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EMBRYOLOGICAL STUDIES ON OVULES OF *MELANDRIUM ALBUM* POLLINATED IN VITRO WITH *LYCHNIS CORONARIA* POLLEN GRAINS

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Placentae of *Melandrium album* excised from ovaries of unopened flowers were pollinated in vitro with pollen grains of *Lychnis coronaria*. Pollen grains germinated, and pollen tubes were found entering the micropyles 18 h after pollination. Double fertilization occurred. Immature and mature hybrid embryos at different stages of development were analyzed up to the third week of culture. Twenty-five days after culture, cotyledonary embryos were isolated from ovules and transferred to MS medium supplemented with 0.5, 1 or 2 mg l^{-1} indole-3-acetic acid (IAA). About 85% of the 115 inoculated embryos died shortly after transfer to medium, due to atrophy of the root and one cotyledon. Eighteen embryos survived; of these, 16 well-formed seedlings with root system were transferred to soil. The hybrid plants grew normally, and their hybridity was confirmed by morphological study. Female and male organs of the intergeneric hybrid were completely sterile.

Key words: *Melandrium album (Silene latifolia), Lychnis coronaria*, hybrid embryo development, intergeneric crosses, in vitro pollination

INTRODUCTION

Melandrium album (syn. *Silene latifolia*) is a dioecious plant possessing a pair of heteromorphic sex chromosomes. Plants of this species are either male (2n = 22, XY), bearing staminate flowers, or female (2n = 22, XX), bearing pistillate flowers. Interspecific or intergeneric crossings are limited mostly by pre- and post-fertilization barriers. *M. album* studies have dealt with methods of overcoming the pre-fertilization barriers through the use of directly pollinated ovule technique. Using this species as a model plant, a complete in vitro ovule pollination, fertilization and embryo rescue system has been developed (Zenkteler, 1969). The large ovary contains around 300 ovules. Its walls should be removed in order to reveal placentae with ovules only. Direct in vitro pollination of the ovule with pollen grains of alien species made it possible to raise interspecific and intergeneric hybrid globular embryos (Zenkteler, 1967; Zenkteler et al., 1975). In our studies on pollinating *M. album* ovules in vitro with pollen grains of *Viscaria vulgaris* and *Silene schafta*, the hybrid embryos developed to the cotyledonary stage (Zenkteler, 1969, 1980, 1999). Plants were developed from those hybrid embryos, but they never formed flowers. Recently, by applying cytogenetic and molecular biology techniques, Zluvova et al. (2004) demonstrated the hybrid origin of *S. latifolia* × *S. viscosa* progeny (plants obtained in vivo).

The purpose of this work undertaken in 2002 was (1) to establish a complete and integrated in vitro pollination, fertilization and embryo rescue system in order to provide a procedure for overcoming both pre- and post-fertilization barriers between *M. album* (a dioecious plant) and *Lychnis coronaria* (a monoecious plant), and (2) to carry out embryological analysis of the pollinated parental female ovules, and of the anthers and ovules of the hybrid female plants.

MATERIALS AND METHODS

The *M. album* and *L. coronaria* plants were grown on an experimental lot in the Botanical Garden of A. Mickiewicz University, Poznań. Pistils of *M. album* were isolated from 190 closed female flower buds 24 or 48 h before opening. The stigmas were cut off, and the ovaries along with part of the calyx and part of the pedicle were disinfected in 70% (v/v) ethanol for 45 sec and in 1% (w/v) chlorinated water for 8–10 min, and

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then rinsed three times in sterile distilled water. After removal of the ovary walls, the placentae were ready for pollination. On the same day, anthers were excised from still-closed flower buds of *L. coronaria* and left to ripen for 2–3 h in a sterile inoculation chamber. Later the pollen grains were scooped out and spread on the surface of the ovules. Pollinated placentae (190) along with a short calyx and a short pedicle were placed vertically in long test tubes (20 cm) partly filled with MS basal medium (Murashige and Skoog, 1962), enriched with 3% (w/v) sucrose but without growth regulators. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were maintained at 23 ± 2°C under continuous cool-white fluorescent light (55 µmol m^{-2} s^{-1}).

Cotyledonary embryos developed after cross pollination were dissected from the ovules and transferred to MS basal medium supplemented with 3% (w/v) sucrose and 1 mg l^{-1} IAA.

At 20, 24 and 72 h after pollination, some of the ovules were removed, stained with aniline blue and examined for pollen tube growth by epifluorescence microscopy. For detailed embryological analysis, pollinated ovules were collected at intervals from 1 to 18 days and fixed in Carnoy's solution for 24–48 h, stored in 70% (v/v) ethanol, then processed by the alcohol-xylene paraplast embedding method and sectioned 10–12 µm thick with a rotary microtome. Slices were double-stained with Heidenhain's hematoxylin and fast green. The preparations were studied under a light microscope (Axioscop, Zeiss).

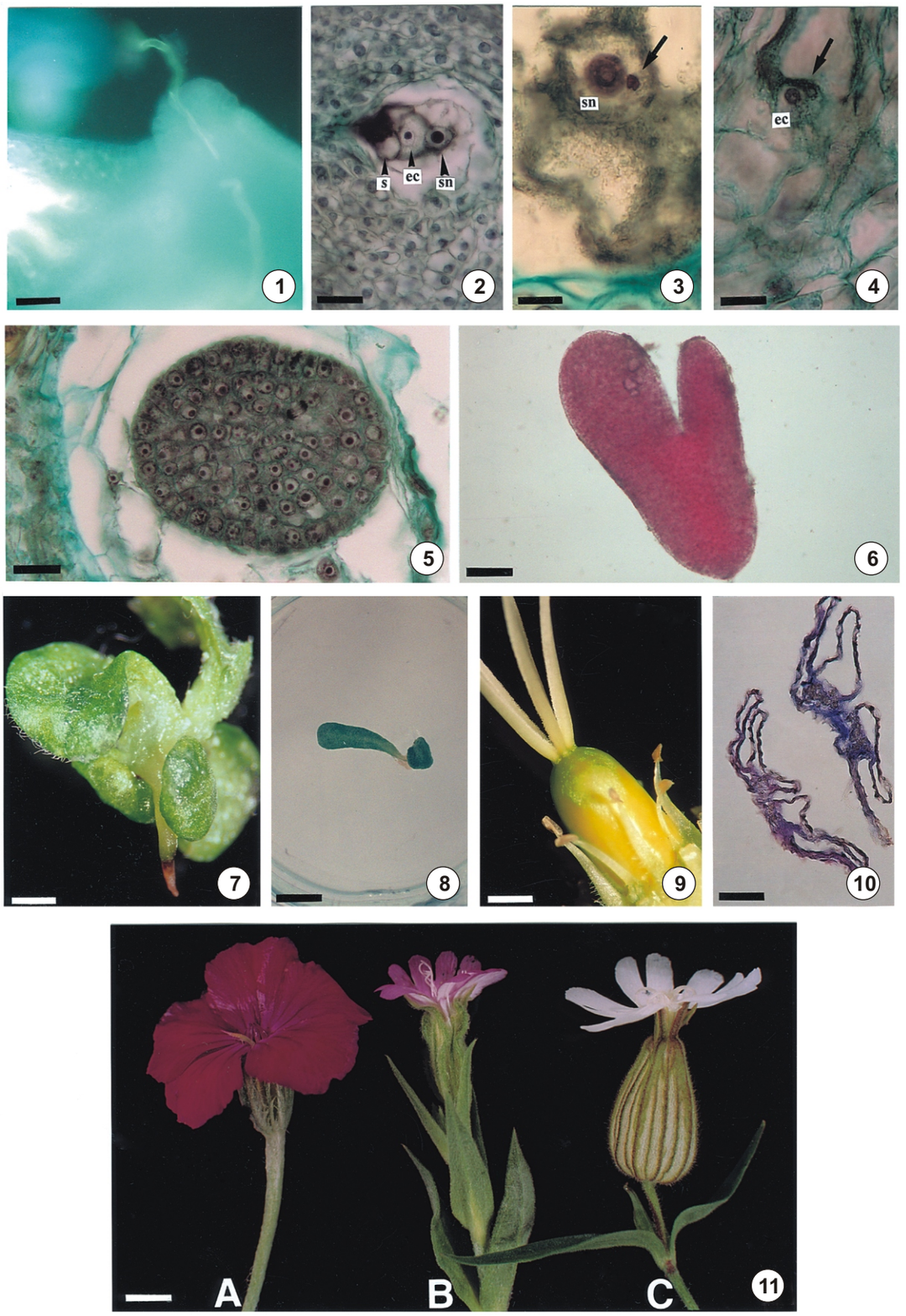
RESULTS

Pollen germination was observed after 6–12 h of culture. Pollen tubes did not follow any particular direction, but several of them entered the ovules through the micropyle (Fig. 1). After 2–3 days of induction some ovules did enlarge. Swelling of ovules were interpreted as a fertilization effect, but not all swollen ovules contained embryos. Those ovules showed the beginning of proliferation of the integument and nucelli. The presence of the pollen tube inside the embryo sac and the destruction of one or both synergids were visible in many of the analyzed ovules (Fig. 2). Microtome preparations of ovules 18–24 h after in vitro pollination showed the presence of male gametes inside the egg cells and in the secondary nuclei (Figs. 3, 4). Between 2 to 10 days following pollination, embryological analysis of the ovules revealed the presence of embryos and endosperm in different stages of development. Maximum embryo development was achieved by 6–8 days after pollination, and at this time the hybrid embryos reached globular to early heart-shaped stage (Fig. 5). Torpedo-shaped embryos obtained from pollinated ovules were observed 14 days after pollination (Fig. 6). In 18-day-old ovules, embryos attained a torpedo shape with poorly developed endosperm cells, and one week later only residues of endosperm could be distinguished.

In *M. album* a single ovary contains around 300 ovules. After in vitro cross-pollination the number of seeds with cotyledonary embryos varied. Of the 210 enlarged ovules that developed between 21 and 28 days following pollination, 115 cotyledonary embryos were obtained and cultivated on MS medium. Of those embryos, only 18 formed green cotyledons, green leaves and well-formed root systems. The remaining embryos showed abnormal growth: usually on the third day of culture, one of their cotyledons became brown and soon shriveled (Fig. 8). The roots developed similar defects (Fig. 7). The embryos with atrophied cotyledons and roots did not develop further, and usually died after 2–3 weeks of culture. A total of 18 embryos survived and continued their growth on MS medium supplemented with 1 mg l^{-1} IAA. Sixteen well-formed seedlings were transferred to soil after 2 months of culture. Four plants died within 3 weeks; the remaining 12 grew normally, wintered, and produced flowers during the next summer months.

Phenotypically the hybrid plants showed features intermediate between the pollen donor and the female parents. The hybridity of the plants was confirmed by morphological study. The flowers of the hybrid plants developed structure intermediate between the parental forms, but smaller. The rose-pink petals of the hybrids were intermediate in color between the two parents (Fig. 11). In each flower bud there was one pistil and 10 stamens. The ovaries were considerably smaller than those of the parents and contained smaller ovules. The ovules were without normally developed

Fig. 1. Pollen tube of *L. coronaria* entering micropyle of ovule of *M. album*. Bar = 25 µm. **Fig. 2.** Remnants of pollen tube of *L. coronaria* inside micropyle region of embryo sac of *M. album* 18 h after in vitro pollination. s – synergid; ec – egg cell; sn – secondary nucleus. Bar = 25 µm. **Figs. 3–4.** 18 h after in vitro pollination. **Fig 3.** Male gametes (arrows) of *L. coronaria* inside secondary nucleus (sn) of *M. album*. **Fig. 4.** Male gametes (arrows) of *L. coronaria* inside egg cell (ec) of *M. album*. Bars = 10 µm. **Fig. 5.** Globular hybrid embryo inside embryo sac of *M. album*, 8 days after in vitro pollination. Bar = 25 µm. **Fig. 6.** Whole mount of cotyledonary hybrid embryo isolated from ovule of *M. album*. Bar = 50 µm. **Fig. 7.** Atrophied root of hybrid embryo, 20 days after culture on MS medium with 1 mg l^{-1} IAA. Bar = 125 µm. **Fig. 8.** Five-day-old hybrid embryo showing one cotyledon fully degenerated. Bar = 0.5 cm. **Figs. 9–10.** Pistil surrounded by stamens (Fig. 9) and rudimentary, empty anthers (Fig. 10) of the female hybrid plant of *M. album* × *L. coronaria*. Bar in Fig. 9 = 125 µm, in Fig. 10 = 50 µm. **Fig. 11.** Flowers of *L. coronaria* (a), hybrid plant *M. album* × *L. coronaria* (b) and *M. album* (c). Bar = 0.5 cm.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integuments and their nucelli degenerated early. No megaspore mother cell or process of meiosis were observed; thus the plants were completely female sterile. The details of the pistils surrounded by stamens are presented in Figure 9. In all hybrid plants the stamens possessed rudimentary anthers, and their development was halted early at the bilobal stage. Compared with the rudimentary stamens of the female plant (*M. album*), the stamens of the hybrids were significantly longer. Embryological analysis of anthers sectioned and squashed at different stages of formation revealed only a mass of homogenous cells surrounded by the epidermis. When the hybrid flowers started to open, all cells inside the microsporangium degenerated. During the following days, only remnants of them could be distinguished (Fig. 10). Thus, the hybrid plants of *M. album* × *L. coronaria* were female and male sterile, and therefore unable to produce progeny. The stems and leaves of the hybrid plants were covered with dense hairs, similarly to the male partner. In general, the hybrid plants were much shorter and more delicate than the robust parental ones. They produced many flowers in July-September 2003.

DISCUSSION

The main significance of the method of in vitro pollination of ovules is that it provides a means of bypassing the pollen/style rejection response in interspecifically incompatible plants. Our results have shown that this technique makes it possible to obtain viable seeds and then plants from crosses between *M. album* and *L. coronaria*, species that do not cross in vivo. Our observations revealed that the reproductive barriers between *M. album* as female and *L. coronaria* as male are prezygotic, and that pollination of ovules can yield hybrid cotyledonary embryos from which hybrid plants can be obtained. However, only 18 of the total 115 cotyledonary embryos were capable of growing and producing plants after transfer to media. In the majority of embryos, one cotyledon and the root died shortly in culture. The application of various combinations of media, and in particular the addition of various growth regulators, did not overcome these defects. In the hybrid plants, stamen development was more advanced in comparison with the female plants. In interspecific hybrids of *Silene latifolia* × *S. viscosa* (Zluvova et al., 2005) the anthers developed much further in comparison with the female parents; according to those authors, this suggested that the *S. viscosa* genome provided the sex determination stamen-promoting function. Moreover, irrespective of some defects in the development of the tapetum and precocious maturation of endotheciums, the hybrids produced few viable pollen grains. Hybrid plants were also developed in interspecific crosses *S. latifolium* × *S. diclinic* (Prentice, 1978), but no information is available on the structure and function of the sexual organs or on the formation of embryos and seedlings. In the intergeneric hybrid *M. album* × *L. coronaria*, both female and male reproductive organs were completely sterile. It seems that they suffer from disruption of coadapted gene complexes, which are commonly assumed to increase the level of developmental stability in plants. The results of our investigations demonstrate the potential of using in vitro culture technique to overcome barriers of incompatibility between dioecious and hermaphrodite species. A more fruitful approach to hybridizing dioecious and monoecious species may be found in molecular methods, particularly those focused on the sex determination mechanism based on the active role of XY chromosomes.

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