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EFFECT OF LOW TEMPERATURE ON ROOTING RATE AND CARBOHYDRATE CONTENT OF *FRITILLARIA MELEAGRIS* BULBS FORMED IN CULTURE *IN VITRO*.

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Fritillaria meleagris (snakeshead) is a bulbous geophyte conventionally propagated by bulbs. Since its multiplication rate in field conditions is very low, *in vitro* propagation is an effective alternative for rapid propagation of this species (Bajaj et al., 1988; Sudha and Seeni, 1994). Dormancy, manifested during the annual life cycle of geophytes, is an essential condition for their normal development. Bulbs of *F. meleagris* formed in culture *in vitro* are also dormant. It has been reported that chilling temperatures of 1-10°C terminate dormancy in lily bulblets regenerated *in vitro* (Stimart et al., 1982; Yae et al., 2001). Numerous investigations have shown that dormancy release is unlikely to rely on a linear control pathway, but is modulated by interconnected metabolic pathways (Rinne et al., 2001). A positive correlation between sugar content and the degree of tolerance to abiotic stresses has been reported for many plant species, suggesting that higher sugar content increases hardiness (Al Hakimi et al., 1995; Kerepesi et al., 1998).

We investigated the effects of culture temperature on breaking of dormancy and accumulation of soluble sugars (sucrose, fructose, and glucose) and polyols in bublets of *F. meleagris in vitro*.

In vitro culture of *F. meleagris* was established as earlier reported by Subotić et al. (2004). Bulbs were formed on solid MS medium with Murashige and Skoog (1962) mineral solution containing 3% sucrose, 0,7 % agar, and (in mg/l): casein hydrolyzate 250, L-proline 250, adenine sulfate 80, and thidiazuron (TDZ, 1.0). Isolated bulbs (0.5-1.2 g) were placed on hormone-free MS medium and cultured at 25°C under conditions of 16 h light / 8 h darkness and intensity of 40 μmol $m^{-2}s^{-1}$ and at 4°C under the same conditions (cold treatment) for six weeks. Each treatment involved 80 to 100 bulbs with three replications. Rooting and the sprouting rate and carbohydrate content of bulbs were examined. Bulblets from each treatment were potted and grown in greenhouse conditions.

For analysis of carbohydrate content samples were frozen with liquid nitrogen and then extracted in three volumes of 80% ethanol to separate the ethanol-soluble fraction containing sugars and ethanol-insoluble starch. The ethanol-soluble supernatant was taken after centrifugation for 15 min at 8000 × g. Separations were performed on a Waters Breeze chromatographic system (Waters, Milford, MA) connected to a Waters 2465 electrochemical detector with a 3-mm gold working electrode and hydrogen referent electrode. Separation of sugars was performed on a CarboPac PA1 column (Dionex, Sunnyvale, CA) measuring 250 x 4 mm and equipped with a corresponding CarboPac PA1 guard column using 0.2 M NaOH as the mobile phase. Sugars were isocratically eluted for 20 min at a flow rate of 1.0 ml·min^{-1} and constant temperature of 30°C. Signals were detected in the pulse mode with the following waveform: E_1= +0.05V for 400 ms; E_2= +0.75 V for 200 ms; E_3= -0.15 V for 300 ms with 180 ms of integration time. The filter timescale was 0.2 s, and the range was 200 to 500 nA for the full mV scale. All sugar standards were obtained from Sigma (Sigma Co., St. Louis, MO).

Cold treatment had a strong positive effect on breaking of the dormancy of *F. meleagris* bulbs formed *in vitro* (Table 1). The rooting rate after six weeks of chilling treatment was significantly higher (60.4%), when compared to the control bulbs grown under standard conditions (32.4%). Shoot and root lengths were also higher for chilled bulbs (Fig. 1). Rooted plantlets from each treatment were acclimatized in greenhouse conditions. Cold treatment was found to be important for the acclimatization of plantlets, since the survival rate of control plantlets was very low (Fig. 2). Several authors have reported the effect of low temperatures on the level of dormancy of *in vitro* regenerated bulblets (Yae et al., 2001; Shin et al., 2002), and the present findings are in agreement with the data recorded by previous authors.

Figs. 1-2. 1. Rooted shoots on MS medium during cold treatment after 6 weeks of cold treatment. 2. Potted "in vitro" plants after 6 weeks of chilling temperatures.

Table 1. Effect of low temperature (4° C) on rooting and sprouting of bulblets of *Fritillaria meleagris*. Each value represents the mean ± standard error. Differences between values followed by the same letters are significant at 5%.

Treatments	Average		
	Shoot	Root	
	length (mm)	N^0	length (mm)
Normal culture conditions (25°C)	20.31 ± 1.41[a]	4.96 ± 0.42[a]	1.49 ± 0.10[a]
4°C	24.54 ± 1.61[b]	8.49 ± 0.75[b]	2.01 ± 0.14[b]

Table 2. Effect of low temperature (4°C) on carbohydrate content in bulbs of *Fritillaria meleagris*. Each value represents the mean ± standard error.

Treatments	Relative water content (%)	Carbohydrate content (mmol/100 g dry weight)			
		Sucrose	Glucose	Fructose	Polyols
25°C	77.64	30.42 ± 1.47	3.54 ± 0.39	2.18 ± 0.18	0.64 ± 0.17
4°C	90.67	32.01 ± 2.11	4.78 ± 0.77	7.73 ± 1.17	1.46 ± 0.16

Metabolism of sugars is closely linked to dormancy and sprouting. The most important biochemical changes occurring during cold treatment of bulbs are quantitative changes in carbohydrate constituents. In many experiments *in vivo*, cold treatment caused a considerable increase in carbohydrate contents, as reported by Oquist et al. (1993) and Galiba et al. (1997). When bulbs are stored at low temperature, there is a net breakdown of starch and accumulation of sucrose from hydrolysis of starch, as reported by Shin et al. (2002) for lily bulblets regenerated *in vitro*. Analysis of carbohydrate content revealed that sucrose was the dominant soluble carbohydrate in bulblets of *F. meleagris*. There was no great increase of sucrose accumulation in bulblets after low temperature storage (Table 2). Monosaccharides (glucose and fructose) are present with significantly lower content than sucrose under standard conditions. Significantly increased accumulation of both glucose and fructose in bulblets was observed after low temperature treatment, but the magnitude of change was much less for glucose than for fructose. The data from our experiments are in accordance with the results obtained for *Lillium* tissue culture (Shin et al., 2002). Sucrose plays a central role in growth and development of plants, and the products of its degradation are important factors, particularly during breaking of dormancy, when internal sprouting can be initiated by utilizing accumulated free sugars (Benkeblia, 2003). The differences of saccharide content reported by previous authors suggest that metabolism of sucrose is not clearly understood. Analysis of polyol content in bulblets of *F. meleagris* revealed more than two times higher accumulation of sugar alcohols after low-temperature treatment. Polyols have been found to be efficient osmolytes, and their accumulation results in an improved tolerance to different abiotic stresses (Ahmad et al., 1979). Cold treatment decreases the water potential of the external medium, causing a considerable increase in content of soluble carbohydrates associated with osmoregulation (Morgan, 1992), and/or protection of cellular membranes (Guy, 1990). Starch degradation requires free water for hydrolysis, but at the same time can be accompanied by the release of bound water protons from starch granules (Kamenetsky et al., 2003).

Thus, low-temperature treatment proved to be an important factor in breaking the dormancy of *F. meleagris*, resulted in increased rooting and sprouting of bulblets. In conclusion, our results also indicate that the accumulation of soluble sugars (mainly monosaccharides) and polyols increases in bulblets of *F. meleagris* in response to cold treatment. It can be suggested that these compounds function as compatible solutes.

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