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Pupae are excellent explants with low microbial contamination and high regeneration frequency for micropropagation of *Freesia ×hybrida* Bailey 'Argenta'

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Abstract
Two separate factorial experiments were conducted to study the effects of explant sources, plant growth regulators, sucrose concentrations, and light conditions on *in vitro* cormlet formation of freesia (*Freesia ×hybrida* Bailey 'Argenta'). Interestingly, it was observed that the pupae had lower contamination levels compared to mother corms. Using 40% sodium hypochlorite solution for 40 min, contamination levels of pupae and mother corms reduced to 19.80 and 46.40%, respectively. Moreover, pupae showed the highest regeneration frequency. In the first experiment, 6.67 cormlets were directly produced per pupa (cold storage-produced corm) on Murashige and Skoog (MS) medium supplemented with 6 mg L^{-1} 1-naphthaleneacetic acid (NAA), 1 mg L^{-1} 6-benzylaminopurine (BA), and 60 g L^{-1} sucrose, when cultures were stored in the dark. In the second experiment, on average, 5.67 shoots were proliferated per pupa explant in the presence of 4 mg L^{-1} BA and 2 mg L^{-1} Kinetin (Kin). Subculturing these shoots on MS medium containing 3 mg L^{-1} BA and 0.5 mg L^{-1} NAA led to production of 3.67 cormlets per shoot. Finally, *in vitro* derived cormlets showed the highest percentage of rooting (77.80%), root number (8.33), and root length (2.13 cm) on MS medium containing 1 mg L^{-1} indole-3-butyric acid (IBA).

Keywords: Direct cormlet formation, *in vitro* shoot proliferation, tissue culture.

Introduction

Freesia is an ornamental cormous plant from the Iridaceae family and due to its fragrance, long vase-life and wide color range of flowers, it is one of the most popular cut flowers in Europe and USA (Anderson, 2007). Market research shows that over 110 million stems of freesia are sold in the UK each year (Gao *et al*., 2009).

The tetraploid cultivars of modern freesia (*Freesia ×hybrida* Bailey) play a key role in cut flower production. However, the conventional propagation systems (seeds and corms) of these cultivars are problematic due to a number of factors including long-term juvenility (Petru *et al*., 1976), shorter inflorescences with less numbers of florets in seedlings, low seed setting rate, reduction in cut flower quality, accumulation of plant diseases in corms, and poor ability of mother corms to produce cormlets (Bajaj and Pierik, 1974). Thus, it would be advantageous to replace conventional propagation systems of freesia with tissue culture methods which have many advantages such as increased

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multiplication rates (Van Aartrijk *et al*., 1990), more flowers per spike in regenerated plants, higher quality flowers, elimination of viruses using apical meristem culture (Van Aartrijk *et al*., 1990), facilitated breeding programs, and maintenance of a large number of elite genotypes (Gao *et al*., 2009).

In many cases, the success of *in vitro* propagation relies on the source of explant. Le Nard and De Hertogh (1993) showed that keeping tulip bulbs at low temperatures (2°C) before flower differentiation resulted in the production of apical bulbs, which prevented flower differentiation. Renau-Morata *et al*. (2013) applied the protocol described for tulip bulbs on unplanted saffron corms and produced new corms. They indicated that these new corms were an appropriate source of explants for *in vitro* culture. Similarly, in a phenomenon known as "pupation", if corms of freesia are kept at temperatures lower than 13°C , they fail to sprout and form new corms on the old ones (Hartsema, 1962).

Different plant growth regulators have been successfully used for micropropagation of freesia. Bajaj and Pierik (1974) found that adventitious organ formation in *Freesia* 'Ballerina' was closely related to the auxin/cytokinin ratio in the medium. In a study, freesia plantlets were multiplied on MS medium supplemented with 2 mg L^{-1} BA and 0.01 mg L^{-1} NAA and then new cormlets were regenerated in the presence of 2 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 60 g L^{-1} sucrose (Zhao, 1989). Foxe *et al*. (1991) showed that the apical shoot tips of six cultivars of *Freesia refracta* (Jacq.) Klatt, proliferated on MS medium containing 2 mg L^{-1} kinetin and 1 mg L^{-1} NAA and subsequent cormlet formation was obtained on these shoots.

High concentrations of sucrose could affect *in vitro* cormlet formation of cormous plants (Sinhaand, 2002; Raja *et al*., 2007). Zhao (1989) used MS medium containing 60 g L^{-1} sucrose for cormlet induction and development on *in vitro* derived plantlets of freesia. Sharafzadehand and Khosh-Khui (2004) reported that 50 g L^{-1} sucrose improved cormlet formation in saffron. The fresh weight and number of cormlets of gladiolus was highest on media supplemented with elevated concentrations of sucrose (Kumar *et al*., 2011).

Various studies have shown the effects of light conditions on the induction and development of storage organs (Ascough *et al*., 2008). Bach *et al*. (2000) showed that darkness could promote *in vitro* cormlet formation and growth in *Freesia refracta* (Jacq.) Klatt explants. Marinangeli *et al*. (1998) reported that bulb induction of *Lilium* was promoted by culture in the dark.

The aim of the present work was to establish an effective micropropagation pathway for direct organogenesis of *Freesia* ×*hybrida* Bailey 'Argenta'. Thus, the influences of different factors such as sources of explant, plant growth regulators, sucrose concentrations, and light conditions were investigated.

Materials and Methods

Plant material

Stock plants of *Freesia* ×*hybrida* Bailey 'Argenta' were grown in a greenhouse. The greenhouse equipment for climatic control was set to produce day and night temperatures of 25±2°C and 20±2°C, respectively. Relative humidity was maintained at approximately 60%. A batch of mother corms was stored at 4°C during the summer (July to September) and after 7 to 8 weeks about 2 pupae (cold storage-produced corms) were produced from the apical bud of these corms. Nodular segments of mother corms and intact pupae were used as sources of explant.

Establishment of aseptic culture

Mother corms: This study used a two-step decontamination procedure for the sterilization of mother corms. In the first step, the mother corms were detached

from their tunics and surface sterilization procedure was done which involved washing them under running tap water for 12 h, immersion in 70% (v/v) ethanol (2 min), soaking in different concentrations (0, 10, 20, 30 or 40%) of sodium hypochlorite solution (using commercial bleach at 5.25% (v/v) of active chloride) for various durations (0, 10, 20, 30 or 40 min), followed by triple rinses with sterile water. In the second step and prior to culture, ready explants were immersed for 0, 10, or 15 min in sodium hypochlorite solution at concentrations of 0, 10, or 15%.

Pupae. One-step decontamination procedure was applied for the sterilization of pupae. They were detached from their tunics and surface sterilization procedure was done which involved washing them under running tap water for 12 h, immersion in 70% (v/v) ethanol (2 min), soaking in different concentrations (0, 10, 20, 30 or 40%) of sodium hypochlorite solution (using commercial bleach at 5.25% (v/v) of active chloride) for various durations (0, 10, 20, 30 or 40 min), followed by triple rinses with sterile water.

Culture conditions

Solidified Murashige and Skoog (MS 1962) basal medium was prepared with 8 g L^{-1} agar; the pH was adjusted to 5.8 with 1N NaOH or 1N HCl prior to dispensing in jars and autoclaving at 121ºC and 1.5 kg cm^{-1} pressure for 15 min. Cultured explants were incubated at 25±1°C under 16/8 h (light/darkness) photoperiod at a light intensity of approximately 45 µ mol m^{-2} s^{-1} emitted by cool-white fluorescent tubes. All cultures were subcultured every 4 weeks on the same media.

Cormlet induction, growth, and development

In the first experiment, nodular segments of mother corms and intact pupae were cultured on MS medium. Sterilized mother corms were vertically cut into 8 equal segments and then put on media. The approximate size of each explant was about 1 cm^2.

In this stage, three factors consisting of: (1) type of explant (mother corm or pupae), (2) different concentrations of plant growth regulators including NAA (0, 2, 4 or 6 mg L^{-1}) or 2,4-D (0, 1.5, 3, or 4.5 mg L^{-1}) in combination with BA (0, 0.5, 1, or 1.5 mg L^{-1}), and (3) two concentrations of sucrose (30 or 60 g L^{-1}) were applied. One batch of cultures was kept continuously in the dark while another was under light conditions (16 h photoperiod at a light intensity of 45 µ mol m^{-2} s^{-1} emitted by cool-white fluorescent lamps).

New cormlets were clearly visible after 5 to 6 weeks in cultures kept under darkness. For further development, newly dark induced cormlets were subcultured on media containing NAA (0, 1, 2, or 3 mg L^{-1}), Kinetin (Kin) (0, 0.5, or1 mg L^{-1}), and two levels of sucrose (30 or 60 g L-1) and kept under light conditions.

After about 35 to 40 and 90 days, number and diameter of regenerated cormlets were determined, respectively.

Shoot proliferation and cormlet formation

In the second experiment, in order to study shoot proliferation on intact pupae they were cultured on MS medium. In the next stage, proliferated shoots were subcultured on new MS media to produce cormlets at the base.

Shoot proliferation experiment consisted of two factors including: (1) BA (0, 2, 4, or 6 mg L^{-1}) and (2) Kin (0, 1, or 2 mg L^{-1}). All media contained 1 mg L^{-1} NAA and 30 g L^{-1} sucrose. Cormlet regeneration experiment also consisted of two factors including: (1) BA (0, 1, 3, or 5 mg L^{-1}) and (2) NAA (0, 0.5, or 1 mg L^{-1}). These media were supplemented with 60 g L^{-1} sucrose.

At the end of this step, number and length of proliferated shoots and number of regenerated cormlets at the base of shoots were determined.

Rooting and acclimatization

After shoot formation, *in vitro* derived cormlets were subcultured on MS media containing IBA (0, 1, or 1.5 mg L^{-1}) or NAA (0, 1, or 1.5 mg L^{-1}) and three weeks later transferred to auxin-free medium. Four weeks later, the percentage of rooted plants was determined as well as the number and length of roots.

Roots of plantlets were rinsed in autoclaved distilled water to remove culture medium and planted in clean plastic containers in a medium with a ratio of 1:1 (v/v) autoclaved perlite and vermiculite. The plantlets were maintained at 25±1ºC with a 16 h photoperiod of 45 µ mol m^{-2} s^{-1} light intensity emitted by cool-white fluorescent lamps. Finally, plantlets were planted in pots containing soil based medium (loamy soil: sand: composted elm leaf, 1:1:1 v/v) in the greenhouse. The greenhouse equipment for climatic control was set to produce day and night temperatures of 25±2°C and 20±2°C, respectively and the relative humidity was maintained at approximately 60%.

Data collection and statistical analysis

This study was carried out as factorial experiments based on a completely randomized design. There were 3 replications (jars) and 3 intact pupae or 3 nodular segments of corms in each replication. Each step of the experiments was repeated twice. Data were analyzed using analysis of variance (ANOVA), and the means were compared using Duncan's Multiple Range Test (DMRT) at 5% level of significance ($P \leq 0.05$) using SPSS Windows version 16.

Results

Sterilization of explant sources

This research indicated that application of one-step sterilization could not satisfactorily remove contamination from mother corms and there was a need for additional treatment with lower concentrations of sodium hypochlorite solution. In contrast, decontaminated pupae explants were obtained at the first step of sterilization.

Using 40% sodium hypochlorite solution for 40 min, the contamination levels of pupae and mother corms was reduced to 19.80 and 46.40%, respectively (Table 1). The application of 15% sodium hypochlorite solution for 15 min reduced the contamination level of mother corms to 13.2% (Table 2).

Cormlet induction, growth, and development

Mother corms did not show a reportable regeneration (Data not shown). However, only nodular segment of corms cultured on MS medium containing 4 or 6 mg L^{-1} NAA, 1 mg L^{-1}BA, and 60 g L^{-1} sucrose could produce 1 to 3 cormlets under darkness. Additionally, pupae produced no cormlets under light, therefore regeneration data were collected from those cultures kept in the dark.

The interaction between the three factors (concentrations of NAA, BA, and sucrose) on cormlet induction from pupa explants was statistically significant. The highest number of cormlets (6.67) were produced per cultured explant on MS medium containing 6 mg L^{-1} NAA, 1 mg L^{-1} BA, and 60 g L^{-1} sucrose in the dark (Table 3; Fig. 1 a and b).

When NAA was replaced with 2,4-D, results indicated that the number of regenerable pupae explants was statistically lower in comparison with NAA treatment. In the case of cormlet formation, the interaction between the three factors was statistically significant. Only cultured pupa explants on MS medium supplemented with 60 g L^{-1} sucrose were able to produce cormlets. The highest number of cormlets per explant (4.67) was obtained on MS medium containing 3 mg L^{-1} 2, 4-D, 1 mg L^{-1}BA, and 60 g L^{-1} sucrose (Table 3).

Application of 2 or 3 mg L^{-1} NAA with 0.5 or 1 mg L^{-1} Kin promoted cormlet growth and development. Only the interaction between

concentrations of sucrose and NAA significantly affected the diameter of cormlets. The highest mean cormlet diameter (13.67 mm) was obtained in the presence of 2 mg L^{-1} NAA, 0.5 or 1 mg L^{-1} Kin, and60 g L^{-1} sucrose (Table 4; Fig. 1 c).

Table 1. Effects of different sterilization treatments on control of contamination in explant sources of *Freesia* ×*hybrida* Bailey 'Argenta'

Sterilization treatments		Percentage of contamination	
Concentration of sodium hypochlorite solution (%)	Time (min)	Pupa	Mother corm
0	0	100 f†	100 c
	10	100 f	100 c
	20	100 f	100 c
	30	100 f	100 c
	40	100 f	100 c
10	0	100 f	100 c
	10	86.40 ef	100 c
	20	86.40 ef	100 c
	30	79.80 def	100 c
	40	72.80 cdef	100 c
20	0	100 f	100 c
	10	59.40 bcde	100 c
	20	53 abcd	100 c
	30	53 abcd	100 c
	40	46.40 abc	93.2 c
30	0	100 f	100 f
	10	53 abcd	100 f
	20	52.80 abcd	86.40 bc
	30	33 ab	86.40 bc
	40	33 ab	72.80 b
40	0	100 f	100 c
	10	39.60 ab	100 c
	20	33 ab	79.80 bc
	30	26.40 ab	53 a
	40	19.80 a	46.40 a

† Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

Table 2. Effects of different sterilization treatments on control of contamination in segmented mother corms of *Freesia* ×*hybrida* Bailey 'Argenta'

Sterilization treatments		Percentage of contamination
Concentration of sodium hypochlorite solution(%)	Time (min)	
0	0	53 abc†
	10	59.40 bc
	15	72.80 c
10	0	39.60 abc
	10	33.20 abc
	15	26.40 ab
15	0	39.60 abc
	10	13.20 a
	15	13.20 a

†Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

Table 3. Effects of different concentrations of NAA or 2,4-D, BA, and sucrose on number of newly produced cormlets from pupa explants of *Freesia ×hybrida* Bailey 'Argenta'

NAA/BA (mg L^{-1}) + Sucrose (g L^{-1})	Mean cormlet number	2,4-D/BA (mg L^{-1}) + Sucrose(g L^{-1})	Mean cormlet number
4/0.5+30	2.67 g†	3/0.5+60	4.33 ab
4/1+30	3.33 efg	3/1+60	4.67 a
6/0.5+30	5.00 bc	3/1.5+60	4.00 bc
6/1+30	4.33 cd	4.5/0.5+60	3.67 c
2/0.5+60	3.67 def	4.5/1+60	3.00 d
2/1+60	4.00 de		
4/0.5+60	5.33 b		
4/1+60	5.67 b		
6/0+60	3.00 fg		
6/0.5+60	5.00 bc		
6/1+60	6.67 a		
6/1.5+60	4.33 cd		

† Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

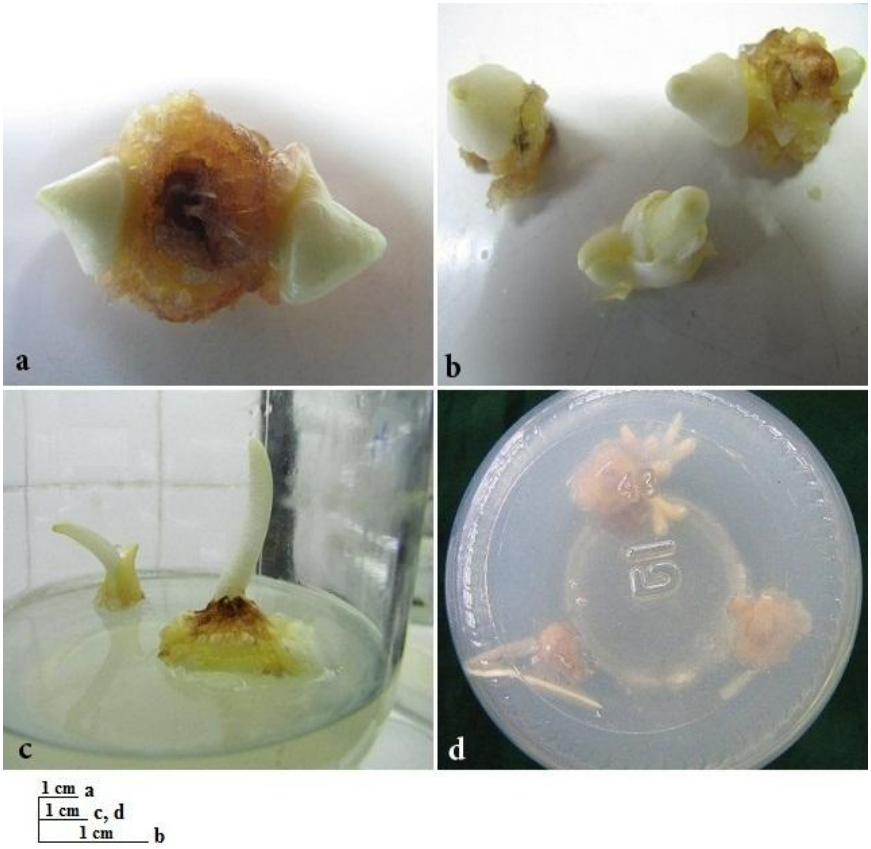

Fig. 1. Direct cormlet formation on intact pupa (a-d). (a) Cormlet formation from axillary buds of pupa on MS medium containing 6 mg L^{-1} NAA, 1 mg L^{-1} BA, and 60 g L^{-1} sucrose. (b) Newly produced cormlets were detached from pupa. (c) Cormlets were subcultured and produced shoot. (d) Roots were developed at the base of cormlets.

Table 4. Effects of different concentrations of NAA, Kin, and sucrose on diameter (mm)of newly produced cormlets on pupa explants of *Freesia* ×*hybrida* Bailey 'Argenta'.

Sucrose (g L^{-1})	NAA (mg L^{-1})	Kin (mg L^{-1})			NAA mean	Sucrose mean
		0	0.5	1		
30	0	7.00 ij†	7.67 ij	7.00 ij	7.66 C	9.27B
	1	7.00 ij	8.67g-j	9.33f-i		
	2	10.00 e-h	11.00 c-f	10.67 d-g	9.77B	
	3	9.00f-j	12.00 ae	12.00a-e		
60	0	7.00ij	8.33 hij	9.00 f-j	11.72 A	11.25A
	1	9.00 f-j	12.00 a-e	12.67a-d		
	2	11.33 b-e	13.67 a	13.67 a	11.88 A	
	3	12.00 a-e	13.00abc	13.33 ab		
Mean		9.04 B	10.79 A	10.95 A		

† Mean values followed by the same letters (small letters for interactions and capital letters for maineffects) are not significantly different at the 5% level (Duncan's Multiple Range Test).

Shoot proliferation and cormlet formation

On average, 5.67 shoots were proliferated per pupa explant in the presence of 4 mg L^{-1}BA and 2 mg L^{-1} Kin which showed significant differences compared to other treatments (Fig.2 a). The lowest mean number of shoots was 1.67 and was obtained only when 2 mg L^{-1}BA was used (Table 5).

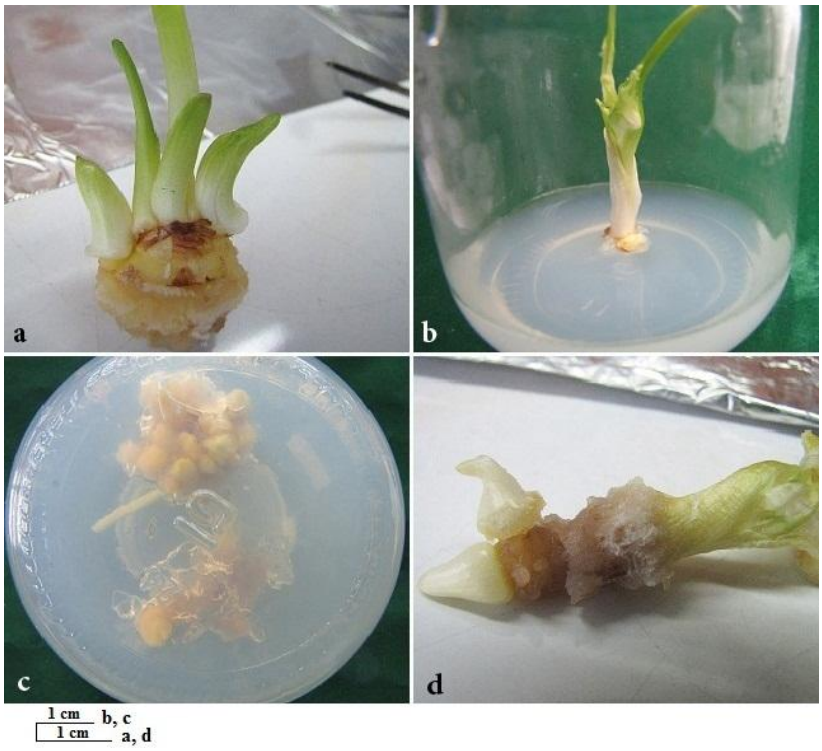

Fig. 2. Shoot proliferation on intact pupa and cormlet formation at the base of shoots (a-d).(a) Shoot proliferation on MS medium containing 4 mg l^{-1} BA and 2 mg L^{-1} Kin. (b) Cormlet development at the base of shoots on MS medium supplemented with 3 mg L^{-1} BA and 0.5 mg L^{-1} NAA. (c) A cluster of regenerated cormlets at the base of shoots. (d) Fully developed cormlets.

Table 5. Effects of different concentrations of BA and Kin on number of proliferated shoots on pupae explant of *Freesia ×hybrida* Bailey 'Argenta'

BA (mg L^{-1})	Kin (mg L^{-1})			Mean
	0	1	2	
0	0.00 f†	0.00 f	2.30 de	0.77 C
2	1.67 e	3.30c	5.30 ab	3.44 B
4	2.67cd	4.67 b	5.67 a	4.33 A
6	2.67 cd	4.67 b	4.67 b	4.00 A
Mean	1.75C	3.16B	4.48 A	

† Mean values followed by the same letters (small letters for interactions capital letters for maineffects) are not significantly different at the 5% level (Duncan's multiple range test).

Subculturing proliferated shoots on MS medium supplemented with 3 mg L^{-1} BA and 1 mg L^{-1} NAA resulted in the regeneration of 3.67 cormlets per shoot (Fig. 2 b-d). The lowest number of cormlets (1.3) was obtained on NAA-free media that contained 3 mg L^{-1}BA (Table 6).

Rooting and acclimatization

Both IBA and NAA significantly improved rooting of newly produced cormlets. The highest percentage of rooting (77.80%), root number (8.33) and root length (2.13 cm) were achieved on MS medium containing 1 mg L^{-1} IBA (Table 7; Fig. 1 d). After 4 weeks, about 60% of in vitro derived cormlets sprouted and acclimatized by transplanting to ex vitro conditions (Fig. 3).

Table 6. Effects of different concentrations of NAA and BA on number of newly produced cormlets at the base of shoots of *Freesia ×hybrida* Bailey 'Argenta'

BA (mg L^{-1})	NAA (mg L^{-1})			Mean
	0	1	2	
0	0.00 e†	0.00 e	0.00 e	0.00 C
1	0.00 e	1.67 d	0.00 e	0.55 B
3	1.30 d	3.67 a	3.30 ab	2.76 A
5	2.00 cd	2.67 bc	3.30 ab	2.66 A
Mean	0.82 B	2.00 A	1.65 A	

† Mean values followed by the same letters (small letters for interactions capital letters for maineffects) are not significantly different at the 5% level (Duncan's multiple range test).

Table 7. Effects of different concentrations of NAA and IBA on rooting of newly produced cormlets of *Freesia ×hybrida* Bailey 'Argenta'

Treatment (mg L^{-1})		Percentage of rooting	Root number	Root length
NAA	0	22.20 b†	3.67 b	1.57 a
	1	55.54 a	5.33 a	1.76 a
	1.5	0.00 c	0.00 c	0.00 b
IBA	0	0.00 b	0.00 c	0.00 b
	1	77.80 a	8.33 a	2.13 a
	1.5	77.80 a	6.00 b	1.93 a

† Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

Figure 3. Acclimatization and transplanting of newly produced cormlets (a and b). (a) Transplanting of plantlets in clean plastic containers with a ratio of 1:1 (v/v) autoclaved perlite and vermiculite. (b) Plantlets were planted in soil based media and pots were placed in a greenhouse.

Discussion

Cormlet induction, growth, and development

- ***Influence of explant source***

To the authors' best knowledge there is no report on the use of freesia pupa as explant source. Mother corms had no reportable regeneration. Conversely, pupae showed the highest frequency of regeneration. This may be due to the presence of young tissues with higher cell division potential and lower natural inhibitors. Moreover, the microbial contamination levels of the pupae were lower than the observed from corms and thus their sterilization treatments were slighter and had fewer effects on regeneration potential.

- ***Influence of cold storage***

As pupae were produced in cold storage, it may be assumed that hormonal balance and enzymatic activity were affected by low temperature (4°C) which in turn may influence better corm regeneration and subsequent growth. Uchikoba *et al*. (2003) showed Freesia protease B and C were found in freesia's new corms kept for 6 months at 4°C. These proteases may supply free amino acids during corms sprouting and the rapid growth phase. Hirata *et al*. (1995) reported that *in vitro* corm formation of freesia was facilitated under low temperature and cormlet fresh weight was increased.

Metabolism of sugars is closely linked to *in vitro* regeneration potential of storage organs. The most important biochemical changes occurring during cold treatment are quantitative changes in carbohydrate constituents. It has been reported that in cold storage, there is a net breakdown of starch and accumulation of sucrose from hydrolysis of starch in lily bulbs (Shin *et al*., 2002; Hong-Mei *et al*., 2005). Thus, it is assumed that the accumulation of soluble sugars in pupae increased by cold treatment and subsequently resulted in elevated regeneration rates.

- ***Influence of plant growth regulators***

This report showed that elevated auxin levels were essential for cormlet formation and development. Similarly, high concentrations of auxin were needed for *in vitro* cormlet formation of *Crocus sativus* L. (Homes *et al*., 1987).

With increase in the concentrations of NAA, the number of newly produced cormlets was increased. In contrast, higher concentrations of 2,4-D was associated with reduction in cormlet number which may be attributed to toxic effects of high concentrations of 2,4-D. Bach (1992) reported that explants of *Freesia ×hybrida* Bailey showed low growth and

regeneration potential on medium containing 2,4-D. Moreover, in this study, increase in size of cormlets in the presence of NAA could be due to the influence of auxins on cell size which consequently leads to more water and nutrients uptake by cells. These results appear to be in agreement with the study of Chrungoo and Farooq (1989) who reported that NAA improve carbohydrate accumulation in corm tissues of freesia.

- ***Influence of sucrose concentrations***

Work presented here shows that the elevated sucrose concentration increased both cormlet number and diameter at the optimal concentrations of NAA, BA and Kin. Based on the study of Ascough *et al*. (2008), carbohydrate could affect storage organs formation by supplying more energy for its formation and growth. Moreover, high carbohydrates levels leads to increase in medium osmolarity, hence creating a 'stress' type environment, that results in storage organ induction as an avoidance response to a perceived unfavorable climate.

Hirata *et al*. (1995) showed that high sucrose concentrations (up to 110 g L^{-1}) facilitated *in vitro* corm formation and development of freesia. Ojha *et al*. (2010) reported that increase in carbohydrate concentrations (up to 60 g L^{-1}) produced the highest number of cormlets in *Gladiolus pacifica* which is in line with the results of this study.

- ***Influence of light conditions***

This study showed that the highest induction frequency and size of cormlets was achieved in the dark. Light quality and duration affect *in vitro* storage organ formation (Ascough *et al*., 2008). It has been claimed that darkness enhanced cormlet formation and growth of *Freesia refracta* (Jacq.) Klatt explants (Bach *et al*., 2000). Subsequently, Bach and Pawlowska (2006) reported that the number of bulbs produced was highest when explants of *in vitro* cultured ornamental bulbous plants were cultured in the dark. Similarly, Kumar *et al*. (2005) reported that *in vitro* regenerated bulblets of lily had higher fresh mass and diameter under continuous darkness.

On the other hand, it has been shown that freesia cormlets are also able to regenerate under both light and dark conditions (Hirata *et al*., 1995). Indeed, Che and Qin (1998) showed that darkness had negative effects on organogenesis of *Freesia*. Such discrepancies in the effect of light on cormlets production may be due to genotypic differences, *ex vitro* environmental conditions of the source plants, or subsequent *in vitro* environmental conditions. Further works are therefore required to study light effects on cormlet formation.

Shoot proliferation and cormlet formation

- ***Influence of plant growth regulators on shoot proliferation.***

In the case of *in vitro* shoot proliferation, there appear to be three major categories of plant response to plant growth regulators: (1) shoot induction and proliferation is only dependent on the use of cytokinins (particularly 1 to 5 mg L^{-1}BA) in medium (Devi *et al*., 2011; Diaz-Vivancos *et al*., 2011); (2) shoot proliferation occurred when only auxins (particularly 0.5 to 1 mg L^{-1} NAA) were added to the medium containing BA (Memon *et al*., 2012); and (3) addition of NAA to medium containing BA could improve shoot production (Madubanya, 2005; Ascough *et al*., 2007; Zeybek *et al*., 2012). Thus, it seems that effect of plant growth regulators on shoot proliferation is completely species-dependent.

From the physiological point of view, cytokinins overcome apical dominance, promote cell division and thus enhance shoot proliferation. This work showed that maximum shoot proliferation was obtained

at higher concentrations of BA and Kin. There are some reports that support the findings of this study. Priyakumari and Sheela (2005) reported that the highest rate of multiple shoots (33.7) of *Gladiolus* 'Peach Blossom' was obtained on MS medium containing BA (4 mg L^{-1}) and NAA (0.5 mg L^{-1}). Karaoglu *et al*. (2007) reported a shoot proliferation from eye buds of *Crocus sativus* L. at high concentrations of BA.

- ***Influence of plant growth regulators on cormlet formation***

Cormlet development at the base of *in vitro* proliferated shoots has been accomplished on different media (Devi *et al*., 2011; Zeybek *et al*., 2012). In this study, cormlets were induced at the base of shoots on the media supplemented with cytokinins. The literature suggests that cormlet formation can be improved by optimizing concentration of cytokinins in the growth media. Some literature reported that cytokinins have a stimulatory effect on *in vitro* storage organ formation. Memon *et al*. (2010) reported the positive role of Kin in cormlet formation of gladiolus. Moreover, Emek and Erdag (2007) achieved proliferated shoots of gladiolus and then subcultured them on media containing 0.1 mg L^{-1}BA that led to cormlet formation at the base of shoots.

Rooting and acclimatization

Although some regenerated cormlets produced roots on auxin-free media. The results indicate that application of auxin improved rooting variables such as percentage of rooting and number and length of roots which supports the data presented by Zhao (1989) who reported that NAA enhanced rooting of *Freesia* cormlets. Some reports demonstrated that both IBA and NAA are necessary for *in vitro* rooting of *Freesia* plantlets (Bajaj and Pierik, 1974).

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