BIOLOGIA PLANTARUM 50 (3): 346-351, 2006

Effects of storage temperature and sucrose on bulblet growth, starch and protein contents in *in vitro* cultures of *Hyacinthus orientalis*

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Abstract

The scale segments of the bulblets of *Hyacinthus orientalis* L. cv. Anna Marie were examined to improve their growth and development with cold-pretreatment and sucrose. The cold-pretreated (4 °C for 4 months) segments showed higher growth and better development of the bulblets on medium without sucrose than ones stored at 20 °C. A rapid decrease in starch content of bulb pieces was found during the first 2 weeks in all cultures and thereafter the content decreased gradually. A scanning electron microscopic observation during the bulblet growth and development showed a gradual decreasing trend of the starch granules from 2 to 16 weeks of the cultures. SDS-PAGE electrophoresis revealed the presence of a characteristic polypeptide of approximately 45 kD, which is assumed to be a major storage protein in the bulblets.

Additional key words: bulblet development, cold-pretreatment, hyacinth, SDS-PAGE, SEM, sucrose.

Introduction

Hyacinth (*Hyacinthus orientalis* L.) is one of the most important bulb ornamental plants, but the natural production rates of their bulblets for multiplication are very slow and the number of the bulblets developed in the scale segments is also very small (Bach 1992). Consequently, many attempts to improve their multiplication have been made by applying various treatments such as low temperatures, different sucrose concentrations, different plant growth regulators (Bach 1992, Le Nard and De Hertogh 1993, Kim and De Hertogh 1997, Yi *et al*. 2002). Some workers (Halmer and Bewley 1982, Heidema *et al*. 1985, Lian *et al*. 2003, Stimart and Ascher l981, Takayama and Misawa 1980) showed that cold pre-treatment and sucrose concentrations have great influence on the initiation and growth of the bulblets or plantlets from the bulb explants of some bulb ornamental plants such as tulips, *Lilium*, hyacinth and gladioli. To understand the relation among storage temperature, sucrose and the bulblet development in hyacinth, we have examined some changes in amount of starch stored in the bulb scale segment, and in protein contents and their profiles.

Materials and methods

Plants and treatments: Bulbs of *Hyacinthus orientalis* L. cv. Anna Marie were obtained from a commercial seed company (*ZaboPlant*, Korte Belkmerweg, The Netherlands). Pretreatment with low temperature was carried out by storing the bulbs at 4 or 20 °C for 4 months. After the bulbs were sterilized as previously described (Yi *et al*. 2002), they were longitudinally cut into 8 pieces and then their outermost scales removed. The scale

Received 21 June 2004, *accepted* 4 April 2005.
Abbreviations: MS - Murashige and Skoog; SDS-PAGE; sodium dodecyl sulfate - polyacrylamide gel electrophoresis.
Acknowledgements: The authors gratefully acknowledge the financial support from the Dong-A University Research Fund in 2002.
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segments with approximately equal sizes were prepared by removing the layered scales from the bulb pieces and the detailed procedures were carried out according to the method of Yi *et al*. (2002).

The bulb pieces were then established *in vitro* in a modified Heller's medium (Heller 1953) containing (per dm^3); 12.5 mg NaFe-EDTA, 1.0 mg glycine, 0.25 mg nicotinic acid, 0.25 mg pyridoxine, 0.05 mg thiamine-HCl, 50 mg myo-inositol. Then the media with or without 2 % sucrose were adjusted to pH 5.4, agar (0.6 %) was added and media were autoclaved. The cultures were incubated in the dark condition at 23 ± 1 °C for 16 weeks. For the quantitative measurements of the percent of bulblet formation, their diameter and length, the mean values were calculated from 3 replications (30 pieces per replication).

Protein extraction and SDS-PAGE analysis: One g of scale segments was pulverized in liquid nitrogen with a mortar and pestle, 0.2 *g* of the pulverized powder was homogenized in 1 cm^3 of an extraction buffer consisting of 1 mM EDTA, 5 mM dithiothreitol and Triton X-100 (pH 7.2). The homogenates were then centrifuged at 10 000 *g* for 40 min at 4 °C and the supernatants used for protein measurements and SDS-PAGE analysis. For quantification of protein, the method of Bradford (1976) was employed using a commercial protein assay kit (*Bio-Rad*, Hercules, CA, USA).

For SDS-PAGE analysis, approximately 20 μg of the total protein extracts were loaded on the gel prepared with a 0.5 % stacking and 12.5 % separating gel (Laemmli 1970). Electrophoresis was run at 200 V using the *Bio-Rad* mini-gel system according to the manufacturer's instructions. The gel was stained with 0.1 % Coomassie Brilliant Blue R-250 (*Bio-Rad*) after electrophoresis and subsequently destained with a mixture of 7 % acetic acid and 10 % methanol.

Starch analysis: Samples obtained from each treatment were cut into small pieces and placed in a flask containing 4 volumes of absolute ethanol followed by heating them at 80 - 85 °C for 15 min. After cooling to room temperature, the samples were homogenized and filtered through a filter paper (*Whatman No*. 2, Maidstone, England) and then dried in a dry oven. Distilled water (2 cm^3) was added to a test tube containing 0.1 *g* of the dried sample, the tube was heated to 100 °C for 30 - 60 min with shakings and then cooled to room temperature. To this tube a stock solution (6 M $HClO_4$) was added to make a solution of 4 M $HClO_4$. Then the solution was centrifuged at 10 000 *g* for 20 min three times. After the supernatant was diluted with distilled water to 0.56 M $HClO_4$, 5 cm^3 of the diluted solution was transferred to a new tube, incubated at 100 °C for 2 h and then its pH arranged to 5.4 with sodium hydroxide. For quantitative measurement of starch, an equal amount (2 cm^3 each) of the pH adjusted sample and the copper reagent were mixed, incubated at 100 °C for 10 min, cooled to room temperature and finally detected with a spectrophoto-meter at 660 nm by adding 2 cm^3 of Nelson's arseno-molybdate reagent (Nelson 1944).

Scanning electron microscopy: For the SEM observation, two-step fixation procedures were conducted. The first fixation was proceeded in a solution of 2.5 % glutaraldehyde at room temperature for 2 h and then the specimens were washed with 0.1 M phosphate buffer (pH 7.2) followed by the second fixation in 2.5 % glutar-aldehyde at room temperature for 2 h and subsequently by 1 % OsO_4 at the same temperature. After washing of the specimen several times, it was then refrigerated at 4 °C overnight and then washed again with the same buffer. For dehydration of the specimen, a series of ethanol treatments from 30 to 100 % were applied and then the specimens were dried at 45 °C for 10 min at 130 kg cm^{-2} in a critical point dryer (*Hitachi HCP-2*, Ibaraki, Japan) after treatment of isoamyl acetate. Thereafter, the specimen was gold-coated in an ion sputter (*Hitachi E-1030*) and finally observed with a SEM (*Hitachi S-4200*) operated at 5 kV.

Results and discussion

Effect of storage temperature and sucrose on change of the bulblet growth: The bulb scale segments pre-treated by storing at two temperatures of 4 and 20 °C for 4 months were cultivated on Heller's medium with and without 2 % sucrose. Some visible initiation of the bulblet formation began at the junction of bulb scale and basal plate of hyacinth after 7 - 8 weeks of the culture. A faster initiation of the bulblets was visually recognized in the cold-pretreated segments on medium without sucrose than in other cultures (data not shown).

The bulblet regeneration was slightly higher in the cold-pretreated cultures without sucrose (80.0 %) than other treatments (73.3 - 76.7 %) (Fig. 1*A*) and the greatest bulblet diameter (Fig. 1*B*) and height (Fig. 1*C*) were determined in the cold-pretreated cultures without sucrose. These observations suggested that the cold storage of the parent bulbs is an efficient way to improve the bulblet growth and development in this hyacinth species.

In general, cold-treatment, energy sources and other

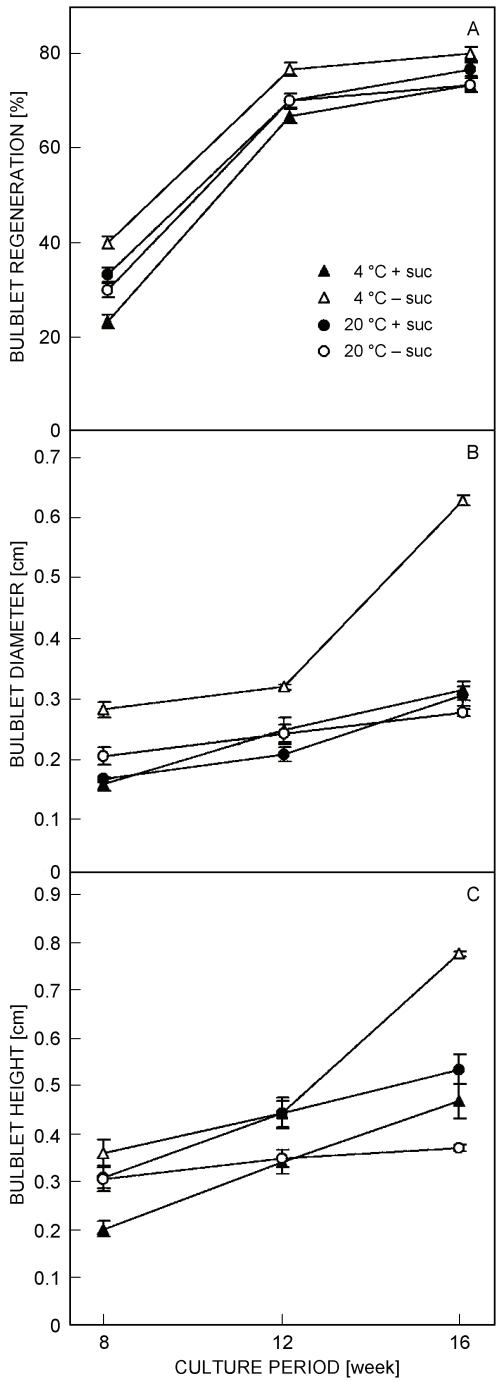

Fig. 1. Effect of cold-pretreatment and sucrose on regeneration of bulblets in the scale segments (*A*), growth of diameter (*B*) and height (*C*) of the bulblets from the scale segments of the hyacinth. Means ± SE were calculated from 3 replicates (30 pieces per replicate for A and at least 70 pieces per replicate for B and C). *Closed triagles* - 4 °C-pretreatment with sucrose, *open triangles* - 4 °C-pretreatment without sucrose, *closed circles* - 20 °C-pretreatment with sucrose, *open circles* - 20 °C-pretreatment without sucrose.

essential nutrients are required for growth and development of not only storage organs such as bulbs, tubers and corms, but also their daughter organs such as bulblets and plantlets (Bach 1992, Fernie and Willmitzer 2001, Fosket 1994, Halmer and Bewley 1982, Xu *et al*. 1998). From our results, one characteristic point is why the cold-pretreated bulbs on medium without sucrose are more effective in growth and development of the bulblets as compared with other treatments. One possible presumption could be derived from physiological processes because growth and development of the bulblets are implicated with sugar metabolism involved with energy source such as starch reserved in their storage tissues and their storage temperature. In general, at low temperature, the consumption rate of the sugar stored in plant storage organs slows down by lowering their respiration (Salisbury and Ross 1992). Based on this physiological aspect, addition of sucrose to the medium could result in excess sugar content in their storage tissues because although the rate of sugar consumption is reduced by cold treatment, sucrose is absorbed and thus accumulated in the tissues. Accordingly, the excess sugar in the tissues would decrease its osmotic potential (Salisbury and Ross 1992) and thus adversely affect growth and development of the bulblets. However, this interpretation remains to be scrutinized by more detailed studies.

Effect of storage temperatures and sucrose on change of starch during bulblet development: The stored starch began to decrease after 2 weeks of the cultures in all treatments and its rapid decrease was observed between 2 and 4 weeks of the cultures (Fig. 2*A*). This finding agreed with other reports that the starch content reserved in the storage organs such as bulbs, corms and tubers, gradually decreased as their regeneration time elapsed. The reports also provided evidence that when the storage organs began to regenerate, they used the reserved starch as an energy source, which had been accumulated during their formation (Fernie and Willmitzer 2001, Jenner 1982, Xu *et al*. 1998). Accordingly, it is thought that the phenomenon on the decrease in the reserve starch is a general pattern in their energy utilization during development of the bulblets (Halmer and Bewley 1982). Other interesting point is that why a rapid transient decrease of starch by temperature pretreatment occurred and why the degree of starch decrease was higher at 20 than at 4 °C (Fig. 2*A*). It seems to result from a transient response of starch metabolic rates to temperature-adaptation process in starch metabolic systems affected by different temperatures (Hochachka and Somero 2002). However, for more detailed understanding about these phenomena, precise studies should be needed.

SEM observation of amyloplasts during the bulb development: Changes in amyloplasts deposited with starch grains were for the first time observed with SEM during the bulblet development (Fig. 3). It is difficult to examine a distinctive difference between cold-pretreated and untreated cultures. Notwithstanding, it is recognizable that the frequency of the starch granules deposited in the amyloplasts was greatly reduced after 16 weeks of the cultures, suggesting that the starch has been utilized as an energy source for growth of the bulblets during their development.

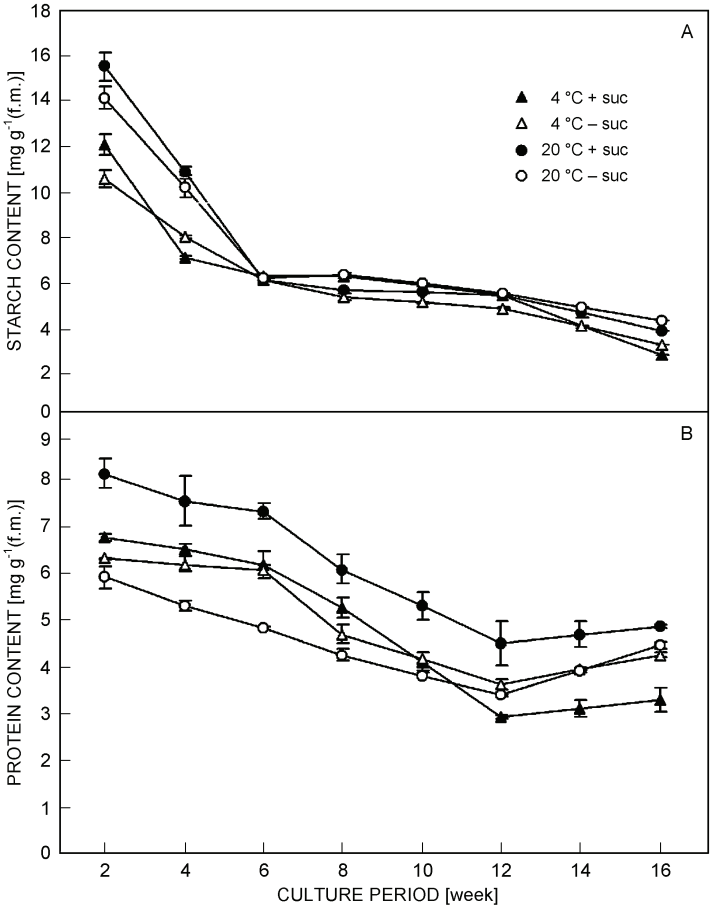

Fig. 2. Changes of starch (*A*) and protein (*B*) contents during development of the bulblets from the scale segments of the hyacinth influenced by cold-pretreatment and sucrose. Mean ± SE were calculated from 3 replicates. The symbols are the same as in Fig. 1.

Effects of storage temperatures and sucrose on changes of proteins during the bulblets development: The total protein contents gradually decreased during the culture and a relatively rapid decrease was observed during first 2 weeks (Fig. 2*B*), suggesting that most of them would be the storage proteins present in the parent bulb scales. No significant difference in the protein changes was determined between treatments. This suggested that storage temperature and sucrose may not influence protein changes during the bulblet development.

Analysis of SDS-PAGE displays the banding patterns of polypeptides and the presence of a strong band corresponding to a polypeptide of 45 kD (Fig. 4). These polypeptides are frequently accumulated during the bulblet development and belong to a family of major storage proteins (Yi *et al*. 2002). Some other weaker bands disappeared after a few weeks of the cultures or remained unchanged throughout the cultures. There was no significant difference in protein patterns between treatments (Fig. 4), suggesting that storage temperature and sucrose may not have influence on changes of the protein profiles during the bulblet development.

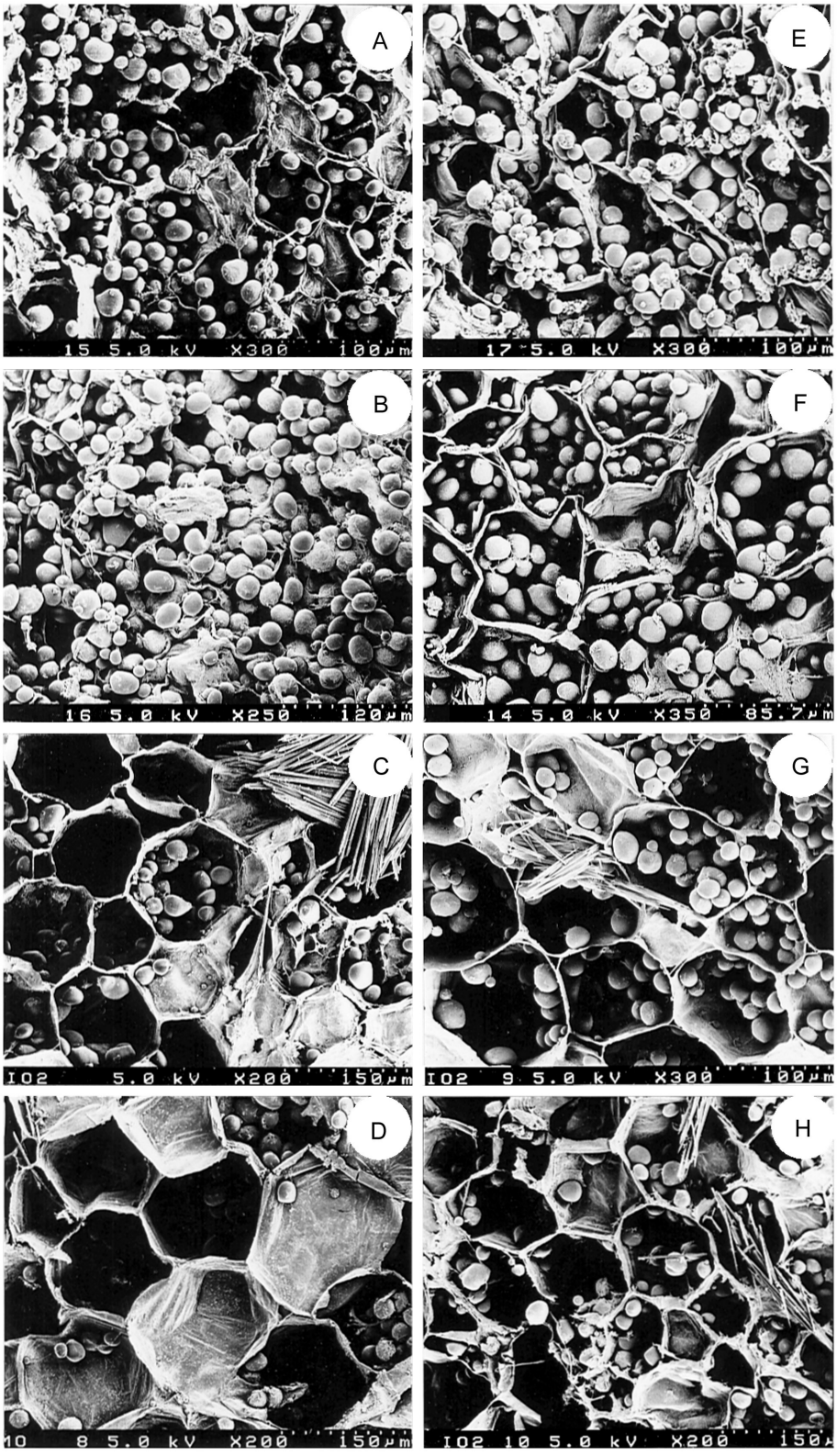

Fig. 3. Scanning electron micrographs of starch granules deposited in the amyloplasts during development of the bulblets from the scale segments of the hyacinth cultivar 'Anna Marie'. Observations were conducted using cold-pretreated (*A-D*) and untreated (*E-H*) cultures without sucrose. Samples were selected at different culture periods; 0 week (*A*,*E*), 2 weeks (*B*,*F*), 8 weeks (*C*,*G*), and 16 weeks (*D*,*H*). Starch storage bodies, amyloplasts containing starch granules were clearly observed. The deposited starch granules began to decrease gradually after 2 weeks of the cultures.

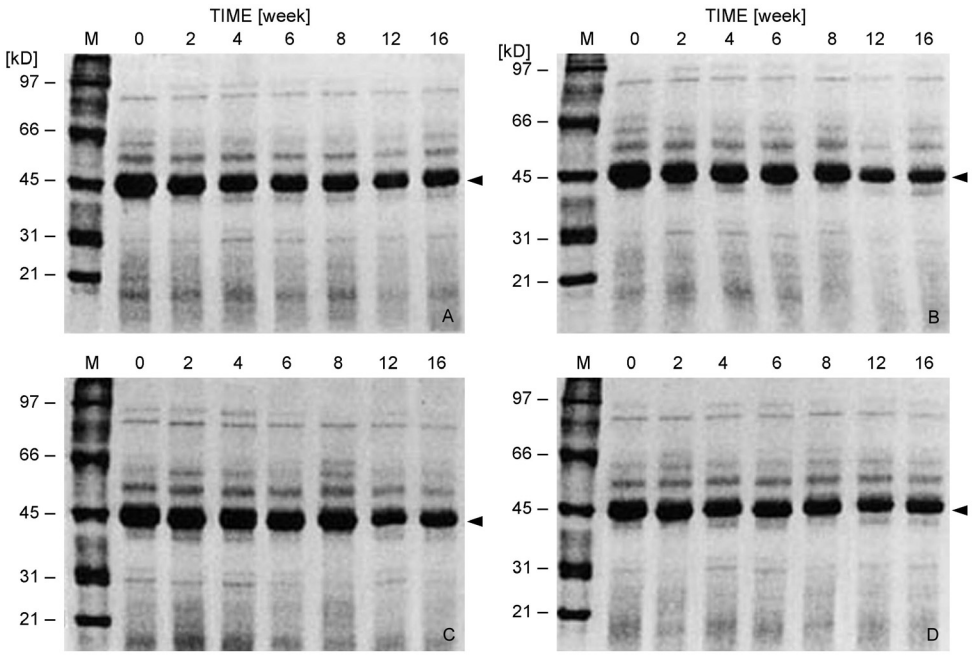

Fig. 4. Protein profiles by SDS-PAGE analysis during development of the bulblets from the scale segments of the hyacinth influenced by storage temperatures and sucrose. These profiles include 4 treatments; 4 °C-pretreatment with sucrose (*A*), 4 °C-pretreatment with no sucrose (*B*), 20 °C-pretreatment with sucrose (*C*), and 20 °C-pretreatment with no sucrose (*D*). M - molecular mass markers. *Arrow-heads* indicate polypeptides described in the text.

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