Effects of Flower Stalk Node Positions and BA Concentrations on Shoot Bud Formation of *Tolumnia* Snow Fairy *in vitro*

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Key words: Tolumnias, Flower stalk culture

Summary

Tolumnia are indigenous to the Caribbean islands, and have extensive habitats in that region. Plants are triangular succulent leaves that form small fan shaped growths and lack of a pseudobulb. The aims of this experiment were developed an efficient tissue culture system for clonal propagation of tolumnias and tested the effects of the plant growth regulators 6-benzyladenine (BA) and flower stalk node positions on shoot development of *Tolumnia* Snow Fairy. After 16 weeks of culture, the 4th node position of flower stalks in *Tolumnia* Snow Fairy showed the highest (57.1%) shoot formation rate. Moreover, there showed higher response on ½ MS salt basal medium supplemented with 4 mg/L BA. And after 9 months of *in vitro* culture, Plantlets were transplanted to greenhouse condition presented 100% of the plantlets of *Tolumnia* Snow Fairy survived after 90 days of transfer.

Introduction

The group of orchids now called *Tolumnia* was one time called *Oncidium* section *Variegata* and was commonly referred to as “equitant oncidiums” (Aldrich, 1994). Plants in this genus are endemic to Caribbean islands. Furthermore, these plants are miniature oncidium that possess

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variable sympodial epiphyte with triangular succulent leaves that form small fan shaped growths and most appearances show of lack pseudobulb (Aldrich, 1994; Baker and Baker, 2006).

Clearly, several plant parts have been used for tissue culture: stem, leaf, floret, and flower stalk (Arditti, 2008). Although these vegetative tissues taken from *in vitro* grown plantlets are able to form embryos at a high frequency, whereas the mother plant may suffer from serious damage after the explants have been taken (Chen and Chang, 2000; Chugh *et al.*, 2009; Mitsukuri *et al.*, 2009a,b). Excision of this developing inflorescence is easy and can conserve the mother plants. Furthermore, floral organs, including flower-stalk tips, micro-inflorescences, florets, flower-stalk buds and internodes of inflorescence, have often been used as explants induce PLBs or shoots, then through PLBs or shoot multiplication can subsequently satisfy the need of mass propagation. The aims of this experiment were developed an efficient tissue culture system for clonal propagation of tolumnias and tested the effects of the plant growth regulators 6-benzyladenine (BA) and flower stalk node positions on mass propagation of *Tolumnia* Snow Fairy.

Materials and Methods

I. Flower stalks culture

1. Plant materials

Tissue culture materials were obtained from the flower stalk nodes of *Tolumnia* Snow Fairy. *Tolumnia* Snow Fairy mature plants were cultivated in pots in net greenhouse from Horticultural Research Station, College of Agriculture and Natural resources, National Chung Hsing University. Flower stalk nodes at an early developmental phase (still burgeon) were selected from inflorescences bearing more 5 flower stalk nodes to investigate the effects of flower stalk node positions on shoot bud formation efficiency. They were counted at lower positions to upper positions, the first flower stalk node position to the fourth flower stalk node position were selected for use as explants. Flower stalks were washed under running tap water and then removed of outer dry scales. The explants were surface-sterilized in 2% sodium hypochlorite for 20 minutes followed by 3 washes with sterilized distilled water.

2. Culture medium and growth conditions

The explants were placed on the surface (one explant per tube) of half strength MS salts medium (Murashige and Skoog, 1962) containing 100 mg/L *myo*-inositol, 170 mg/L NaH_2PO_4, 30

g/L sucrose, 80 mg/L adenine and 8 g/L agar supplemented with, 0.2 mg/L α-naphthaleneacetic acid (NAA), 1 mg/L kinetin and different concentration of 6-benzyladenine (BA) (1, 2, 4 and 8 mg/L). The culture mediums without BA treatment were considered as control. In effects of flower stalk nodes positions and BA concentrations, for each treatment, about 7 explants were cultured. In each culture vial, one piece was inoculated. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl prior to autoclaving and autoclaved at 121°C for 20 min. All the cultures were maintained in a culture room at 25± 2°C under a photoperiod of 12 h provided by cool white fluorescent with 56 μmol $m^{-2}s^{-1}$ of light intensity.

3. Observation

Flower stalk nodes were subcultured every 5 weeks during culture period. After 8 weeks of culture, shoot bud formation percentage, flower stem (peduncle form) percentage, contamination percentage and survival percentage were recorded and evaluated. Cultures were examined and photographed with a stereozoom microscope (SZH, Olympus).

II. Plantlets culture and transplantations

1. Plant materials

Six-month-old *in vitro*-grown plantlets of *Tolumnia* Snow Fairy was obtained from direction and multiple shoots.

2. Culture medium and growth conditions

Explants were cultured on half strength MS salts medium (Murashige and Skoog, 1962) containing 100 mg/L *myo*-inositol, 170 mg/L NaH_2PO_4, 20 g/L sucrose, 150 mL/L coconut water, 1 g/L peptone, 1 g/L activated charcoal and 8 g/L agar. The pH of the medium was adjusted to 5.2 with 0.1 N NaOH or HCl prior to autoclaving and autoclaved at 121°C for 20 minutes. All the cultures were maintained similarly with flower stalk culture condition. Explants were subcultured every 12 weeks during culture period.

3. Acclimatization and transplantation

After 9 months of *in vitro* culture, fully developed plantlets were transferred to white plastic pots containing with sphagnum moss. The potted plantlets were then maintained in the fan and pad greenhouse. Pots were maintained in greenhouse and which were irrigated once or twice every one week and every two months. Plants were irrigated with 0.5 g/L Peter’s professional 20N-20P-20K general purpose water soluble fertilizer liquid feed.

4. Observation

After 9 months of *in vitro* culture from first time of subculture, the number of leaves, the number of plantlets and the plant height were evaluated. After 60 and 90 days of cultivated, the number of leaves, the number of plantlets, the height and new shoots were recorded and evaluated on survived plants.

Results

After 16 weeks of culture, the effect of flower stalk node positions of *Tolumnia* Snow Fairy and BA concentrations on shoot induction could be explained in Table 1. The 4th node position with 4 mg/L BA showed the best survival rate (100%). After 8 weeks of culture, the highest shoot formation achieved using the 2nd node position with 4 mg/L BA (Fig. 1A). However, after 16 weeks of culture, among all node position tested, the 4th node position induced the highest frequency of shoot formation, especially in the 4th node position with 4 mg/L BA. Among the different level of BA concentrations evaluated the best was 4 mg/L BA which resulted in 57.1% on shoot formation.

Direct shoot was form from single node of the 4th node position with 2 mg/L BA after 8 weeks of culture (Fig. 1B). Multiple shoot was formed at the 3rd and 4th node position, respectively (Fig. 1C-D). No shoot bud formation and flower stem (peduncle form) formation were found at the 1st node position and all node position when cultured on BA free medium. In addition, the highest percentages of flower stem (peduncle form) formation were found with 4 and 8 mg/L BA (Fig. 1E; Table 1). However, the frequency of flower stem (peduncle form) production increased with increased upper location of node position. After 16 weeks of culture, flower stalk node explants taken from the 4th node position tended to induced flower stem (peduncle form) before turning to vegetative shoot (Fig. 1F). The highest percentage of contamination was observed in 100% of the 1st node position with 1, 2 and 4 mg/L BA and BA free medium.

The cultures were then maintained for 9 months under ½ MS salt basal medium for rooting grow about three times of subculture (Fig. 2A). *Tolumnia* Snow Fairy gave better results in term of number of roots (Fig 2B). 100% of the plantlets of *Tolumnia* Snow Fairy (Fig. 2C) survived after 90 days of transfer (Table 2). There showed new shoot formation from rhizomatous enlargement at the base of old shoot (Fig. 2D)

Table 1. Effects of flower stalk node position and BA concentration on shoot bud formation of *Tolumnia* Snow Fairy after 16 weeks of culture *in vitro*.

Node position[z]	BA (mg/L)	Survival (%)	Shoot formation (%)	Flower stem[y] (%)	Contamination (%)
1	0	0.0	0.0	0.0	100.0
	1	0.0	0.0	0.0	100.0
	2	0.0	0.0	0.0	100.0
	4	0.0	0.0	0.0	100.0
	8	14.3	14.3	0.0	85.7
2	0	14.3	14.3	0.0	57.1
	1	14.3	14.3	0.0	85.7
	2	14.3	14.3	0.0	71.4
	4	42.9	42.9	0.0	57.1
	8	28.6	28.6	0.0	71.4
3	0	57.1	28.6	0.0	42.9
	1	28.6	14.3	0.0	71.4
	2	28.6	14.3	0.0	71.4
	4	14.3	0.0	0.0	85.7
	8	42.9	28.6	0.0	42.9
4	0	42.9	42.9	0.0	28.6
	1	71.4	42.9	28.6	28.6
	2	28.6	28.6	0.0	57.1
	4	100.0	57.1	42.9	0.0
	8	57.1	14.3	42.9	28.6

[z] They were counted at lower positions to upper positions, the first flower stalk node position to the fourth flower stalk node position were selected. (7 samples per treatment)

[y] Flower stem : Peduncle form.

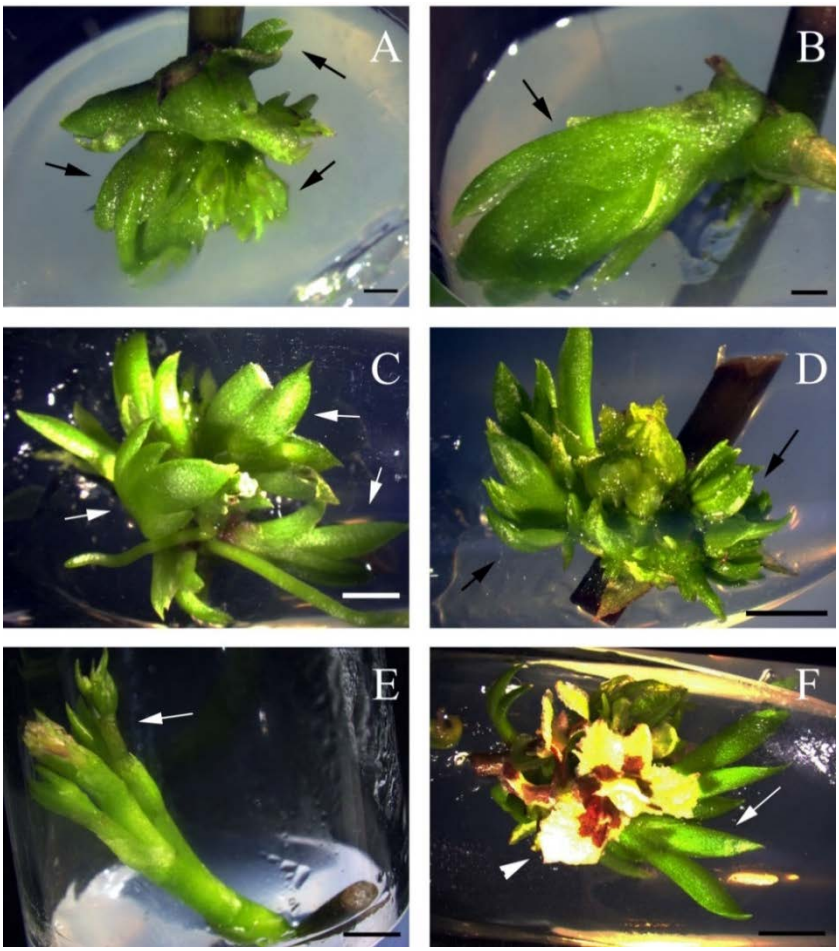

Fig. 1. Effects of flower stalk nodes positions and BA concentrations of *Tol.* Snow Fairy
(A) Shoot bud formation at 2nd node position with 4 mg/L BA after 8 weeks of culture (*arrow*) (bar = 1 mm); (B) Shoot bud formation at 4th node position with 2 mg/L BA after 8 weeks of culture (*arrow*) (bar = 1 mm); (C) Shoot bud formation at 3rd node position with 2 mg/L BA after 16 weeks of culture (*arrow*) (bar = 3 mm); (D) Shoot bud formation at 4th node position with 4 mg/L BA after 16 weeks of culture (*arrow*) (bar = 3 mm); (E) Flower stem (peduncle form) formation at 4th node position with 8 mg/L BA after 8 weeks of culture (*arrow*) (bar = 3 mm) and (F) Flower stem (peduncle form) formation and turned to induced shoot formation at 4th node position with 4 mg/L BA after 16 weeks of culture (*arrow*) (bar = 4 mm)

Table 2. The survival and growth of plantlets of *Tolumnia* Snow Fairy derived from flower stalk node culture in greenhouse

Durations[z]	Survival (%)	Plant height[x] (cm)	No. of leaves[x]	New shoot[w]/plant
1 days	100.0[y]	4.6 ± 0.2	11 ± 0.2	0.0
60 days	100.0	4.6 ± 0.1	12 ± 0.1	0.0
90 days	100.0	4.9 ± 0.1	12 ± 0.1	0.5

[z] After transplanting

[y] 170 plantlets were used.

[x] Means of totally plantlets ± Standard error.

[w] New shoot : which grows from rhizomatous enlargement at the base of old shoot.

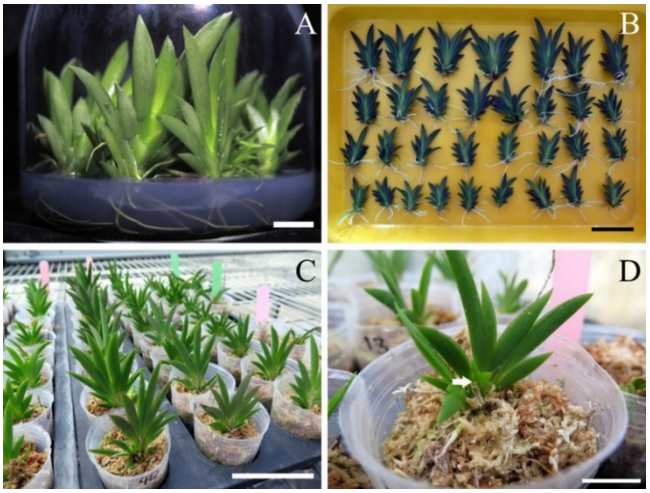

Fig. 2. Transplantation and growth at greenhouse of plantlets derived from flower stalks node of *Tol.* Snow Fairy

(A) *Tol.* Snow Fairy plantlets on regeneration medium (bar = 1 cm); (B) *Tol.* Snow Fairy transplantation (bar = 5 cm); (C) *Tol.* Snow Fairy in greenhouse after 3 months of transplantation (bar = 5 cm) and (D) New shoot formation from rhizomatous enlargement at the base of old shoot (*arrow*) (bar = 1 cm)

Discussions

Most of 100% contamination rates were found at the 1st node position of *Tolumnia* Snow Fairy. Although, Mitsukuri *et al.* (2009a) found among explants of *Ponerorchis graminifolia* Rchb.f. from reproductive stages plants which presented highest survival whereas the explants located next to vegetative stages plants will showed the higher rates contamination as similar results were also reported by Mitsukuri *et al.* (2009b). Who presented that when flower stalks of *Habenaria radiate* were cultured that also showed higher percentage of contamination. Moreover, it is hard to obtain aseptic explants from mature plant though normal surface sterilization steps for *in vitro* culture like the results of *Paphiopedilum* Armeni White and *Paphiopedilum* Deperle (Liao *et al.*, 2011).

When shoot buds were formed which presented some bud first emerged from the node and swelled into globular structure, then initiated adventitious bud and finally which produced numerous axillary shoot from the base after 2-3 months, which is in agreement with previous studies on *Phalaenopsis* (Chen and Piluek, 1995) and *Vanda spathulata* (Decruse *et al.*, 2003). In addition, most of none buds formed at the lower position (1st and 2nd nodes position), which might be due to degeneration or dormancy, similar results were also reported by Vendrame *et al.* (2007) and Mitsukuri *et al.* (2009a). Higher percentages of flower stems (peduncle form) were observed in *Tolumnia* Snow Fairy at the 4th nodes position. This could be probably due to flowering stimulant which may exist in flower stalk tissue. These finding are in agreement with the reports of *Phalaenopsis* (Tanaka *et al.*, 1988). However, higher rates of shoot formation were increased after 16 weeks of culture that showed nodal positions taken from the position near the upper location tended to induced flower stem (peduncle form) before turning to multiple shoot around peduncle like *Phalaenopsis* (Tsao *et al.*, 2008).

BA is better for shoot formation by induced the cytoplasmic zone of apical meristem to enlarge and enhanced leaf development as reported in *Cymbidium forrestii* (Paek and Yeung, 1991) and *Geodorum densiflorum* (Sheelavantmath *et al.*, 2000). There showed higher response on ½ MS salt basal medium supplemented with 4 mg/L BA. These results presented first the frequency of flower stem (peduncle form) production increased with increased upper location of node position and later the number forming of new shoots and the number of new shoots per explants increased with time. Similar results were also reported (Tsao *et al.*, 2008; Wu and Chen, 2008; Tsao *et al.*, 2011). Plantlets after transplants that showed slowly growth may be due to the transplantation time not

suitable for vegetative development phase, whereas first transplanted in winter season, which could explain why they are growing slowly in 90 days of transplantation.

Conclusions

The aims of this experiment were developed an efficient tissue culture system for clonal propagation of tolumnias and tested the effects of the plant growth regulators 6-benzyladenine (BA) and flower stalk node positions on mass propagation of *Tolumnia* Snow Fairy. After 16 weeks of culture, the 4th node position of flower stalks in *Tolumnia* Snow Fairy showed the highest (57.1%) shoot formation rate. Moreover, there showed higher response on ½ MS salt basal medium supplemented with 4 mg/L BA. However, nodal position taken from the position near the upper location tended to induced flower stem (peduncle form) before turning to multiple shoot around peduncle. On the other hand, the percentages of contamination were observed in 100% of the 1st node position explants. Plantlets establishment and transplantations derived from flower stalk node were cultured and transplanted to greenhouse condition presented 100% of the plantlets of *Tolumnia* Snow Fairy survived after 90 days of transfer and which showed about 0.5 new shoot per plant.

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節位與 BA 濃度對劍葉文心蘭花梗節培養芽體形成之影響

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摘要：劍葉文心蘭原產於加勒比海群島並廣泛的分佈於該區域，帶有三角形之肉質葉，葉片成扇形排列，無假球莖之小型文心蘭。本研究目的為建立對劍葉文心蘭有效的組織培養方法，並進行不同濃度的 BA 試驗，及花梗節位對 *Tolumnia* Snow Fairy 大量培養的影響，培養 16 週後，*Tolumnia* Snow Fairy 的最佳繁殖節位為第四節，芽體形成率最高，達 57.1%，而最佳培養基則為 1/2 MS + 4 mg/l BA；瓶內培養 9 個月後，馴化移植至溫室中栽培，移植 90 天後，*Tolumnia* Snow Fairy 的存活率為 100%。

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