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Investigation of callogenesis and indirect regeneration of *Freesia* × *hybrida* Bailey 'Argenta'

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ABSTRACT

An investigation was conducted to study the effects of explant sources, plant growth regulators, carbohydrates and light conditions on indirect cormlet regeneration and the induction of embryogenic callus of freesia (*Freesia* × *hybrida* Bailey 'Argenta'). Sections of two different types of explants, corms and pupae (cold storage-produced corms), were placed on Murashige and Skoog (MS) media containing different concentrations of plant growth regulators. The results showed that the highest percentage of callus induction (100%), the highest callus growth (15 mm diameter) and the best type of calli were achieved for pupa explants grown on the medium that contained 4 mg L^{-1} 1-naphthaleneacetic acid (NAA) and 2 mg L^{-1} 6-benzylaminopurine (BAP) in the dark. Increasing BAP up to 3 to 4.5 mg L^{-1} resulted in the maximum number of regenerated cormlets from 1 cm^2 calli (2 cormlets) under light conditions. Overall, the best rooting of regenerated cormlets was achieved on MS media supplemented with 1 mg L^{-1} indole-3-butyric acid (IBA). In the next stage, high quality calli were subcultured on MS media containing sorbitol, sucrose, maltose and mannitol (0, 5, 10 and 15 g L^{-1}). The results indicated that 15 g L^{-1} maltose was able to induce the highest percentage of embryogenic callus, with an average of 88.9% on media containing 2 mg L^{-1} BAP and 1 mg L^{-1} NAA.

Key words: organogenic callus, embryogenic callus, indirect cormlet formation

Abbreviations:

MS – Murashige and Skoog medium, NAA – 1-naphthaleneacetic acid, BAP – 6-benzylaminopurine, IBA – indole-3-butyric acid, Kin – kinetin

INTRODUCTION

Freesia is an ornamental bulbous plant from the Iridaceae family that has recently become one of the most popular cut flowers in Europe and the USA due to its fragrance, long vase life and the wide colour range of the flower (Anderson 2007). Market research shows that over 110 million freesia stems 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 the cut flower production of this genus. However, the conventional propagation systems (seeds and corms) of these cultivars are accompanied by many disadvantages such as long-term juvenility, shorter inflorescences with fewer numbers of florets in the seedlings, a low seed setting rate due to self-incompatibility, self-sterility and aneuploidy of different cultivars, a reduction in cut flower quality, and the accumulation of plant viruses in corms by successive cultivation and also the poor ability of

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mother corms to produce cormlets (3 to 6 corms per year) (Bajaj and Pierik 1974). Thus, the conventional propagation systems in *Freesia* and other bulbous and cormous plants can be replaced with modern systems of tissue culture with many advantages such as more flowers per spike, higher quality flowers, increased multiplication rates (Takayama and Misawa 1982, Van Aartrijk et al. 1990), the elimination of viruses using apical meristem culture (Van Aartrijk et al. 1990), facilitated breeding programs, and the maintenance of a large number of elite genotypes (Gao et al. 2009).

On the other hand, in molecular breeding, the establishment of callus induction and an indirect regeneration pathway is very crucial for the successful introduction of foreign genes into the plant genome (Azria and Bhalla 2011).

Indirect cormlet formation of *Freesia* via callus induction has been investigated in various studies, though not recently. In the presence of 5 mg L^{-1} NAA and 3 mg L^{-1} BAP, calli were produced from explants of *Freesia* after five weeks. Auxins were essential for callus induction while cytokinins were optional (Bajaj 1989, Ascough et al. 2008). In another study, corm segments of *Freesia* produced regenerable calli on a medium containing 5 mg L^{-1} NAA and 1.2 mg L^{-1} BAP (Bajaj and Pierik 1974). Hussey (1975) reported that corm explants of *Freesia* were able to produce callus on media containing different concentrations of NAA (0.5 to 8 mg L^{-1}) and 2,4-D (0.1 to 2 mg L^{-1}). In the next stage, 0.5 to 8 mg L^{-1} BAP led to shoot proliferation from calli tissues.

Somatic embryogenesis is a process with several advantages over regeneration by organogenesis, such as improvement in the *in vitro* multiplication rate of ornamental geophytes and the probable single-cell origin, thus avoiding chimeras in the regenerated plants (Skirvin et al. 1993). We can propagate the embryogenic culture in a bioreactor and use this culture for cryopreservation and genetic modification (Paek et al. 2001).

Since the work by Haberlandt, sucrose has been the most widely used carbohydrate *in vitro*. However, sucrose is not always the best carbohydrate to achieve regeneration (Blanc et al. 1999). Several reports have shown that the ability of the cells to induce somatic embryogenesis and the development of somatic embryos was influenced by the carbohydrate sources and concentrations used in the culture media. Maltose, glucose and fructose have been widely compared with sucrose to determine their effect on somatic embryogenesis in several species (Tokuhara and Mii 2003, Jheng et al. 2006).

The aim of the present work was to establish an effective micropropagation pathway for indirect organogenesis of *Freesia* × *hybrida* Bailey 'Argenta'. Thus, the effects of different factors such as explant sources, plant growth regulators and light conditions were investigated. Moreover, using friable calli, we studied the impacts of types and concentrations of four carbohydrates on the early stages of indirect somatic embryogenesis in this cultivar. We report here the effect of four carbohydrates with a view to highlight their roles in somatic embryogenesis induction and discuss their impacts on callus physiology.

MATERIAL AND METHODS

Plant materials

We purchased *Freesia* × *hybrida* Bailey 'Argenta' as a pot plant and kept pots 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%. During the experiment, corms were removed from the pots. A batch of corms was stored at 4°C during the summer and about two pupae (cold storage-produced corms) were produced from the apical bud of corms after seven to eight weeks. We did not put the other batch of the harvested corms in the cool storage, but rather directly used them in the tissue culture. These two batches of plant material were used as the primary explant sources.

For decontamination, corms and pupae were detached from their tunics and thoroughly washed under running tap water for 12 h, dipped in 70% (v/v) of ethanol for two minutes and then soaked in 40% sodium hypochlorite solution (using commercial bleach at 5.25% of active chloride) for 40 min. Corms and pupae were then rinsed three times in sterile distilled water.

Culture conditions

Murashige and Skoog (1962) MS basal media supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agar were prepared and the pH was adjusted to 5.8. Autoclaved media were distributed in jars and solidified. Cultured explants were incubated at 25 ± 1°C under a 16/8 h (light/darkness) photoperiod at a light intensity of approximately 45 μmol m^{-2} s^{-1} emitted by cool-white fluorescent tubes. All cultures were subcultured every four weeks on the same media.

Callus induction

For callus induction, the segments of pupae as well as mother corms were used as explants. Sterilized pupae and mother corms were vertically cut into eight equal segments and then put on the media. The approximate size of each explant was about 1 cm^2. Because of vertical cutting, each explant shared whole parts of the corm from apical bud to basal plate. This method of cutting led to the production of completely similar explants.

At this stage, the three factors consisted of: (1) the type of explant (pupae or mother corm), (2) different concentrations of NAA (0, 2, 4 or 6 mg L^{-1}) or 2,4-D (0, 1, 2 or 3 mg L^{-1}), and (3) different concentrations of BAP (0, 1 or 2) applied. Each treatment contained only one type of auxin plus BAP.

After about five weeks, several factors including the percentage of callus induction, callus growth (mm), and type of calli were determined.

In order to measure the callus growth, the calli were transferred to a sterilized petri dish and the diameter of the calli mass was measured by a ruler that was placed under the petri dish.

The produced calli were separated according to all the visual factors that could be associated with the type of callus such as colour, freshness, friability and compactness. The best types of calli were yellow-brownish or cream coloured, fresh, friable and compact (Fig. 1). The groups were as follow:

- Colour (Scale of 1 through 4): 1 = yellow-brownish or cream coloured callus / 4 = white callus.
- Freshness (Scale of 1 through 4): 1 = translucent and fresh callus / 4 = dry and necrotic callus.
- Friability (Scale of 1 through 4): 1 = friable callus that was easily spread by forceps / 4 = non-friable callus that must be divided by scalpel.
- Compactness (Scale of 1 through 4): 1 = compact callus / 4 = dispersed callus.

Cormlet regeneration

We studied indirect cormlet formation from the yellow-brownish, translucent and compact calli produced from the pupae explants cultured on MS medium containing 2 mg L^{-1} BAP and 4 mg L^{-1} NAA. This step was carried out with three factors including: (1) different concentrations of NAA (0.5, or 1 mg L^{-1}), BAP (0, 1.5, 3 or 4.5 mg L^{-1}) and Kin (0, 1 or 2 mg L^{-1}).

Subculturing these selected calli on the MS medium supplemented with the mentioned plant growth regulators resulted in the formation of two different groups of calli, including organogenic calli (with differentiated bodies) and non-organogenic calli (without any differentiation).

After about six weeks new cormlets were indirectly formed on the calli. At the end of this part of the experiment, we measured the number of indirectly regenerated cormlets from each 1 cm^2 piece of callus.

Somatic embryo formation

The influences of various types and concentrations of carbohydrates on the formation and development of the embryogenic callus were assessed. The initial plant material consisted of yellow-brownish, translucent, friable and compact calli produced from pupae explants cultured on MS medium containing 2 mg L^{-1} BAP and 4 mg L^{-1} NAA.

This experiment consisted of two factors, including: (1) type of carbohydrate (sorbitol, sucrose, mannitol and maltose) and (2) concentrations of carbohydrates (0, 5, 10 or 15 g L^{-1}). These carbohydrates were individually added to the medium containing only 2 mg L^{-1} BAP and 1 mg L^{-1} NAA.

Five weeks after subculturing, we measured the percentage of embryogenic calli and type of calli. They were separated into two groups including embryogenic calli (white translucent calli with small and globular embryoids that subsequently produced bipolar embryos) and non-embryogenic calli (only showed globular aggregates and did not go to embryogenesis).

Acclimatization

New shoots and roots were respectively emerged from *in vitro* derived cormlets, respectively. 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 an auxin-free medium. Four weeks later, the roots of the plantlets were rinsed in autoclaved distilled water to remove agar and planted in clean plastic containers with a ratio of 1:1 (v/v) autoclaved perlite and vermiculite. The containers were placed in a room set at 25 ± 1°C with a 16 h photoperiod of 45 µmol m^{-2} s^{-1} light intensity emitted by cool-white fluorescent lamps. Finally, the plantlets were planted in soil-based media and the pots were placed 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, and the relative humidity was maintained at approximately 60%.

Statistical analysis

This study was carried out as factorial experiments based on a completely randomized design with three replications. Each step of the experiments was repeated twice and the average of three explants was used as the value of each replication. Data were analysed using analysis of variance (ANOVA), and the means were compared using Duncan's multiple range test (DMRT) at a 5% level of significance ($P \leq 0.05$) using SPSS Windows version 16.

RESULTS

Callus induction

The interaction between three factors (explant type, different concentrations of BAP and various concentrations of NAA) was observed in the percentage of callus induction. The results showed that in both type of explants (pupae and mother corms), callus induction was promoted with an increase in the concentrations of NAA and BAP. Pupae explants showed the highest percentage of callus induction (100%) on the media containing 4 or 6 mg L^{-1} NAA + 2 mg L^{-1} BAP or 6 mg L^{-1} NAA + 1 mg L^{-1} BAP (Tab. 1). It was significantly different from the control (medium free of plant growth regulators). According to Table 1, the highest percentage (88.9%) of callus induction in segments of mother corms was achieved on MS medium supplemented with 4 or 6 mg L^{-1} NAA and 2 mg L^{-1} BAP. However, there was no statistically significant difference between the pupae and mother corms with this treatment.

The interaction between three factors (explant type, different concentrations of BAP and various concentrations of NAA) was observed in callus growth. Pupae segments showed the highest amount of callus growth (15 mm) on the MS medium

Table 1. The effects of explant type, NAA and BAP on callus induction, growth and quality

Explant	Plant growth regulators (mg L^{-1})		Callus induction (%)	Callus growth (mm)	Callus quality group
	BAP	NAA			
Pupa	0	0	0 g*	0 g	-
		2	0 g	0 g	-
		4	44.43 ef	7 e	4
		6	55.56 de	9 c	4
	1	0	0 g	0 g	-
		2	44.43 ef	8 d	3
		4	88.9 ab	14 ab	2
		6	100 a	14 ab	2
	2	0	0 g	0 g	-
		2	66.7 cd	13 b	3
		4	100 a	15 a	1
		6	100 a	14 ab	1
Mother corm	0	0	0 g	0 g	-
		2	0 g	0 g	-
		4	0 g	0 g	-
		6	33.3 f	5 f	4
	1	0	0 g	0 g	-
		2	0 g	0 g	-
		4	55.56 de	8 d	3
		6	77.8 bc	10 c	3
	2	0	0 g	0 g	-
		2	0 g	0 g	-
		4	88.9 ab	10 c	3
		6	88.9 ab	10 c	2

*Mean values followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan's multiple range test). No callus (-), Group 1 with the highest quality, Group 2, Group 3 and Group 4 with the lowest quality

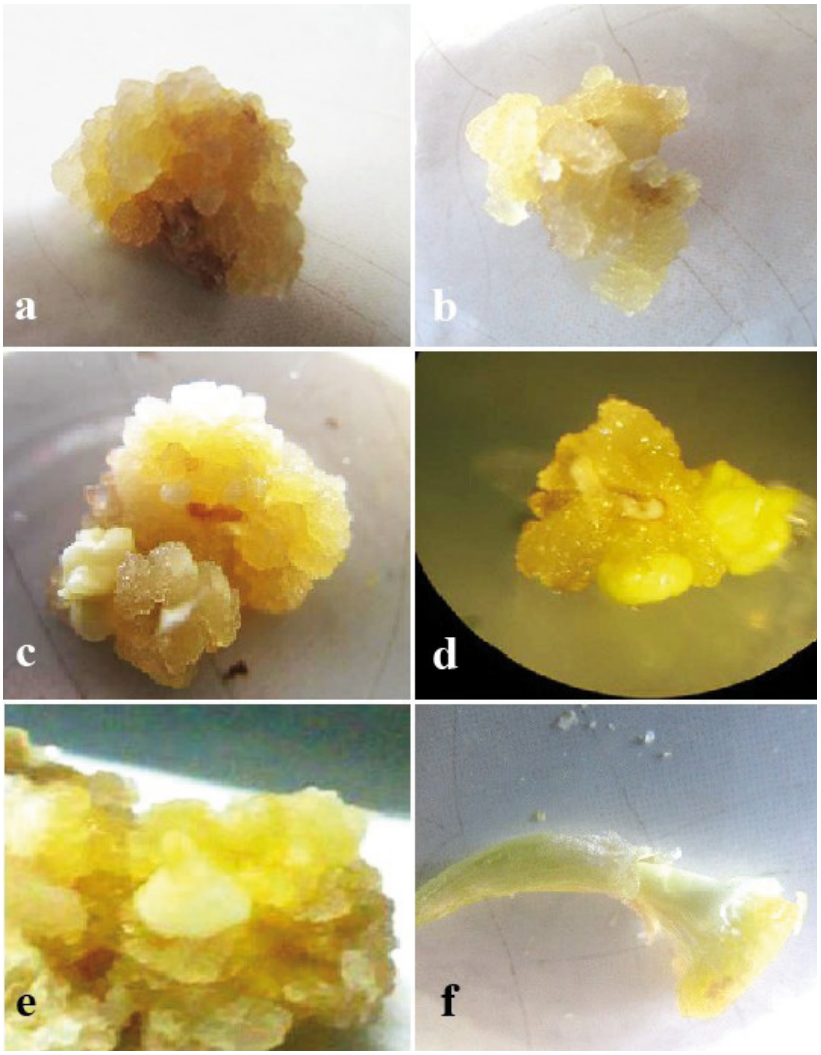

Figure 1. Callus induction and cormlet regeneration. (a) Callus of the highest quality (yellow-brownish coloured, fresh, friable and compact), (b) callus of the lowest quality (white, non-friable, and dispersed), (c and d) indirect cormlet regeneration from the callus after subculturing on MS medium containing 4.5 mg L^{-1} BAP and 2 mg L^{-1} Kin, (e and f) growth of regenerated cormlets after subculturing on MS medium containing 4.5 mg L^{-1} BAP and 2 mg L^{-1} Kin

Table 2. The effects of 2,4-D and BAP on callus induction, growth and quality in pupae explants

Plant growth regulators (mg L^{-1})		Callus induction (%)	Callus growth (mm)	Callus quality group
BAP	2,4-D			
0	0	0 c*	0 e	-
	1	0 c	0 e	-
	2	0 c	0 e	-
	3	0 c	0 e	-
1	0	0 c	0 e	-
	1	0 c	0 e	-
	2	77.8 a	11 c	2
	3	88.9 a	13 a	1
2	0	0 c	0 e	-
	1	0 c	0 e	-
	2	55.54 b	9 d	2
	3	77.8 a	12 b	2

*Mean values followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan's multiple range test). No callus (-), Group 1 with the highest quality and Group 2 with the lowest quality

containing 4 mg L^{-1} NAA and 2 mg L^{-1} BAP (Tab. 1). However, calli derived from segments of mother corms grew slowly and showed the lowest callus growth (5 mm) on the MS medium containing 6 mg L^{-1} NAA and without BAP. The results showed that the application of 4 or 6 mg L^{-1} NAA and 1 or 2 mg L^{-1} BAP led to the formation of calli with the best type (Tab. 1 – Fig. 1a).

The results showed that the effect of 2,4-D on callus induction was lower than NAA and about two third of the treatments showed no response. In addition, the segments of mother corms could not produce callus. The highest percentage of callus induction (88.9%) was obtained for pupae explants on the MS medium supplemented with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP (Tab. 2). However, there were no statistically significant differences between the mentioned medium and media containing 1 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D as well as 2 mg L^{-1} BAP + 3 mg L^{-1} 2,4-D. The maximum growth of calli (13 mm) and the best type was obtained in explants cultured on the MS medium containing 3 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP. This treatment showed significant differences compared to the others.

Cormlet regeneration

Subculturing the calli on MS media containing higher concentrations of BAP (0, 1.5, 3 or 4.5 mg L^{-1}) led

Table 3. The effects of plant growth regulators on the number of regenerated cormlets from portions of the callus

Plant growth regulators						
NAA (mg L^{-1})	BAP (mg L^{-1})	Kin (mg L^{-1})			Mean	Mean
		0	1	2		
0.5	0	0 c*	0 c	0 c	0 C	0.41 A
	1.5	0 c	0 c	0 c		
	3	0 c	1 b	1 b	0 C	
	4.5	0 c	1 b	2 a		
1	0	0 c	0 c	0 c	0.83 B	0.66 A
	1.5	0 c	0 c	0 c		
	3	0 c	1 b	2 a	1.33 A	
	4.5	1 b	2 a	2 a		
Mean		0.12 C	0.62 B	0.87 A		

*Mean values followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan's multiple range test)

Table 4. The effects of different concentrations and types of carbohydrates on the induction of embryogenic calli

Type of carbohydrate	Concentration (g L^{-1})	Embryogenic callus induction (%)	Callus quality group
Sorbitol	0	0 a*	-
	5	0 a	-
	10	0 a	-
	15	0 a	-
Sucrose	0	0 b	-
	5	0 b	-
	10	0 b	-
	15	66.67 a	1
Maltose	0	0 c	-
	5	55.56 b	1
	10	77.8 ab	1
	15	88.9 ab	2
Mannitol	0	0 b	-
	5	0 b	-
	10	77.8 a	1
	15	77.8 a	1

*Mean values followed by the same letters within the type of carbohydrate are not significantly different at $P \leq 0.05$ (Duncan's multiple range test). No callus (-), Group 1: globular calli and Group 2: embryogenic calli

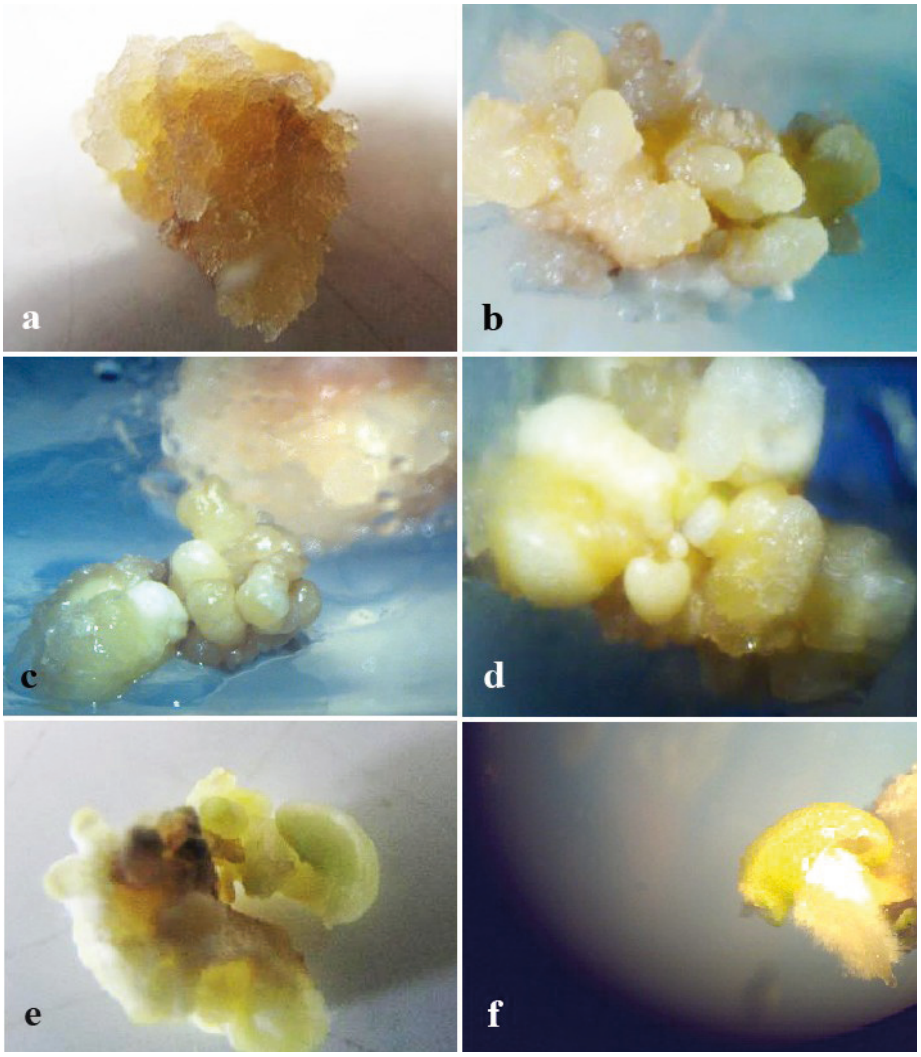

Figure 2. Different stages of induction of embryogenic callus. (a) Callus of the highest quality (yellow-brownish coloured, fresh, friable and compact) that was induced on MS medium containing 4 mg L^{-1} NAA and 2 mg L^{-1} BAP and used as the initial plant material, (b) embryogenic nodular callus with globular bodies that was induced after subculturing on MS medium supplemented with 15 g L^{-1} maltose, (c, d) development of embryoids, (e) shoot emergence, (f) root emergence

to cormlet regeneration. Interaction between BAP and Kin was observed in the number of regenerated cormlets. The highest number of cormlets (2) from each 1 cm^2 portion of the calli was achieved on the MS media containing 4.5 mg L^{-1} BAP + 1 or 2 mg L^{-1} Kin + 1 mg L^{-1} NAA as well as on media supplemented with 4.5 L^{-1} BAP + 0.5 mg L^{-1} NAA + 2 mg L^{-1} Kin or 3 mg L^{-1} BAP + 1 mg L^{-1} NAA + 2 mg L^{-1} Kin (Tab. 3 – Fig. 1c-e). However, there were no statistically significant differences between the application of 0.5 or 1 mg L^{-1} NAA (0.41 A and 0.66 A) (Tab. 3).

The effects of different carbohydrate sources on the formation of embryogenic calli

Following the morphological development of calli using a microscope, we found that embryogenic nodular callus was induced under dark conditions. The highest percentage of embryogenic calli induction (88.9%) with the highest quality was observed on the MS medium supplemented with 15 g L^{-1} maltose (Fig. 2b-f). However, the percentage of callus induction showed no statistically significant differences between this treatment and applications of 15 g L^{-1} sucrose, 5 or 10 g L^{-1} maltose, and 10 or 15 g L^{-1} mannitol (Tab. 4).

Rooting and acclimatization

IBA and NAA significantly improved the rooting parameters of *in vitro* derived cormlets. The results showed that the highest percentage of rooting (77.8%), root number (8.33) and root length (2.13 cm) were obtained on MS media containing 1 mg L^{-1} IBA (Tab. 5). Eventually, about 60% of

Table 5. The effects of different concentrations of NAA and IBA on rooting parameters of *in vitro* derived cormlets

Type of auxin	Concentration (mg L^{-1})	Rooting parameters		
		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 c	0 c	0 b
IBA	0	0 b	0 c	0 b
	1	77.80 a	8.33 a	2.13 a
	1.5	77.80 a	6 b	1.93 a

*Mean values followed by the same letters within the type of auxin are not significantly different at $P \leq 0.05$ (Duncan's multiple range test)

Figure 3. Transplanting and acclimatization of *in vitro* derived cormlets. (a) Transplanting of plantlets in clean plastic containers with a ratio of 1:1 (v/v) autoclaved perlite and vermiculite. (b) Plantlets were sown in soil based media and pots were placed in a greenhouse

in vitro derived cormlets were acclimatized after transplanting (Fig. 3).

DISCUSSION

Callus induction and cormlet regeneration

With pupae explants, the percentage of callus induction and quality of calli was higher than the mother corms. To our best of knowledge there is no report on the use of pupae as an explant source for the micropropagation of freesia. Obviously, they showed the highest callus induction, growth and quality. This can be due to the presence of young tissues with higher carbohydrate content and cell division potential. Moreover, the contamination levels of the pupae were lower than the corms and thus their sterilization treatments were slighter and had fewer effects on the regeneration potential.

Furthermore, as pupae were produced in cold storage, it may be assumed that hormonal balance and enzymatic activity were affected by cold temperature (4°C). In a study, Freesia protease B and Freesia protease C were found in freesia new corms kept for six months at 4°C, which may supply free amino acids during corm sprouting rapid growth (Uchikoba et al. 2003). In addition, the most important biochemical changes occurring during cold storage are quantitative changes in carbohydrate constituents. It has been reported that in cold storage there is a net breakdown of starch and the accumulation of sucrose from hydrolysis of starch in lily bulbs (Shin et al. 2002, Hong-Mei et al. 2005). Thus, we assumed that the accumulation of soluble sugars in pupae could be increased during cold storage.

The results show that high concentrations of auxin were required to induce callus. Many studies have shown various plant requirements of auxin (type and concentration) for callus induction. For example, *Asparagus* shoot tips need 0.5 mg L^{-1} NAA for callus induction (Takatori et al. 1968) while *Lilium* explants may need no auxin (Sheridan 1968). Despite this, gladiolus explants need more than 5 and up to 9 or 10 mg L^{-1} NAA (Kamo and Joung 2007). Emek and Erdag (2007) used longitude segments of *Gladiolus anatolicus* (BOISS.) STAPF and reported that the highest percentage of callus induction (75%) was achieved on an MS media

containing 8.5 mg L^{-1} NAA. Wilfert (1971) showed that the highest quality calli of gladiolus explants were produced after four to five weeks on MS media supplemented with 5 mg L^{-1} NAA and 0.5 mg L^{-1} Kin. Bajaj and Pierik (1974) reported that explants of freesia corms cultured on media containing 5 mg L^{-1} NAA and 1.2 mg L^{-1} BAP were able to produce callus.

The results showed that the combination of NAA and BAP was needed for callus induction. Our findings indicated that the highest percentage of high quality callus induction was obtained by the application of higher concentrations of NAA (Tab. 1). It has been proven that auxins are the most important group of plant growth regulators for callus induction. Therefore, NAA could enhance cell growth and increase the callus mass. Bajaj (1989) investigated the interaction between NAA (0, 0.1, 1, and 5 mg l^{-1}) and BAP (0, 0.6, 3, and 6 mg l^{-1}) on callus induction in freesia and reported that unlike BAP, NAA was necessary for callus induction.

Explants cultured on BAP-free media showed the lowest percentage of callus induction and quality. Having a key role in cell division, we indicated that BAP was needed for callus induction and increasing callus growth. Also, BAP makes callus cells as storage for more nutrient uptake. Furthermore, cytokinins such as BAP are anti-senescence compounds, and we concluded that the presence of BAP in the media could produce the highest quality of calli and maintain their freshness.

It was clear that the type of auxin was very important for callus induction. The application of NAA was associated with the highest percentage of callus induction and the highest quality of calli, while 2,4-D reduced these parameters. The main reason may be due to the chemical structure of NAA and 2,4-D, which differently affect cell division and growth.

Various studies have also reported that NAA is the best auxin for callus induction in bulbous and cormous plants such as *Freesia*, *Crocus* and *Gladiolus* (Bajaj and Pierik 1974, Stimart and Ascher 1982, Stefaniak 1994, Kim et al. 1997, Bach et al. 2000, Ebrahimzadeh et al. 2000). Bach (1992) showed that after receiving picloram, the ability of *Freesia* × *hybrida* explants to induce callus was higher compared to the 2,4-D treatment. Furthermore, explants treated with picloram showed the best longevity and regeneration potential. We assume that the chemical structure of NAA is more compatible with different genotypes of cormous plants and therefore that this group of plants are more amenable to NAA compared to other auxins such as 2,4-D. However, we found that media free of 2,4-D exhibited no callus and it indicated that the presence of one type of auxin was needed for callus induction.

The investigation of proper light conditions for callus induction showed that the highest amount and quality of calli were obtained under dark conditions. In most micropropagation systems of cormous plants (*Freesia*, *Crocus* and *Gladiolus*), callus induction was achieved under darkness and our findings are in line with these studies (Bajaj and Pierik 1974, Stimart and Ascher 1982, Stefaniak 1994, Kim et al. 1997, Bach et al. 2000, Ebrahimzadeh et al. 2000).

Pupa explants produced the best calli and also exhibited the best indirect organogenesis. However, during this research only *de novo* cormlets were regenerated from calli and no shoot was proliferated. These findings are different from many studies that have reported indirect shoot regeneration from calli. We assume that the ratio of endogenous hormones in callus can be the source of this difference. This means that perhaps the concentration of NAA in callus cells is high and inhibits indirect shoot regeneration. Therefore, an investigation on the hormone balance of callus cells along with an application of higher concentrations of more powerful cytokinins could help us to clear up this matter.

As mentioned above, high concentrations of NAA were needed for direct cormlet formation on pupa explants, while calli were able to produce cormlets only after receiving BAP and Kin. This can be due to the presence of differences in endogenous hormone concentration between pupa and callus tissues. Many researchers have applied high concentrations of cytokinins for regeneration from callus (Hussey 1975, Petru et al. 1976, Ascough et al. 2008).

The effects of different carbohydrate sources on the formation of embryogenic calli

A review of the literature shows that some studies have been conducted on the embryogenesis of *Freesia*. However, the influences of carbohydrates on the induction of embryogenic calli of freesia have not yet been studied.

Wang et al. (1990) cultured young inflorescence segments of *Freesia refracta* (Jacq.) Klatt on an MS medium containing 2 mg L^{-1} indole-3-acetic acid (IAA), 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA.

One week later, pale-yellow translucent nodular calli were achieved. Subculturing these calli on MN_6 media supplemented with 2 mg L^{-1} IAA and 3 mg L^{-1} BAP led to the formation of globular embryos. In another study, Wang et al. (1996 and 1998) reported direct somatic embryogenesis of this species from young inflorescence segments on an MN_6 media containing 2 mg L^{-1} IAA and 3 mg L^{-1} BAP. Gao et al. (2009) used inflorescence explants of *Freesia* × *hybrida* and found that direct or indirect somatic embryogenesis was dependent on the concentration of plant growth regulators. For direct somatic embryogenesis, 2 mg L^{-1} IAA with 1 mg L^{-1} BAP was used while the embryogenic callus was achieved on an MS medium containing 5 mg L^{-1} BAP and 1 mg L^{-1} 2,4-D for indirect somatic embryogenesis.

Using a microscope, we followed the morphological changes in calli and found that 15 g L^{-1} maltose was the best treatment for the induction of the embryogenic calli with friable morphotypes. Thus, these calli are very suitable for transformation to produce genetically uniform and stable transgenic plants. Scott et al. (1995) assumed that the beneficial effect on barley somatic embryogenesis of maltose came from its slow hydrolysis, which limited cell carbon nutrition. The maltose effects might also be due to low cell uptake, leading to a drop in the available carbohydrates and hence creating a signal that reorients the development programs (Koch 1996). Blanc et al. (2002) reported that maltose reduced the soluble sugar content by around 60% in *Hevea brasiliensis* callus and they further suggested that the regulation of endogenous hexose contents at a low level, through slow maltose hydrolysis, was a key element of the biochemical signal leading this callus towards somatic embryogenesis.

Rooting and acclimatization

Although subculturing several *in vitro* derived cormlets on an auxin-free medium resulted in the rooting of cormlets, it was obvious that the application of auxin was needed to improve rooting variables such as percentage of rooting and number and length of roots. It has been shown that the presence of IBA or NAA is crucial for the *in vitro* rooting of freesia plantlets (Bajaj and Pierik 1974). Zhao (1989) also reported that NAA could enhance the rooting of cormlets of freesia.

Transplanting of plantlets from an *in vitro* environment to greenhouse conditions means changing the conditions from the heterotrophic to autotrophic state. Moreover, various abiotic stresses such as light, drought and temperature adversely affect plantlet growth. Thus, hardening, which increases tolerance to these stresses, is required for acclimatization. However, special care is needed.

In the greenhouse, the relative humidity must be high and light intensities low for several days until the plantlets become autotrophic (Debergh and Read 1991). Subsequently, plantlets can be grown under lower humidity and higher light intensity. In this work, about 60% of *in vitro* derived cormlets via organogenesis were acclimatized after transplanting. It seems that the acclimatization of *in vitro* plantlets is not a concern in various genera of the Iridaceae family and most of them have been easily transferred to *ex vitro* conditions (Ascough et al. 2008). Moreover, some bulblets, such as *Lilium,* do not require hardening treatments even though they contain leaves and stems (Novak and Petru 1981).

CONCLUSIONS

1. In this study we focused on the indirect regeneration of *Freesia* × *hybrida* Bailey 'Argenta' which is considered as a prerequisite for genetic engineering programs. Thus, our findings can be further used to establish an efficient genetic transformation system for this cultivar.
2. We found that pupae were excellent explants with low microbial contamination and high regeneration frequency for callogenesis and indirect regeneration of this popular cut flower. The results showed that NAA and BAP were the best plant growth regulators to achieve these purposes.
3. This study showed that 15 g L^{-1} maltose was the best treatment for the induction of embryogenic calli with friable morphotypes.

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AUTHOR CONTRIBUTIONS

A.P. – designed and performed experiment, M.K.K. – discussed the results and commented on manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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