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Effect of cultivation conditions on morphological and biochemical characteristics of lily explants *in vitro*

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Abstract

Regeneration of bulblets from excised bulbscales has become the preferred method for vegetative propagation of lilies. For optimal *ex vitro* growth and survival during *in vitro* propagation of lilies bulblets need to have both a period of high temperature as well as cold storage. The objective of the present experiments was to determine the optimal sugar concentration in the medium and duration of pre-cold storage period for lily explants of Asiatic lily clone 'NS-94' and Trompet lily cv. 'Mezhare' and to relate morphological characteristics with peroxidase and polyphenol oxidase activities. Morphological parameters of lily microplants were affected by cultivation conditions, e.a., low temperature storage, length of pretreatment period before cold treatment, as well as by light regime and sucrose concentration in the medium. Growth of bulblets was strongly affected by cold treatment, leading to a significant decrease of relative mass. Root formation was inhibited by cold treatment. This effect was more pronounced in cv. 'Mezhare' than clone 'NS-94'. Formation of both leaves as well as bulblets was suppressed by cold storage of lily microplants. Cold treatment resulted in an increase of oxidative enzyme activity in bulblet tissues. Prolongation of duration of pre-cold period strongly increased peroxidase and polyphenol oxidase activity in bulblets of clone 'NS94', the effect was more pronounced in minimum light conditions. In contrast, for cv. 'Mezhare', higher enzyme activities were in bulblets of lily explants showing shortest duration of pre-cold period, especially in the light. A decrease in peroxidase activity appeared to be a good indicator for cold-induced slow growth of lily explants. In part this may be associated with increased antioxidative capacity in conditions of slow growth.

Key words: cold treatment, *in vitro* propagation, lily, morphological parameters, peroxidase, polyphenol oxidase.

Introduction

Regeneration of bulblets from excised bulbscales has become the preferred method for vegetative propagation of lilies. However, the necessity for specific conditions during tissue culture resembling those of natural growth of geophytes has been described. Thus, *in vitro* cultivated varieties of *Lilium speciosum* are dormant during incubation at 20 to 25 °C and need a cold treatment resembling a rest period in natural conditions prior to planting *ex vitro* (Aguettaz et al. 1990; Langens-Gerrits et al. 2003). It was suggested that development of dormancy occurs at temperatures higher than 15 °C (Delvallée et al. 1990). In contrast, lilies of the Asiatic and Trompet groups develops normally in tissue culture at warm

temperatures. We developed a protocol for micropropagation of lilies *in vitro* from small bulblets developing on scale explants (Jakobsone, Andersone 1997). However, for optimal *ex vitro* growth and survival, lily bulblets of these groups need to have both a period of high temperature as well as cold storage (Higgins, Stimart 1990; Ievinsh et al. 2003).

Peroxidase and polyphenol oxidase are enzymes catalysing oxidation of various substrates, mainly of phenolic nature, by means of hydrogen peroxide and oxygen, respectively. Attempts have been made to correlate the changes of oxidative enzymes during cultivation in tissue culture with developmental processes e.g. as indicators of explant viability or as biochemical markers of morphogenic capacity (Bouazza et al. 1993; Andersone, Ievinsh 2002). Peroxidase was shown to be involved in the dormancy of onion bulbs (Benkeblia, Selselet-Attou 1999) and in garlic microbulblets (Arguello et al. 2001). However, there is no information available on how oxidative enzymes are related to morphological changes *in vitro* during low temperature storage. Therefore, the objective of the present experiments was to determine the optimal sugar concentration in the medium and duration of the pre-cold storage period for lily explants and to relate morphological characteristics with peroxidase and polyphenol oxidase activities.

Materials and methods

Asiatic lily (*Lilium* L.) clone 'NS-94' and Trompet lily cv. 'Mezhare' cultures were produced using bulb scale explants (Jakobsone, Andersone 1997). Lily bulblets cultivated for ten months on agar-solidified Murashige and Skoog (1962; MS) medium supplemented with 3 % (w/v) sucrose were used as a source for the experiments. The cultures were grown in test tubes in a growth cabinet at 25 °C with a photoperiod of 16 h (white fluorescent lamps, 6 W m^{-2}). During this period, active growth of bulblets, leaves and roots, as well as proliferation of bulblets occurred. Lily bulblets without leaves and roots were used as initial material for experiments and were transplanted in test tubes on agar-solidified modified MS medium supplemented with 0.08 mg l^{-1} 1-naphthalene acetic acid and 2 %, 4 % or 6 % (w/v) sucrose as a source of reduced carbon. Explants were cultivated in a growth cabinet at 25 °C at the same conditions as previously for one to three weeks to test the effect of duration of the pre-cold storage period. Then explants were transferred to a cold room at 5 °C for nine weeks in dark or minimum light conditions. Control cultures were kept at the above mentioned conditions in a growth cabinet for an additional nine weeks.

Several morphological parameters were measured to characterize the physiological state of lily plantlets after cold storage, including an increase in relative fresh mass of bulblets, number of roots, number of leaves, and number of bulblets per explant. The relative increase of bulblet fresh mass was calculated based on the increase within the nine weeks of experiment divided by the initial bulblet mass.

Bulblet tissues were ground with mortar and pestle under liquid nitrogen. Soluble protein was extracted from frozen material using 25 mM HEPES buffer, pH 7.2 as described previously (Kruzmane et al. 2002). Polyphenol oxidase activity was measured spectrophotometrically using pyrocatechol as a substrate (Gauillard et al. 1993). Peroxidase activity was determined spectrophotometrically using guaiacol as a hydrogen donor.

For each treatment, 24 explants in three replicates were used. Statistical analysis and correlations were performed with KaleidaGraph® 3.6.4. (Synergy Software).

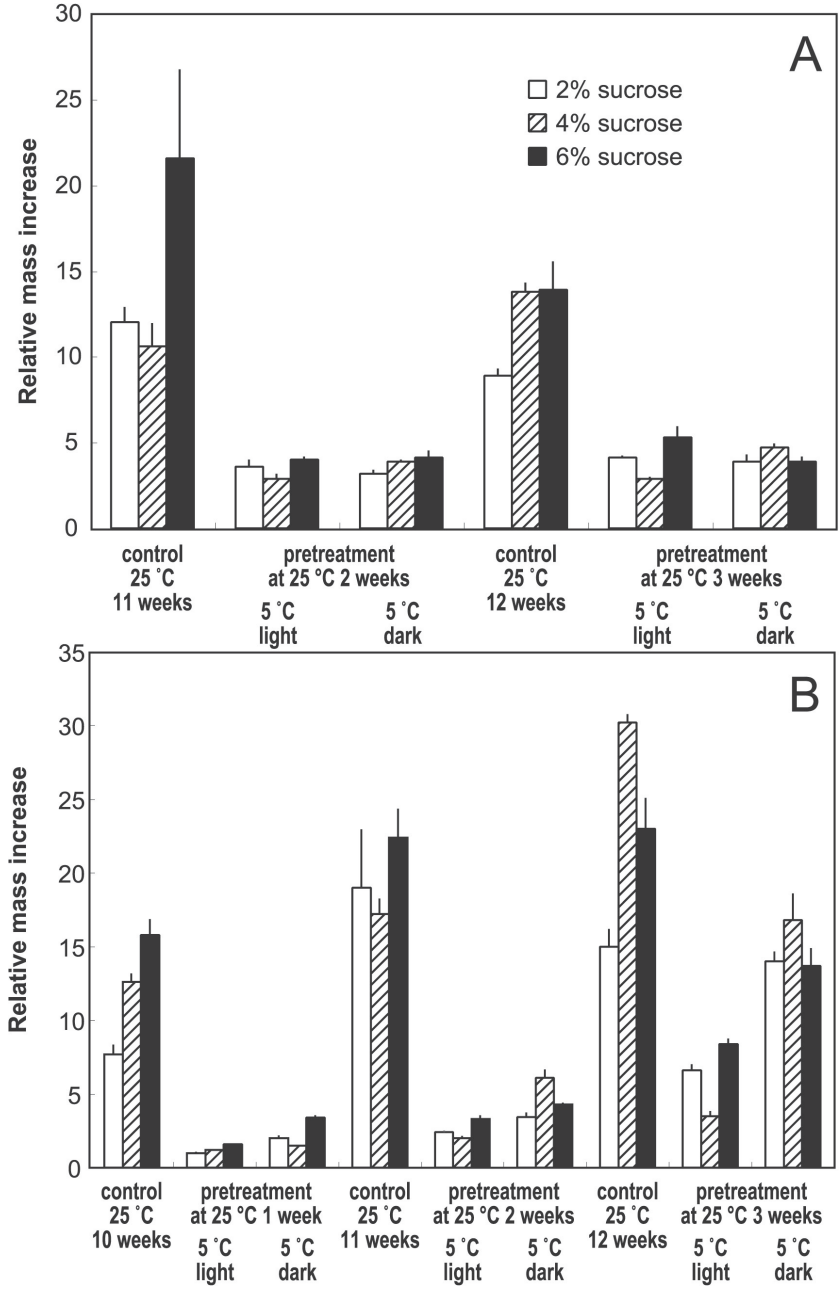

Fig. 1. Effect of pretreatment period at 25 °C and sucrose concentration in the medium on the relative mass increase of lilium explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Relative fresh mass increase of bulblets was calculated based on the increase within the period of experiment divided by the initial bulblet mass. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

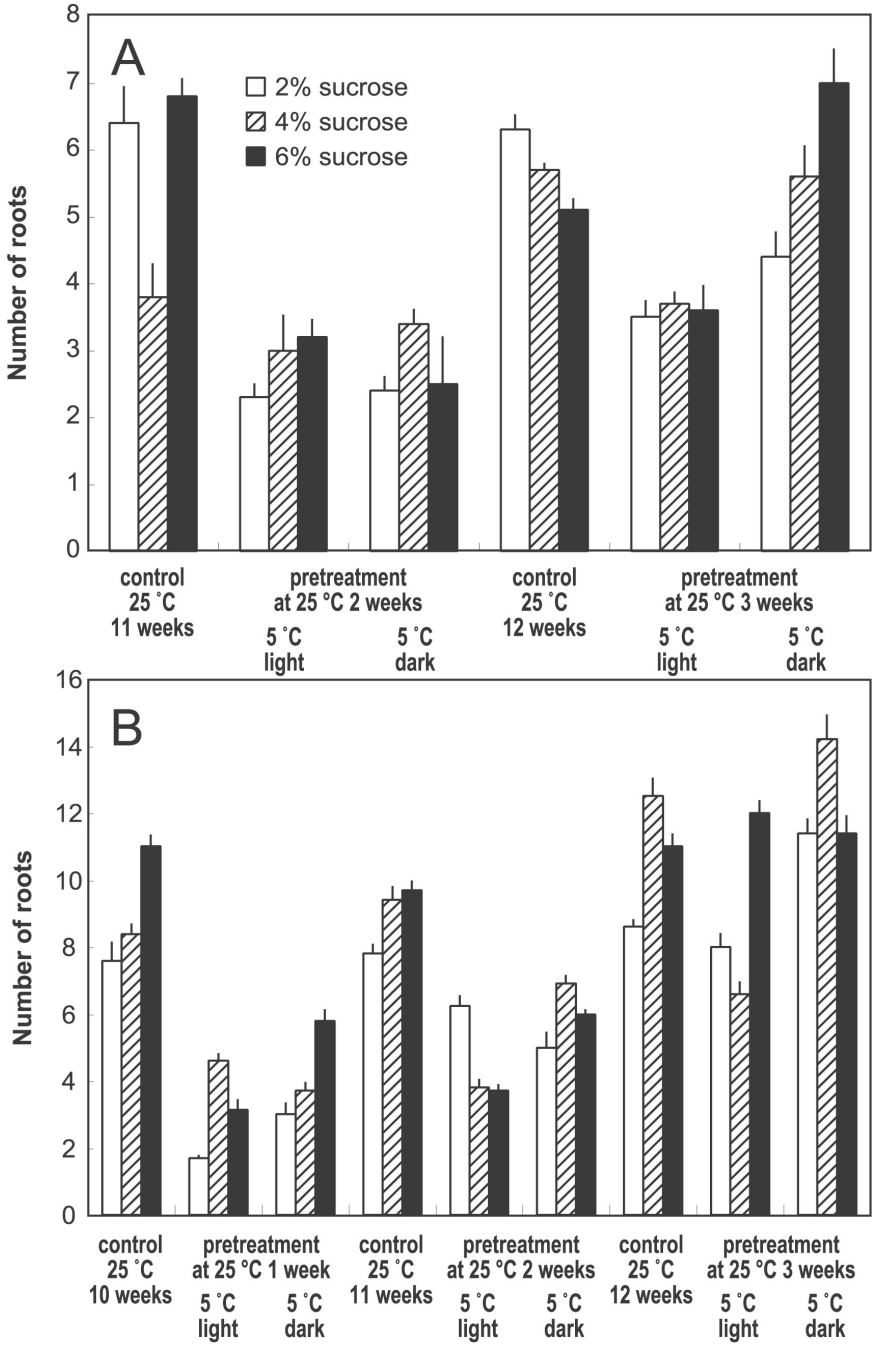

Fig. 2. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on number of roots of lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

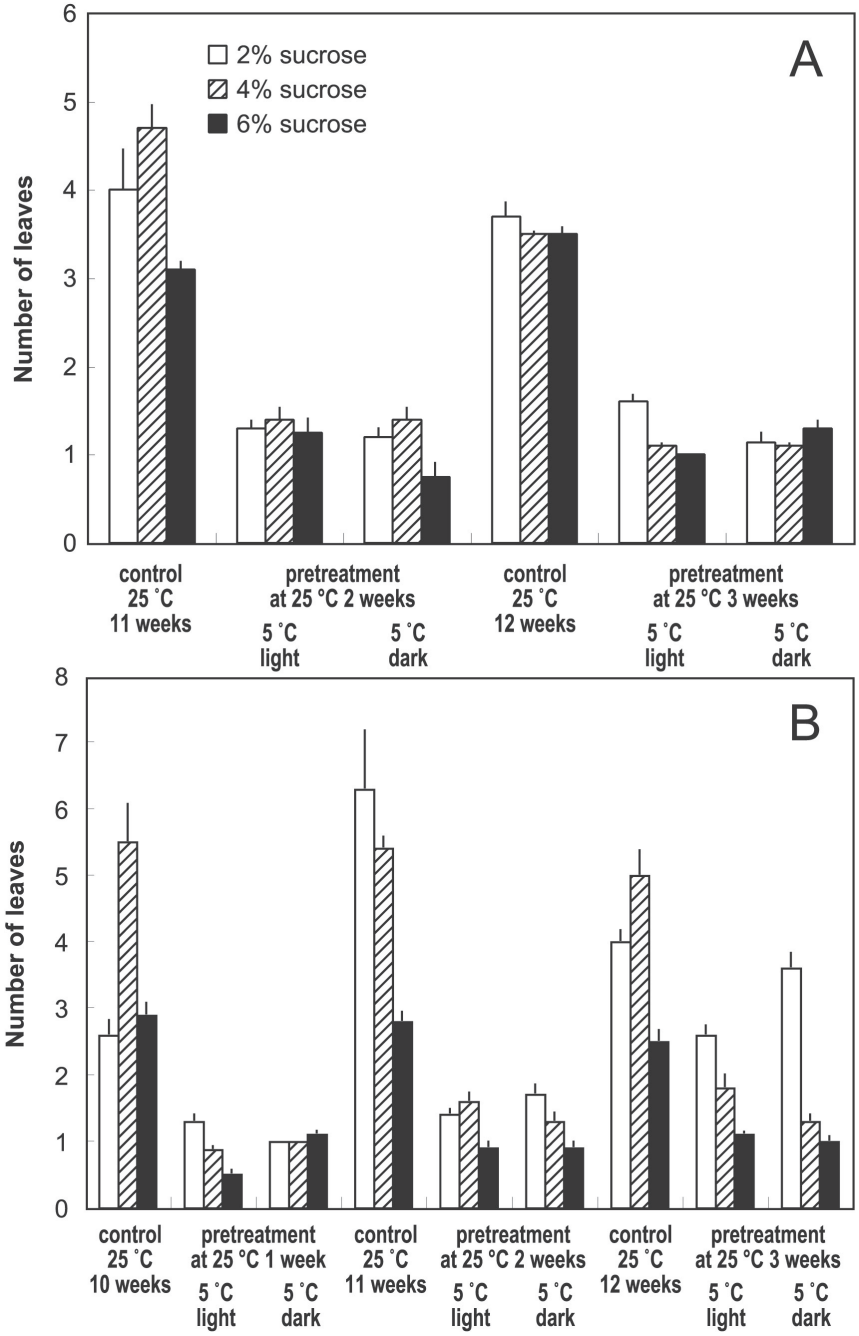

Fig. 3. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on number of leaves of lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

Results

The optimal sugar concentration in the medium and duration of the pre-cold storage period for lily explants was determined in the present experiments. High temperature-induced dormancy was practically absent for both tested lily cultivars – they developed normally at 25 °C. A moderate effect of prolonged cultivation at 25 °C was evident only as a tendency of depressed bulblet growth in clone 'NS-94' at high sucrose concentration (Fig. 1A, 4A). In cv. 'Mezhare', prolonged high temperature induced activation of bulblet growth and multiplication (Fig. 1B, 2B, 4B).

In general, the morphological parameters of lily microplants were affected by cultivation conditions, e.a., low temperature storage, length of pretreatment period before cold treatment, as well as by light regime and sucrose concentration in the medium. Growth of bulblets was strongly affected by cold treatment, leading to a significant decrease of relative mass (Fig. 1). In clone 'NS-94' the relative mass increase was not affected by length of a pretreatment period before cold storage (Fig. 1A). In contrast, prolongation of the pretreatment period significantly stimulated a mass increase of cv. 'Mezhare' bulblets, especially in the dark (Fig. 1B). Root formation was inhibited by cold treatment (Fig. 2). This effect was more pronounced in cv. 'Mezhare' than clone 'NS-94'. However in cv. 'Mezhare', the inhibitory effect of cold treatment was strongly diminished by a prolongation of pretreatment period before cold storage (Fig. 2B). Formation of both leaves (Fig. 3) as well as bulblets (Fig. 4) was suppressed by cold storage of lily microplants. For cv. 'Mezhare', prolongation of pretreatment period before cold storage had a tendency to diminish the suppressive effect of cold treatment on organ formation. This was especially pronounced for bulblet formation as well as for leaf formation at the low sucrose concentration.

Peroxidase and polyphenol oxidase activity was measured in lily bulblets at the end of the experiment. In parallel with morphological parameters, oxidative enzyme activities as well were affected by cultivation conditions. In general, cold treatment resulted in increase of oxidative enzyme activities in bulblet tissues. A longer duration of the pre-cold period strongly increased peroxidase and polyphenoloxidase activity in bulblets of cv. 'NS94'; the effect was more pronounced in minimum light conditions (Fig. 5A, 6A). In contrast, for cv. 'Mezhare', higher enzyme activity was observed in bulblets of lily explants with the shortest duration of pre-cold period, especially in the light (Fig. 5B, 6B).

The effect of sucrose concentration on oxidative enzyme activity was not clearly pronounced. Bulblets of explants of both cultivars tended to have higher polyphenol oxidase activity in their bulblets with increasing sucrose concentration in the medium (Fig. 5).

There was a strong negative correlation between peroxidase activity and relative mass increase for both lily cultivars ($r^2 = -0.81$, $p < 0.001$, clone 'NS94'; $r^2 = -0.77$, $p < 0.001$, cv. 'Mezhare'). For clone 'NS94', the peroxidase activity was also highly negatively correlated with the number of leaves ($r^2 = -0.81$, $p < 0.001$) and the number of bulblets ($r2 = -0.78$, $p < 0.001$). For cv. 'Mezhare', peroxidase activity was strongly negatively correlated with the number of roots ($r^2 = -0.72$, $p < 0.001$) and the number of bulblets ($r^2 = -0.67$, $p < 0.001$). Polyphenol oxidase activity also was negatively correlated with morphological parameters, although to a lesser extent ($r^2 = -0.64$ for mass increase, $r^2 = -0.63$ for number of bulblets, $r^2 = -0.65$ for numer of leaves). The correlation was similar for both cultivars.

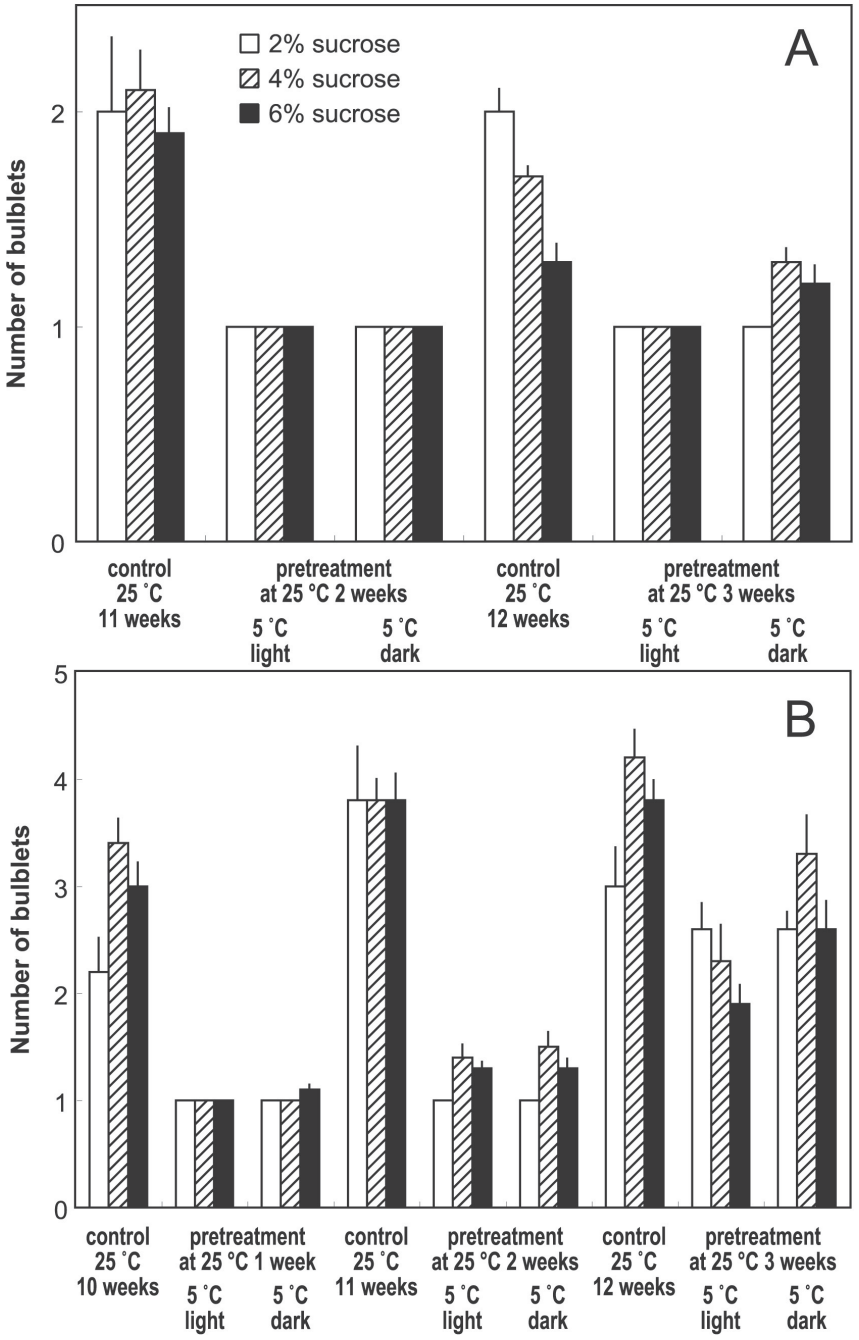

Fig. 4. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on number of bulblets of lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

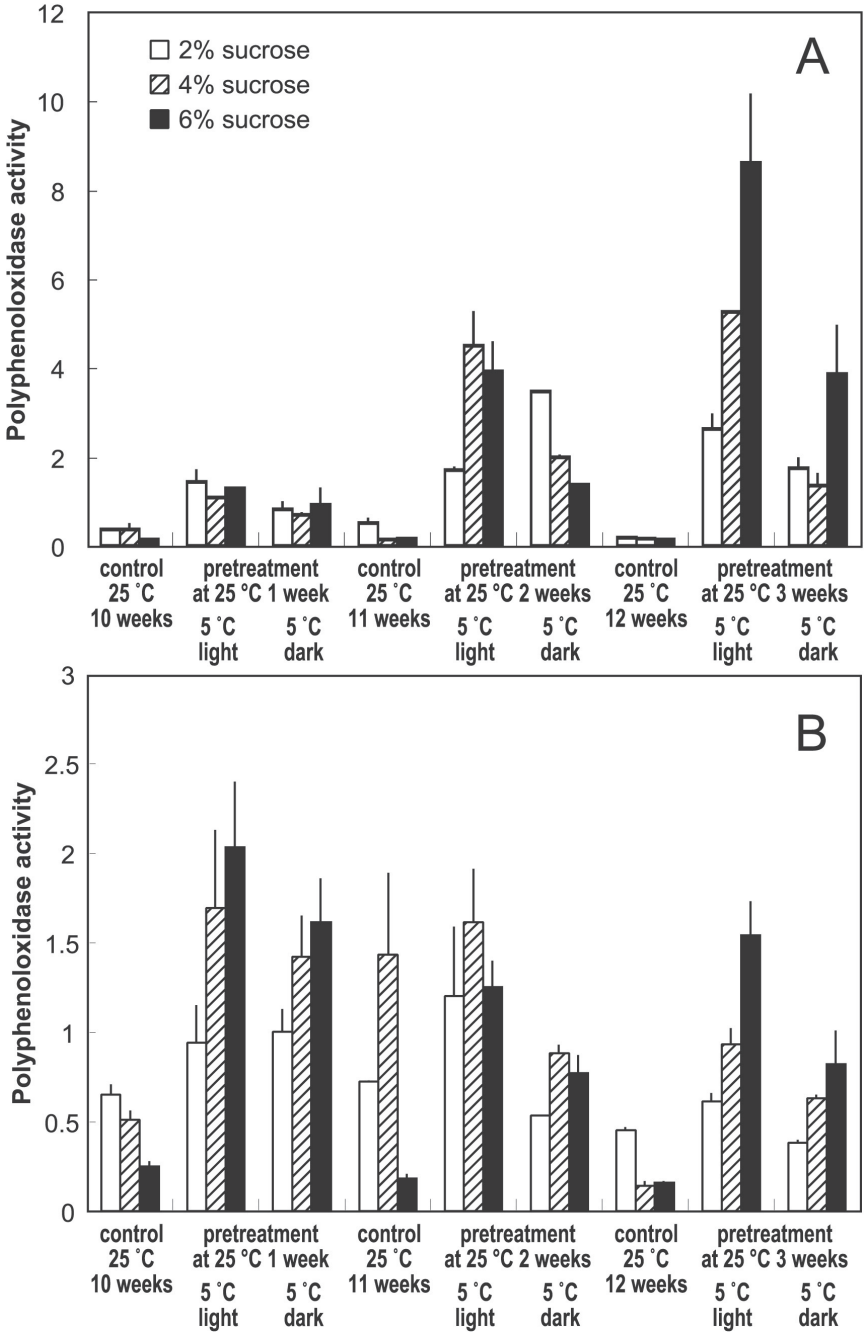

Fig. 5. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on polyphenol oxidase activity in lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

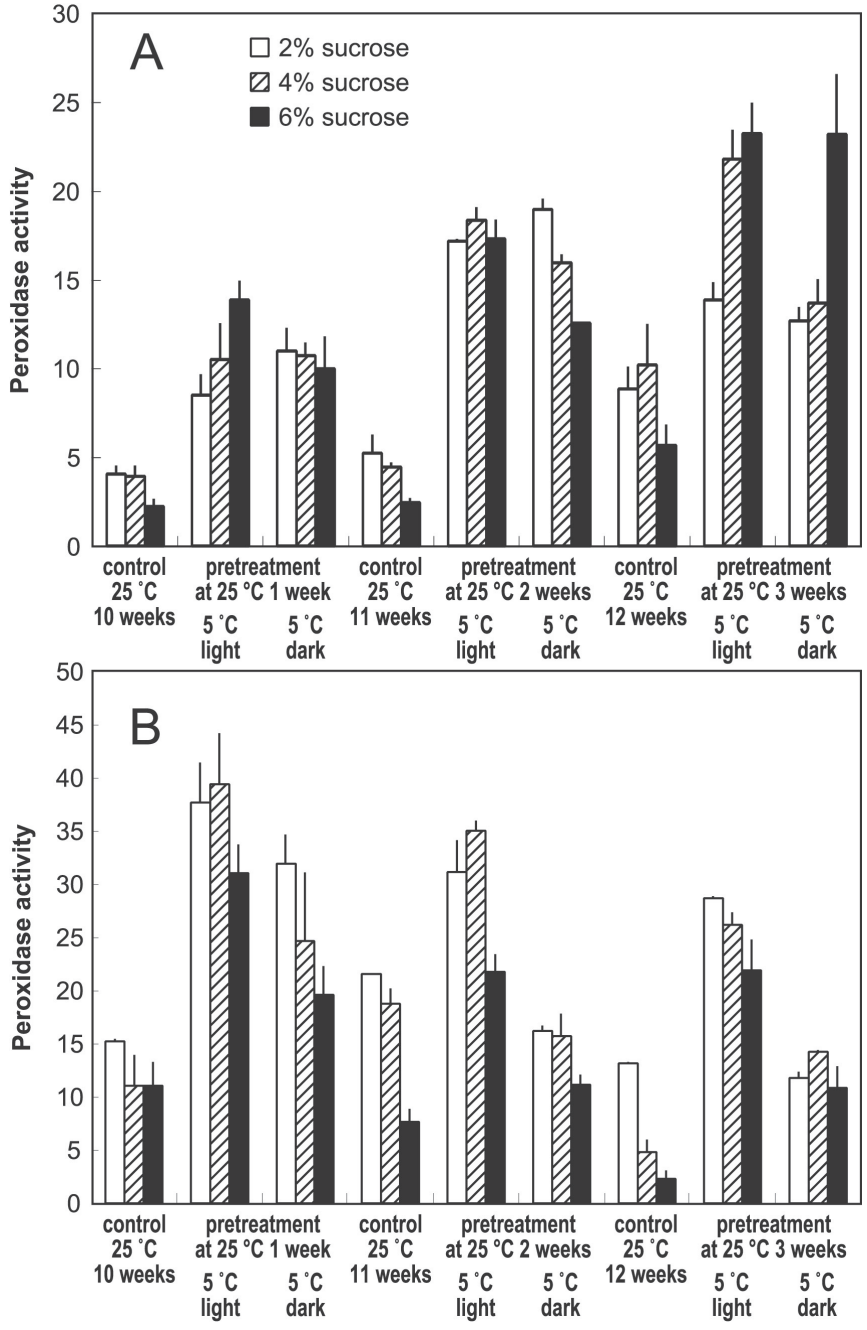

Fig. 6. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on peroxidase activity in lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

Discussion

During propagation of plants with a seasonal lifestyle using tissue culture, a serious problems could arise because of lack of specific environmental signals for maintaining the normal sequence of developmental events. For lilies, sensitivity to high temperature-induced dormancy is species- or even cultivar-dependent. Our results clearly showed that both a high temperature period followed by low-temperature incubation are necessary prerequisites for establishment of a normal developmental sequence of lily explants during tissue culture. While high temperature-induced dormancy was practically absent for Asiatic lily 'NS-94' and for Trompet lily 'Mezhare', the length of the high-temperature period was important for certain morphological characteristics of explants after the cold period. Both lilies remained in the juvenile stage at 25 °C as elongation of stems was not observed. The adult phase of *Lilium* begins stem growth initiated by cold treatment (Langens-Gerrits et al. 2003).

In general, 'NS-94' was relatively tolerant to an increase of preincubation period in respect to growth and development with the exception of root formation in dark at the high level of sucrose in the cultivation medium. However, a longer preincubation period in clone 'NS-94' enhanced enzyme activity in bulblets after cold storage. In cv. 'Mezhare', a prolonged preincubation period reversed the inhibitory effect of cold storage on plantlet growth and development together with a reversal of the increase in enzyme activities. Thus, it can be concluded that lilies of Asiatic and Trompet groups have different requirements for cultivation conditions during tissue culture.

In the present experiments an increase of sucrose concentration in the cultivation medium had no consistent effect on morphological parameters of lily explants and on oxidative enzyme activities in bulblets. For other species of the genus *Lilium*, a high concentration of sucrose during *in vitro* cultivation together with cold treatment has been observed to promote bulblet growth (Yamagishi 1998). Sucrose had little or no effect on shoot and bulblet growth of *in vitro* cultivated lily microplants at 25 °C in light (Bonnier, Van Tuyl 1997). However, viability as well as a regrowth *ex vitro* was increased at higher sucrose concentration. Most probably, the sensitivity of lily explants to changes in sucrose concentration in the medium is a species-specific trait.

Cold treatment-induced slow growth is a period physiologically resembling cold acclimation leading to initiation of flowering. In general, cold acclimation increases tolerance to oxidative stress due to increased ability to scavenge activated forms of oxygen (Kuroda et al. 1990; Bridger et al. 1994; Scebban et al. 1998). In our experiments, cold incubation increased both peroxidase and polyphenol oxidase activity in lily bulblets in parallel with decreased growth and development of plantlets. This effect was more pronounced in clone 'NS-94'.

An increase of peroxidase activity during cold incubation might be suggested as a result of enhanced antioxidative capacity due to increased generation of active oxygen species (Okuda et al. 1991). The most important part of chilling injury in natural conditions is associated with photoinhibition (Wise, Naylor 1987). During growth in tissue culture in darkness with high concentrations of exogenous carbohydrate supplied, this should be of less importance. Indeed, in lily 'Mezhare', cold-induced peroxidase activity was significantly higher in light-grown bulblets than in dark-grown. This was also the case for polyphenol oxidase activity in 'Mezhare'. In contrast, the enzyme activity in 'NS-94' during cold storage

were not affecetd by the light regime.

In the present experiments, a decrease in peroxidase activity appeared to be a good indicator for cold-induced slow growth of lily explants. In part this may be associated with increased antioxidative capacity during slow growth. Our data are in accordance with the fact that a transition from growth to wintering includes an increase in the antioxidative system concomitant with an increase in the cold resistance (Kuroda et al. 1990). In this respect it is interesting to note that in woody plants proteins related to cold acclimation are connected to the dormancy status of the plants (Wisniewski et al. 1996; Rowland, Arora 1997).

Acknowledgements

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Kultivēšanas apstākļu ietekme uz liliju eksplantu morfoloģiskajām un bioķīmiskajām īpašībām *in vitro*

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Kopsavilkums

Liliju veģetatīvajā pavairošanā vsibiežāk izmantotā metode ir sīksīpoliņu reģenerācija uz atdalītām sīpola zvīņām. Optimālas *ex vitro* augšanas nodrošināšanai un izdzīvošanai *in vitro* apstākļos liliju sīksīpoliņi jāeksponē gan augstā temperatūrā, gan arī aukstumā. Aprakstīto eksperimentu mērķis bija noteikt optimālās barotņu cukura koncentrācijas un pirms aukstuma perioda ilgumu Āzijas liliju klona 'NS-94' un trompešliliju šķirnes 'Mežāre' eksplantiem, kā arī saistīt morfoloģiskās īpašības ar peroksidāzes un polifenolu oksidāzes aktivitāti. Liliju mikroaugu morfoloģiskos parametrus ietekmēja tādi kultivēšanas apstākļi kā uzglabāšanā zemā temperatūrā, priekšapstrādes perioda ilgums pirms aukstuma uzglabāšanas, gaismas režīms un saharozes koncentrācija vidē. Sīpoliņu augšanu būtiski ietekmēja aukstuma uzglabāšana, izraisot būtisku relatīvās masas samazināšanos. Aukstums inhibēja arī sakņu veidošanos, un šī ietekme bija izteiktāka šķirnei 'Mežāre', nekā klonam 'NS-94'. Mikroaugu uzglabāšana aukstumā apspieda gan lapu, gan sīpoliņu veidošanos. Aukstuma apstrāde izsauca oksidatīvo fermentu aktivitātes pieaugumu sīpoliņu audos. Siltuma inkubācijas perioda paildzināšana pirms aukstuma apstrādes būtiski paaugstināja peroksidāzes un polifenolu oksidāzes aktivitāti klona 'NS-94' sīpoliņos, un šis efekts bija vairāk izteikts minimālās gaismas apstākļos. Pretēji tam, šķirnei 'Mežāre' augstākas fermentu aktivitātes bija novērojamas to liliju eksplantu sīpoliņos, kuriem bija īsākais siltuma inkubācijas periods, it īpaši, gaismā. Pazemināta peroksidāzes aktivitāte izrādījās labs indikators aukstuma inducētajai liliju eksplantu lēnajai augšanai. Tas daļēji varētu būt saistīts ar pretoksidatīvās sistēmas aktivāciju lēnās augšanas apstākļos.