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Formation of Calli from Isolated Microspore Cultures of Asiatic Hybrid Lily 'Connecticut King'

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Summary

Calli from microspores were induced for the first time in *Lilium* species. The uninucleate microspores of the Asiatic hybrid lily 'Connecticut King' were isolated and cultured in liquid medium, containing half strength of MS macronutrients, full strength of micronutrients, Fe - EDTA and vitamins of MS, 100 mg・liter^{-1} cosamino acids, 500 mg・liter^{-1} glutamine, 1 mg・liter^{-1} picloram and 0.25 M sucrose or maltose. Microspore viability and development of cultured microspores were influenced by the carbohydrate sources in culture medium. A relatively high viability rate of microspores was observed in the maltose medium, compared with that of sucrose. Cell divisions of microspores and callus formation were found in the maltose medium, whereas in the sucrose medium, a large number of swollen microspores containing many starch grains remained undivided. Hence, maltose is the preferred carbohydrate source for microspore culture of 'Connecticut King'.

Key Words: maltose, microspore culture, sporophytic development.

Introduction

Haploid and doubled haploid plants have been obtained in *Lilium* species by anther culture (Sharp et al., 1971; Gu and Cheng, 1982; Arzate - Fernández et al., 1997), but diploid plantlets, which probably originated from somatic cells of anther wall, are also produced (Yamagishi, 1995). Our previous study indicated that both haploid and diploid plantlets were regenerated from anther cultures of the Asiatic hybrid lily 'Connecticut King' (Han et al., 1997), but the origin of these diploids was obscure. Isolated microspore culture is a suitable way to obtain gametophyte - derived plants (Bhojwani and Razdan, 1983). Moreover, isolated microspore culture and plant regeneration techniques are potentially applicable for other biotechnological applications (Jähne and Lörz, 1995).

To obtain the plants from isolated microspores, microspores must undergo sporophytic development, or mitosis, resulting in multinucleated or multicellular microspores instead of normal pollen formation. Induction of sporophytic development in cultured microspores has been reported for the Asiatic hybrid lily 'Whilito', but only multinucleate microspores were obtained (van den Bulk et al., 1992). This paper describes effects of carbohydrate sources and media renewal on microspore viability and callus formation of isolated microspores in the Asiatic hybrid lily 'Connecticut King'.

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Materials and Methods

Plant material

The Asiatic hybrid lily 'Connecticut King', whose flower buds (20 to 30 mm in length) containing microspores at the mid - to late - uninucleate stage, growing under natural conditions in the field were harvested from plants 50 to 70 days after sprouting.

Culture media

The culture medium consisted of half strength macronutrients, full strength of Fe - EDTA, micronutrients and vitamins of MS (Murashige and Skoog, 1962), 100 mg・liter^{-1} casamino acids (Difco Laboratories, USA), 500 mg・liter^{-1} glutamine, and 1 mg・liter^{-1} picloram. As a carbohydrate source, 0.25 M sucrose or maltose was added to the basal liquid medium, referred to as sucrose - and maltose - medium, respectively. The media were adjusted to pH 5.8, using 0.1 N NaOH, then sterilized by passing through a membrane filter (pore size 0.45 μm, Toyo Roshi Co., Ltd. Japan).

Isolation and culture of microspores

After storing at 4 ℃ for 4 days, flower buds were surface - disinfected with 70% ethanol for 1 min and with a commercial bleach containing 2% active chlorine for 15 min. The sterilized buds were rinsed three times

with sterilized distilled water and the dissected anthers cut transversely into 1 mm length. Anther segments were then placed into a 100-ml Erlenmeyer flask, containing 20 ml of liquid sucrose- or maltose-medium and stirred for 15 min with a magnetic stirrer (RC-2 Tokyo Rikakikai Co., Ltd. Japan). The liquid medium containing microspores and anther debris was filtered through a nylon sieve (pore size 150 μm). The microspores were immersed in the same fresh medium and centrifuged twice at 100 $\times$ g for 5 min. The microspores were resuspended and the density was adjusted to 3×10^5 microspores$\cdot$ml^{-1} using a haemocytometer (Kayagaki Irikakogyo Co., Ltd. Japan). Three ml of microspore suspension was placed into a 50 $\times$ 15 mm sterilized plastic Petri dish, sealed with a double layer of Parafilm (Iwaki Glass Co., Ltd. Japan), and incubated at 25 ℃ in the dark. At 1, 3, 5, and 7 days after initiation of the culture, the microspores were collected by centrifuging the suspensions at 100 $\times$ g for 5 min. The pellets were resuspended in the same fresh medium. Each treatment was triplicated.

Cytological observations

Microspore viability was determined at 0, 10, and 20 days after the initiation of the culture, using fluorescein diacetate (FDA) staining method (Heslop-Harrison et al., 1984). About 50 μl of 0.05% FDA solution was added to 1 ml microspore suspension and 300 to 400 microspores per 3 samples were stained, and observed under a fluorescent microscope (Olympus BHF wavelength: 395-410 nm); those which fluoresced under UV light were considered viable. The starch in the microspore was stained by a solution, containing 0.2 mM I_2 and 0.3 mM KI. Microspore development was observed under a light-inverted microscope (Olympus CK2).

Results

Effect of carbohydrate sources on the microspore viability

About 60% of the microspores was viable at the initiation of culture; the percentage decreased with culture time in both sucrose- and maltose-media. The viability was 20.3% and 11.7% in sucrose-medium, and 42.8% and 21.3% in maltose-medium after 10 and 20 days of culture, respectively (Fig. 1). The viability of the microspore in maltose-medium was approximately two-fold higher than that in sucrose-medium.

Effect of media renewal on the microspore viability

The microspore viability was increased by renewal (subculturing) with fresh sucrose-medium, whereas no improvement occurred with the maltose-medium after 10 days of culture (Fig. 2A). No improvement of microspore viability occurred by renewal of both media after 20 days of culture (Fig. 2B). More microspores subcultured in the maltose-medium were consistently

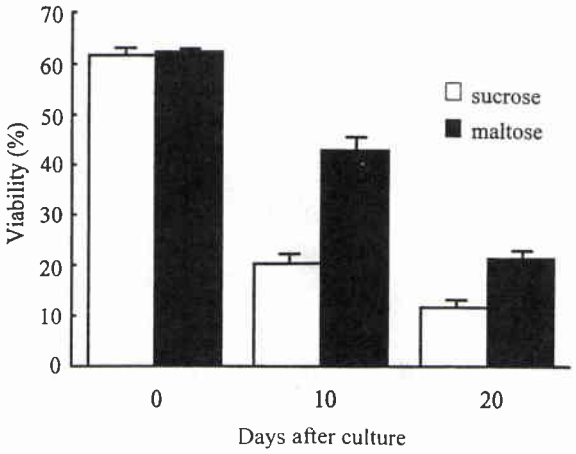

Fig. 1. Effects of sucrose and maltose on the viability of in vitro cultured microspores.

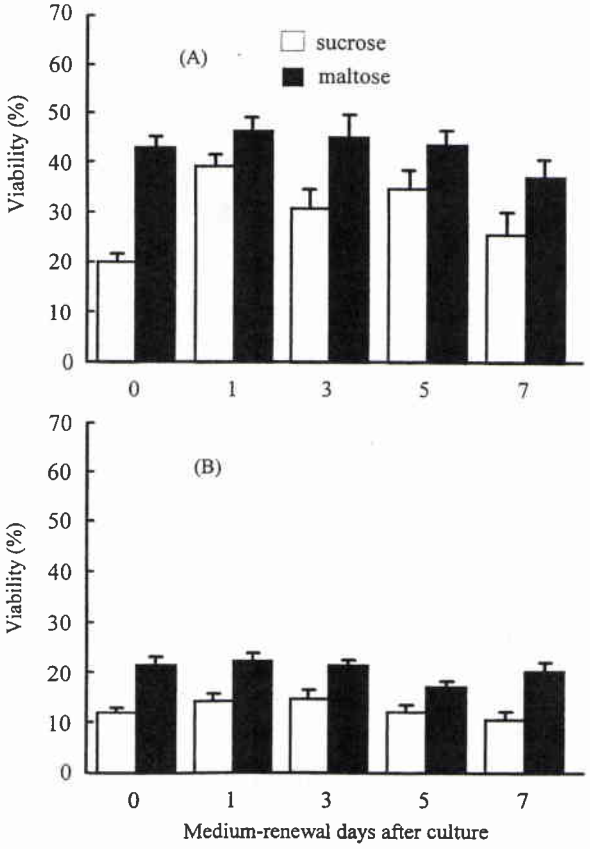

Fig. 2. Effects of medium renewal (subculturing) on the viability of cultured microspores. The microspore viability was measured 10 days (A) and 20 days (B) after culture.

viable than those subcultured on the sucrose-medium in all treatments (Fig. 2A and 2B).

Effect of carbohydrate sources on the development of cultured microspores

Microspore development was influenced by the carbohydrate sources in the media as numerous swollen microspores were observed 3 weeks after culture in

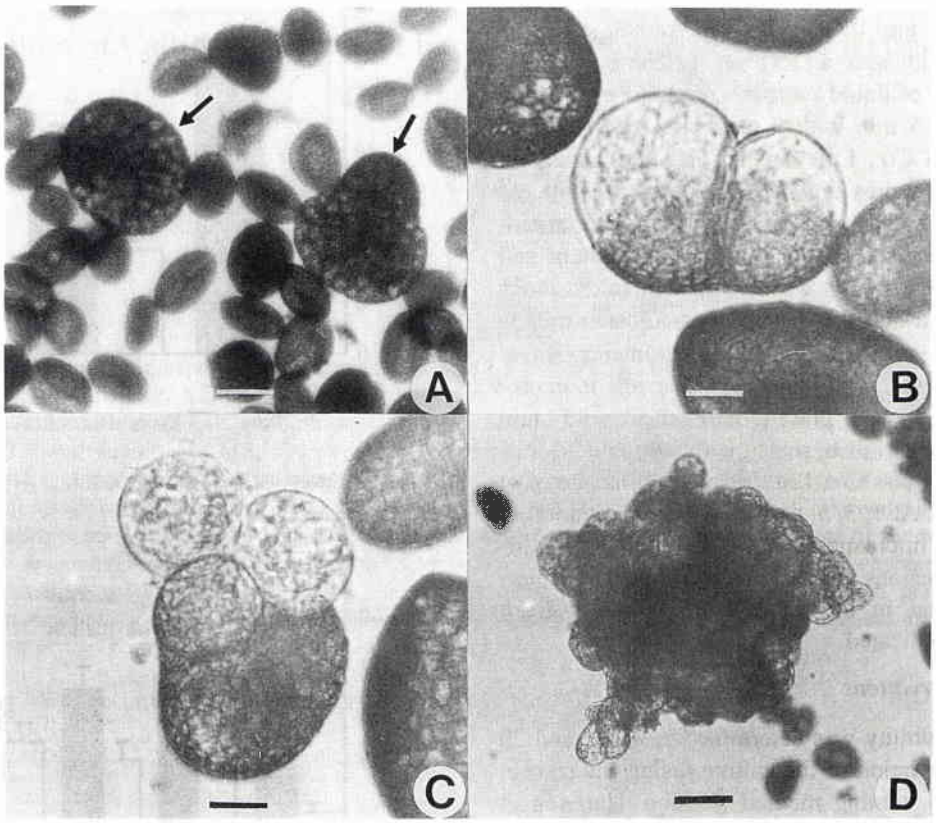

Fig. 3. Photomicrographs of developing isolated microspores during in vitro culture. (A) Swollen microspores (arrows) containing starch grains in sucrose-medium (bar = 65 μm). (B) A dividing microspore in maltose-medium (bar = 15 μm). (C) A microspore dividing into a multicellular structure in maltose-medium (bar = 15 μm). (D) A microspore-derived callus in maltose-medium (bar = 100 μm).

sucrose-medium (Fig. 3A). The swollen microspores contained many starch grains but remained undivided even after 5 weeks of culture. In contrast, the microspores cultured in maltose-medium did not form starch grains but began to divide after two weeks of culture (Fig. 3B and 3C). The division of microspores continued, forming calli after 4 weeks of culture (Fig. 3D). Two to 9 cell masses developed per Petri dish. However, the cell division in the calli almost stopped after 5 weeks of culture.

Discussion

We know no report on callus and/or embryo formation nor plant regeneration by in vitro culture of isolated microspores of *Lilium* species. van den Bulk et al. (1992) reported that uninucleate microspores of the Asiatic hybrid lily 'Whilito' developed into multinucleate microspores containing up to eight-nuclei when they were cultured in NLN-medium (Lichter, 1982) with 13% sucrose, but no further division of the multinucleate microspores occurred. In this study, multicellular microspores and callus of the Asiatic hybrid lily 'Connecticut King' were successfully induced when the uninucleate microspores were cultured in the maltose medium. Similar results have been reported on the microspore culture of barley, in which a relatively higher viability and development of embryoids or calli were observed in the maltose medium but not in the media containing sucrose, glucose or fructose (Scott and Lyne, 1994a). The initial division of rice microspores was promoted in the maltose medium than it was with sucrose (Xie et al., 1995). Based on the results of both this and other studies, maltose is a more suitable carbon source than sucrose for microspore viability and division in some plant species.

Scott and Lyne (1994b) found that the addition of sucrose or glucose to the medium was toxic on microspore cultures of barley; these effects may have resulted from the accumulation of toxic metabolites of sucrose or glucose. Renewal or subculturing of the culture medium seems to be an effective way to overcome this unfavorable culture condition. Hansen and Svinnset (1993) reported that the renewal of sucrose-containing medium after 3 days of culture markedly improved the production of embryos in microspore culture of *Brassica napus* ssp. *rapifera*. Our results show that the viability of the microspores of 'Connecticut King' cultured in sucrose-medium was improved by medium renewal (Fig. 2A), although no cell division occurred. Therefore, the medium renewal during microspore culture may be a selection to maintain the viability of cultured microspores.

Tanaka et al. (1980) showed that in cultured microspores of *Lilium longiflorum*, sucrose is essential for normal development and formation of mature pollen. However, the 0.5 M sucrose accelerated starch accumulation in microspores. In our trial, the sucrose induced a large number of undivided, swollen microspores to accumulate numerous starch grains, whereas those in the maltose actively divided and rarely synthesized starch grains. The accumulation of starch has been demonstrated to be closely related to the growth and development of cultured cells and tissues (Ho and Vasil, 1983; Landry and Smyth, 1988; Stamp, 1987) but simultaneously, impairs protoplast division in pea (Lehminger-Mertens and Jacobsen, 1989). Gram et al. (1996) reported that cell division is prevented below or above an optimum starch level. Thus, the inhibition of cell division in microspores of 'Connecticut King' cultured in the sucrose-medium might be related to the excess accumulation of starch, which may possibly be prevented by the stress treatment such as starvation (Sangwan et al., 1997). Because no plantlets were regenerated from calli, further experiments involving the starvation treatment are under way to improve the cell division with the hope of regenerating plantlets from cultured microspores of lily.

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単離したアジアティックハイブリッド‘コネチカットキング’の小胞子培養および小胞子由来のカルス形成

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摘　要

ユリにおいて単離小胞子からのカルスの誘導に初めて成功した．圃場で栽培した‘コネチカットキング’から花らい(長さ2-3cm)を採取し，やくから1核期の小胞子を単離し，25℃，暗黒下で培養した．小胞子の生存と発達に及ぼす炭素源(ショ糖およびマルトース)と培地更新の影響をMS培地を用いて検討した．培地の炭素源は培養小胞子の生存率および発達に影響した．小胞子の生存率はショ糖よりもマルトースを添加した培地で高かった．培地の更新は小胞子の生存率を改善しなかった．ショ糖培地では，多数の小胞子がデンプン粒を蓄積し，それは培養を継続しても分裂しなかった．一方，マルトース培地では，デンプン粒を含む，膨張した小胞子はほとんどなく，小胞子の分裂が観察された．これらの小胞子は分裂を繰り返し，多細胞小胞子やカルスに発達した．