutagenic treatment of millions of cells with physical or chemical mutagens. *In-vitro* culture techniques also allow free selection of some desired variants, which can then be selected in the field (Ahloowalia *et al.*, 1996). Thus this study was undertaken to develop reliable protocols for *in-vitro* propagation of *Crossandra* var. Danica through shoot-tip and callus culture techniques. These protocols could then be used as the baseline for creating novel genotypes of *Crossandra* var. Danica using somaclonal variation and *in-vitro* induced mutation.

MATERIALS AND METHODS

Explants

Tender leaves, immature stems and actively growing apical and axillary shoot tips of *Crossandra infundibuliformis* var. Danica were collected from a 6 month old mother plant stock for culture initiation.

The explants were thoroughly washed under running tap water for 30 min. They were then surface sterilized with 70% ethanol for 10 sec, followed by immersion for 20 min in 10% Chlorox to which 2 drops of Tween-20 was added. They were then rinsed thrice in sterilized distilled water in order to remove all traces of the disinfectant.

Callus initiation

Pieces of tender leaves (about 1 cm² size including mid ribs) and immature stem sections (about 1 cm) were excised aseptically and cultured on MS medium (Murashige and Skoog, 1962) supplemented with combinations of sucrose (2 and 3%), kinetin (1, 2, 3 and 5 mg/l) and 2,4-D (1, 2, 3 and 5 mg/l) for callus initiation. The cultures were incubated in

the dark. After 4 weeks in culture, percentage of explant survival and percentage callus initiation were recorded and the extent of callus growth was ranked visually.

Callus multiplication

Calli collected from the initiation medium were sub-cultured on different combinations of kinetin (0.5, 2, 3 and 5 mg/l) and 2-4, D (0.5, 1, 3 and 4 mg/l) at 2% sucrose level for callus proliferation. The cultures were incubated in complete dark.

After 8 weeks in culture, percentage callus proliferation was recorded and the extent of callus proliferation was ranked visually.

Establishment of shoot tip cultures

Shoot tips were excised aseptically and cultured on modified MS medium, where the concentration of macro elements of MS basal medium was reduced to half strength and supplemented with different combinations of cytokinin (kinetin or BAP) and auxin (IAA).

Each treatment combination consisted of 10 replicates. Survival rate, regeneration percentage and average number of shoots per explant were recorded, 4 weeks after culturing.

Multiplication of shoots

The aim of this step was to determine the optimum concentration of BAP for shoot multiplication. Hence selected significant level of BAP for shoot establishment was further elaborated without IAA.

In this study, explants were obtained from one-month-old stock cultures that were grown in selected establishment medium.

Four weeks after transferring to multiplication media, average number of shoots produced per explant and average length of shoots were recorded.

Shoot elongation

The multiple shoot clusters were dissected and cultured on hormone-free MS medium. The average length of shoots was recorded after 3 weeks in culture.

Induction of rooting

The isolated shoots were transferred to MS media supplemented with different concentrations of IBA (0-5 mg/l) for rooting. After 4 weeks in culture, average number of roots per explant and percentage of rooted cultures were recorded.

The selected rooting medium was further modified by adding 0.2% activated charcoal and the rooted cultures were sub cultured on the modified medium.

Acclimatization of plantlets

The rooted plantlets were transferred to the laboratory and maintained at room temperature for another week, to enhance the acclimatization process. Then they were transferred to plastic pots (12×9 cm) containing a mixture of sterile garden soil, sand and cattle manure (1:1:1) and maintained for another 5-6 weeks before being transferred to normal plant house conditions.

Culture incubation

The shoot cultures were maintained at $26 \pm 1^\circ\text{C}$ temperature and 16 h photoperiod (cool white fluorescent tubes, $60 \mu\text{E}/\text{m}^2/\text{s}$ at the culture level).

Data analysis

The effects of different treatments for callus initiation, callus multiplication and plant regeneration were quantified as percentages. Number of shoots per explant, shoot length and number of roots per explant for different treatments were subjected to Analysis of Variance (ANOVA) followed by Least Significant Difference (LSD) test to determine significance of difference between mean values.

RESULTS AND DISCUSSION

Callus initiation

In both types of explants, callus initiation was high in media containing 1.0-2.0 mg/l kinetin and 1.0 mg/l 2, 4-D with 2% sucrose (Table 1 and 2). Based on the percentage callus initiation and extent of callus growth, the immature stems were selected as the ideal explant and the best medium for callus initiation was shown to be the MS medium supplemented with 1 mg/l kinetin and 1 mg/l 2-4, D with 2% sucrose (Fig. 1a).

Dodds and Roberots (1985) have reported that the addition of 2, 4-D (0.2-2.0 mg/l) induced callus formation in many plant tissues. They have further reported that, the addition of cytokinin (0.5-2.0 mg/l) could enhance callus initiation in many tissues. The cytokinin and auxin requirement for callusing generally vary with the plant species. For good callus production from shoot segments and shoot tips of Sandal wood, the presence of kinetin together with 2, 4-D is required (Lakshmi Sita *et al.*, 1979).

Table 1. Effect of 2, 4-D and kinetin (with 2% sucrose) on callus initiation in immature stems and tender leaves of *Crossandra* var. Danica after 4 weeks in culture.

2, 4-D (mg/l)	Kinetin (mg/l)	2% sucrose in MS media					
		Immature stems			Tender leaves		
		% explant survival	% callus initiation	Callus growth ranking	% explant survival	% callus initiation	Callus growth ranking
1	1	100	100	+++	100	40	++
2	1	100	60	++	90	0	-
3	1	90	0	-	80	0	-
5	1	50	100	+	100	0	-
1	2	100	70	++	100	55.5	+++
2	2	90	77.7	++	80	50	+
3	2	60	66.6	+	90	0	-
5	2	40	0	-	90	0	-
1	3	90	66.6	+	70	0	-
2	3	80	80	+	70	71.42	+
3	3	80	75	+	50	0	-
5	3	40	0	-	30	0	-
1	5	50	60	+	30	100	+
2	5	40	100	++	30	100	+
3	5	50	100	++	40	75	+
5	5	20	0	-	20	0	-

Each treatment consisted of 20 replicates
 - no callus, + poor callus, ++ fair callus, +++ good callus

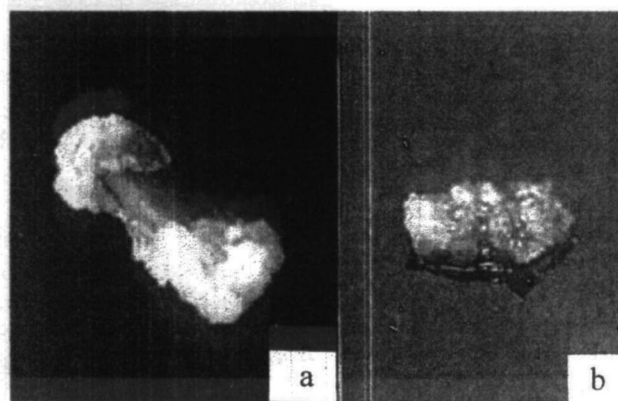


Fig. 1. a) Callus initiation in immature stems cultured in MS medium with 1 mg/l kinetin and 1 mg/l 2, 4-D. b) Multiple callus masses in MS medium with 5 mg/l kinetin and 4 mg/l 2, 4-D.

Table 2. Effect of 2, 4-D and kinetin (with 3% sucrose) on callus initiation in immature stems and tender leaves of *Crossandra* var. Danica after 4 weeks in culture.

2, 4-D (mg/l)	Kinetin (mg/l)	3% sucrose in MS media					
		Immature stems			Tender leaves		
		% explant survival	% callus initiation	Callus growth ranking	% explant survival	% callus initiation	Callus growth ranking
1	1	90	66.6	+	80	0	-
2	1	100	0	-	70	57.14	+
3	1	80	0	-	40	0	-
5	1	80	0	-	50	40	+
1	2	80	87.5	+	60	100	+
2	2	70	42.85	+	40	75	+
3	2	50	0	-	20	0	-
5	2	40	0	-	20	0	-
1	3	70	85.7	++	20	0	-
2	3	60	66.6	++	0	0	-
3	3	40	0	#	10	0	-
5	3	10	0	#	0	0	#
1	5	10	10	+	0	0	#
2	5	0	0	#	0	0	#
3	5	0	0	#	0	0	#
5	5	0	0	#	0	0	#

Each treatment consisted of 20 replicates

- no callus, + poor callus, ++ fair callus, +++ good callus, # explant degenerated

Callus multiplication

After transferring the calli to the multiplication media, newly proliferated callus masses were observed in some of the media containing various levels of 2, 4-D and kinetin (Table 3).

Callus proliferation was highest in the medium supplemented with 4 mg/l 2, 4-D and 5 mg/l kinetin after 8 weeks in culture (Table 3). The calli were nodular in shape and white in colour at the early stages of development. At a later stage (after another 8 weeks), the colour of callus changed from white to pale yellow (Fig. 1b). When selected culture media were kept for another 4 weeks in complete darkness, initiation of roots was observed in the peripheral region of callus proliferated in the multiplication medium. Doi (1981) observed similar results in proliferated callus masses in tea anther culture. This may be due to the high concentration of 2, 4-D present in callus multiplication medium.

Browning of callus growing in multiplication media was minimized by incorporating PVP (2 g/l) in the media tested.

Table 3. Effect of different combinations of 2,4-D and kinetin (with 2% sucrose) on callus proliferation in *Crossandra* var. *Danica* after 8 weeks in culture.

Growth regulators (mg/l)		% callus proliferation	Ranking of callus proliferation
Kinetin	2,4-D		
0.5	0.5	0	-
2.0	0.5	0	-
3.0	0.5	0	-
5.0	0.5	10	+
0.5	1.0	0	-
2.0	1.0	30	+
3.0	1.0	20	+
5.0	1.0	40	+
0.5	3.0	0	-
2.0	3.0	10	+
3.0	3.0	20	+
5.0	3.0	60	++
0.5	4.0	0	-
2.0	4.0	30	+
3.0	4.0	30	+
5.0	4.0	90	+++

Each treatment consisted of 20 replicates

- no callus, + poor callus, ++ fair callus, +++ good callus

Establishment of shoot tip culture

It was evident that the cytokinins did not have any effect on the survival of *in-vitro* grown shoot tips of *Crossandra* var. *Danica* (Tables 4 and 5).

The type of cytokinin present in the medium affected the average number of shoots per explant. BAP gave rise to a higher number of shoots per explant when compared to kinetin. Thus, BAP was more effective than kinetin, with respect to breaking the apical dominance of shoot tips. This is in accordance with the results of Amin and Jaiswal (1992) who reported the effect of cytokinins on *in-vitro* response of apical bud explants from mature trees of *Artocarpus heterophyllus*.

Boxus, 1997 has reported that although incorporation of cytokinins in culture media prevents apical dominance, it induces axillary branching and the mean number of axillary shoots per explant varies depending on the type of cytokinin introduced.

Table 4. Effect of different combinations of cytokinin (Kinetin) and auxin (IAA) on shoot tip establishment in *Crossandra* var. Danica after 8 weeks in culture.

Growth regulators (mg/l)		Survival %	% bud breaking	Mean number of shoots/explant
Kinetin	IAA			
2	0	60	50	1.80
3	0	90	60	1.50
4	0	80	50	2.40*
5	0	60	66.67	1.70
2	1	70	80	1.50
3	1	60	25	2.00
4	1	60	66.67	1.60
5	1	80	80	1.20
2	3	70	66.67	1.40
3	3	60	33.33	1.70
4	3	60	50	1.30
5	3	60	40	0.80
CV (%)				15.73
LSD (0.05)				0.43

Each treatment consisted of 10 replicates * significant at 0.05 level

Table 5. Effect of different combinations of cytokinin (BAP) and auxin (IAA) on shoot tip establishment in *Crossandra* var. Danica after 8 weeks in culture.

Growth regulators		Survival %	% bud breaking (% regeneration)	Mean number of shoots/explant
BAP (mg/l)	IAA (mg/l)			
2	0	80	75	6.00
3	0	80	100	6.40*
4	0	60	80	4.00
5	0	60	60	4.70
2	1	70	85.70	4.90
3	1	70	62.85	5.30
4	1	80	87.5	5.10
5	1	70	57.14	4.40
2	3	60	60	4.80
3	3	70	57.14	4.40
4	3	70	57.14	4.60
5	3	60	66.67	5.00
CV (%)				8.82
LSD (0.05)				0.13

Each treatment consisted of 10 replicates * significant at 0.05 level

Combination of BAP and IAA gave rise to a higher regeneration and increased number of shoots per explant than the combination of kinetin and IAA (Table 4 and 5). However, 3 mg/l BAP without IAA was superior ($p=0.05$) to other treatments for *in-vitro* establishment of shoot tips of *Crossandra* var. Danica giving 100% regeneration as well as an average number of 6.40 shoots per explant (Table 5).

Multiplications of shoots

Inhibition of shoot growth and significant reduction in shoot numbers were observed in the lowest (0.5 mg/l) and highest (2.5 mg/l) concentrations of BAP tested (Table 6). In the medium without hormone, multiplication and growth of shoots was very low. The highest shoot multiplication and growth of shoots ($p=0.05$) observed in the medium containing 1.5 mg/l BAP (Fig. 2). In this treatment, the mean number of shoots produced per explant after 8 weeks in culture was 9.80 (Table 6).

Table 6. Effect of the concentration of BAP on multiplication and growth of shoots of *Crossandra* var. Danica after 4 weeks in culture.

BAP (mg/l)	Mean number of shoots per explant	Mean shoot length (cm)	Callus formation at the explant base
0.0	4.70	0.50	+
0.5	6.00	0.70	+
1.0	6.80	0.70	+
1.5	9.80*	2.50*	+
2.0	7.00	1.40	+
2.5	2.30	0.40	+
CV (%)	16.43	9.98	
LSD (0.05)	0.48	0.26	

Each treatment consisted of 10 replicates

* significant at 0.05 level

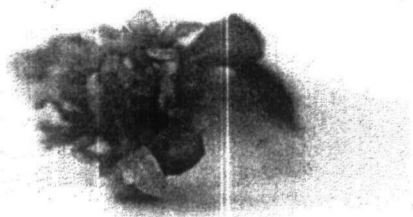


Fig. 2. Shoot multiplication in *Crossandra* in 1/2 MS medium containing 1.5 mg/l BAP after 8 weeks in culture.

Shoot length increased with increasing concentrations of BAP (up to 1.5 mg/l) in the medium and it declined again when the concentration was below 1.5 mg/l. The maximum mean shoot length of 2.40 cm ($p=0.05$) was obtained at 1.5 mg/l BAP. These shoots were used for rooting experiments, as shoots from the other treatments were too small.

This study indicates that a high BAP level is required to achieve *in-vitro* multiplication of *Crossandra* var. *Danica*. This observation is in agreement with the results obtained by Mulwa and Bhalla (2000), where higher levels of BAP (2 mg/l) were needed for shoot multiplication in *Macadamia tetraphylla*.

In *Gravillea scapigera*, another ornamental Protaceous plant, incorporation of 0.1 mg/l BAP in the medium resulted in a 2-3 fold increase in shoot multiplication and shoot elongation of up to 20 mm was observed (Bunn and Dixon, 1992). Furthermore, the use of 0.1 mg/l BAP and 1.0 mg/l kinetin in the medium resulted in a 5-6 fold increase in shoot multiplication of *Gravillea scapigera* (Bunn and Dixon, 1992) indicating the requirement of a higher level of cytokinin in the multiplication medium. Sahoo and Chand (1998) and Lemos and Blake (1996) also reported optimal bud break for nodal cultures of *Vitex negundo* and *Annona muritica* in media containing 2 mg/l BAP. In this study, BAP levels above 2 mg/l resulted in abnormal, hyperhydric and stunted shoots. Therefore 1.5 mg/l BAP was considered to be the optimum for shoot multiplication and initial shoot development in *Crossandra* var. *Danica*.

Shoot elongation

The multiple shoot clusters elongated with an average length of 3.6 cm in hormone free MS medium after 20 days in culture (Fig. 3a).

Induction of rooting

Hormone free MS medium (control treatment) could induce sufficient roots from *in-vitro* grown shoots (with 95% success) after 45 days in culture (Fig. 3b). The necessary endogenous hormone balance required for rooting (which is present in the tissues transfer to rooting media) could have been maintained in the hormone-free medium. Increasing levels of IBA in rooting media induced calli at the basal portion of shoot cultures which inturn interrupted *in-vitro* rooting in this genotype (Table 7).

Roots hardened with many lateral roots when the rooted cultures were separated and transferred to the modified rooting medium with 0.2% activated charcoal for another 3 weeks. With the introduction of charcoal, the dark environment of the cultures may have promoted rooting. Ranasinghe *et al.* (2000) have observed similar results for *in-vitro* root induction in *Cryptocoryne wendtii*, an endemic aquatic, ornamental plant species.

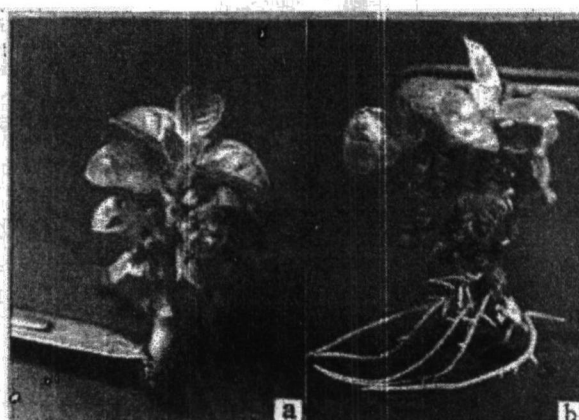


Fig. 3. a) An elongated shoot of *Crossandra* in a hormone free, MS medium after 20 days in culture. b) A rooted shoot of *Crossandra* in a hormone free, MS medium after 45 days in culture.

Table 7. Effect of different concentrations of IBA on *in-vitro* root formation in *Crossandra*.

IBA (mg/l)	Root ability		Remarks
	Average number of roots per explant	% rooted	
0.0	5.6	90	Good root growth and no basal callus
0.5	1.1	20	Poor root growth with basal callus
1.0	-	0	No root growth and high callus formation at explant base
2.0	-	0	No root growth with high callus formation at explant base
5.0	-	0	Explant degenerated

Each treatment consisted of 10 replicates

Acclimatization of plantlets

Acclimatization of plantlets was conveniently achieved by transferring them to plastic pots containing a mixture of sterile garden soil, sand and cattle manure (1:1:1). Within 10-12 weeks of transfer to plastic pots, the plants showed healthy growth with 95% survival rate under normal plant house conditions (Fig. 4).

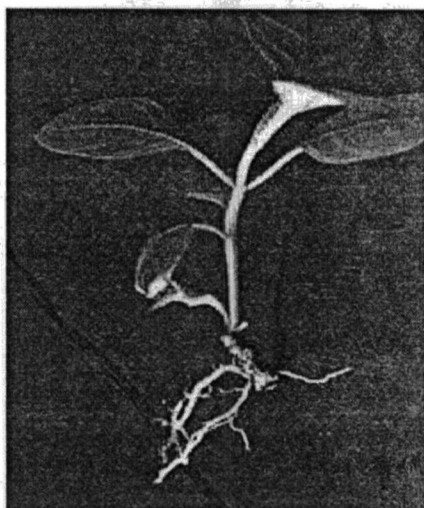


Fig. 4. Shoot tip culture derived *Crossandra* plant let - 3 weeks after acclimatization.

CONCLUSIONS

The present study indicates that *in-vitro* propagation of *Crossandra infundibuliformis* var. *Danica* through callus and shoot tip cultures is feasible. Therefore, the protocols established in this study can be used as a baseline for future studies on somaclonal variation and *in-vitro* induced mutation of this important plant.

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