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Interspecific crosses of lily by in vitro pollinated ovules

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Abstract. Because of pre- and post-fertilization barriers, successful interspecific crosses have not been reported for *Lilium* using Asiatic hybrid as the mother to cross with Oriental or *Lilium longiflorum*. Using an in vitro ovular pollination method, where pollen tubes directly penetrate isolated ovules, this study demonstrated that 1) the Brewbaker and Kwack agar plate containing sucrose (10%), with the isolated ovules being placed ca. 1 mm apart from the pollen, is optimal for pollination, 2) interspecific crossing barriers do not occur during penetration of the pollen tubes into the micropyle, 3) micropylar penetration can be obtained in all kinds of intra- and inter-specific cross combinations of lily cultivars, 4) multiple penetration occurs, in which the penetrating pollen tubes pass the embryo sac without entering the synergid cells, 5) a small amount of pollen tube penetrates the synergid cells in the A x O cross, and 6) completion of pollen tube penetration (8-12 h after germination) is much earlier than the division of the generative cell in sperm cells (17 h after germination), which may be the primary cause for the failure in fertilization.

Keywords: In vitro ovular pollination; Interspecific crosses; *Lilium*; Micropylar penetration; Pollen tube; Sperm cells; Synergid.

Abbreviations: **A,** Asiatic hybrid; **O,** Oriental hybrid; **L,** *Lilium longiflorum*; **MP,** micropylar penetration; **HAG,** hour after germination.

Introduction

Genus *Lilium* comprises about 85 species that are classified into seven sections, and more than half of these species originate from Asia (Beattie and White, 1993; De Jong, 1974). There are three important modern commercial lilies: the Asiatic hybrid (A), derived from crosses within the *Sinomartagon* section), the Oriental hybrids (O), derived from crosses within the *Archelirion* section), and the *longiflorum* group (L), (Van Creij et al., 1993). Most of the commercial cultivars were bred and selected in The Netherlands (Van Tuyl et al., 1997), and are only suitable for cultivation in a moderate climate. Owing to this, the subtropical lily industry is hampered by the plant's poor tolerance of subtropical culture conditions, which causes poor bulb growth and low quality cut flowers (Chin et al., 1996). Therefore, widening the genetic basis by introducing heat-tolerance together with disease resistance (the botrytis resistance of Orientals, the virus resistance of Asiatics, and the fusarium resistance of trumpets) and special flower colors and forms from wild species into commercial lily assortments is very important in subtropical lily breeding programs (Chi et al., 1999). For these purposes, interspecific crosses have to be made. Some of these interspecific crosses in the groups L x A, L x O, O x L, and O x A have been successful (Van Tuyl et al., 1997), but the A x L and A x O crosses have not. Therefore, no hybrid plant of the latter crossing types has been obtained, even though different in vitro methods have been exploited.

It has been proposed that the limiting hindrance factors of interspecific crosses in *Lilium* are primarily fertilization barriers that can be separated into pre-and post-fertilization barriers (Van Tuyl et al., 1991). To overcome pre-fertilization barriers, the cut-style method, the grafted style method, the placenta pollination method, and the in vitro ovule pollination method have been tested (Asano and Myodo, 1977a, b; Janson et al., 1993; Van Tuyl et al., 1991; Willemse et al., 1995). Embryo rescue, ovary-slicing, and ovule culture have been tried to overcome post-fertilization barriers (Asano, 1980; Okazaki et al., 1992, 1994; Van Tuyl et al., 1991). By overcoming both barriers, interspecific lily crosses have been made more efficient, resulting in increased numbers of hybrid plantlets in a single interspecific cross and more successful interspecific combinations (Van Tuyl et al., 1991, 1997).

Recently, in an L x A cross using the in vitro ovular pollination method, where L was *L. longiflorum* "Gelria" and A was the Asiatic hybrid "Enchantment," Willemse et al. (1995) showed micropylar penetrations of 8-17%; however, fertilization was not successful (Plyushch et al., 1995; Willemse et al., 1995). The purpose of this research was to optimize the conditions for in vitro ovular pollination and to study at which stage the barriers may occur in those crosses.

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Materials and Methods

Culture Media

The BK (Brewbaker and Kwack, 1963) medium for in vitro pollination contained (in 1 L of double distilled water) 5 g Difco agar, 0.3 g H_3BO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g KNO_3 and 0.3 g $Ca(NO_3)_2 \cdot 4H_2O$, adjusted to pH 6. With KOH, sucrose at 5 to 12% was included. The ovule culture medium consisted of full strength Murashige and Skoog (1962) basal salts and vitamins, and was supplemented with 0.4% phytagel, 6% sucrose, and 0.01 mg/l NAA at pH 5.8.

Plant Materials

The cultivars from three different groups, namely Oriental hybrids (O)-Stargazer, Asiatis hybrids (A)-Connecticut King, and *Lilium longiflorum* (L)-Gelria, were used to perform interspecific crosses (A x O, O x A, O x L, L x O, A x L, L x A) and Oriental hybrids (O)-Stargazer, Casa Blanca, Asiatis hybrids (A)-Connecticut King, Orlito, and *Lilium longiflorum* (L) - Gelria, Indian Summer, were used to perform intraspecific crosses (O x O, L x L and A x A). Pollen from Stargazer was used for the time course studies of generative cell division and pollen tube penetration. The combination Mont Blanc x Stargazer (A x O) was used to check whether the pollen tube contents could be transferred into the synergid cells. The pollen and ovules were obtained from flowers collected from the greenhouse one day after anthesis. Greenhouse temperatures varied from 15°C at night to 20-25°C during the day with a summer peak of 30-35°C.

Staining

To follow the transfer of cytoplasm into the synergid, 1 µl of 0.01% DIOC 6 (3,4-dihexyloxacarbocyanin iodide) in 7% sucrose was applied to the pollen mass. To identify the sperm nuclei, 1 µl of buffered 10^{-4} DAPI (4,6-diamino-2-phenylindole dihydrochloride) in citrate buffer (pH 4.0) was added. After staining, the ovules were cleared with glycerol according to Willemse and Keijzer (1990) and examined with a Nikon fluorescene microscope.

Results and Discussion

In vitro Ovular Pollination Applied to Intra- and Inter-Specific Crosses

In this study, the in vitro ovular pollination method was tried for reciprocal interspecific crosses among the three groups of commercial lily cultivars. During these experiments, different conditions—including the distance between pollen and the isolated ovules and concentration of sucrose in the media—were tested. Figure 1 shows the pollination set-up in Stargazer x Gelria (O x L) cross as an example. To facilitate pollination, a row of pollen mass from Stargazer (ca. 4 × 0.5 cm) was spread onto the surface of the BK agar plate (15 ml in a 9 cm petri dish) to promote germination and enhance straight orientation of the pollen tubes. Fifty to one hundred ovules were dissected from the placenta of Gelria and immediately placed around the pollen mass at 1 mm apart, where they were kept half-immersed in the medium. For comparison, sucrose at a final concentration of 5, 7, 10, or, 12% was supplemented. The plates were incubated at 20°C, and the percentage of micropylar penetration (MP) was determined by counting the number of penetrated ovules under a binocular microscope (40× magnification) at 24 h after germination (HAG). As shown in Figure 2, pollen tube penetration was observed in each of the crosses, ranging from 2.6% in O x A crosses to 62% in L x O crosses. Several points were noticed in these experiments. First, pollen from O exhibited better penetrability (26-62%) than that from L (11-28%) and A (2.6-17%). Second, a higher percentage of penetration was always observed in the presence of 10% sucrose than in the lower concentrations, while concentrations higher than 12% caused detrimental effects (data not shown). Similar effects of sucrose have also been observed in the in vitro stigma pollination of lily, where 7% of sucrose was shown to be the most effective (Van Tuyl et al., 1991).

The results of theses experiments are consistent with the finding of Willemse et al. (1995) suggesting that the *Lilium* ovules show a non-specific micropylar attraction. In other words, interspecific crossing barriers do not occur during penetration of the pollen tube into the micropyle.

Timing of Generative Cell Division

Division of a generative cell to produce two sperm cells is required for subsequent fertilization (Keijzer et al., 1988). To monitor the time of generative cell division, the Oriental hybrid Stargazer pollen were cultured on the BK plate at 20°C and examined microscopically after DAPI staining at intervals of 1 h. Most of the pollen started to germinate after 1-2 h of incubation, and some of them started entering the metaphase at 16 h after germination (HAG) (Figure 3). Then, at 17 HAG, the number of pollen tubes that have sperm cells reached a maximum (3%) within the following 2 h (Figure 4). No further increase in the number of pollen tubes containing sperm cells was observed after 23 HAG. These results are similar to the findings of Janson et al. (1993) that sperm cell formation initiates at 18 h after cut-style pollination (in *L. longiflorum* cv. Gelria × *L. longiflorum* cv. White American cross) and 16 h after stigma pollination. Van Roggen et al. (1988) also found that the generative cell divided into two sperm cells as soon as pollen tubes were 2-4 mm long, i.e., before pollen tubes reach micropyle after cut-style pollination (Van Roggen et al., 1988). These findings suggest that the initiation of sperm cell formation is independent of the species of *Lilium* or the pollination methods used.

In addition, no difference in generative cell division was caused by different concentrations of sucrose in the medium of those experiments (Data not shown).

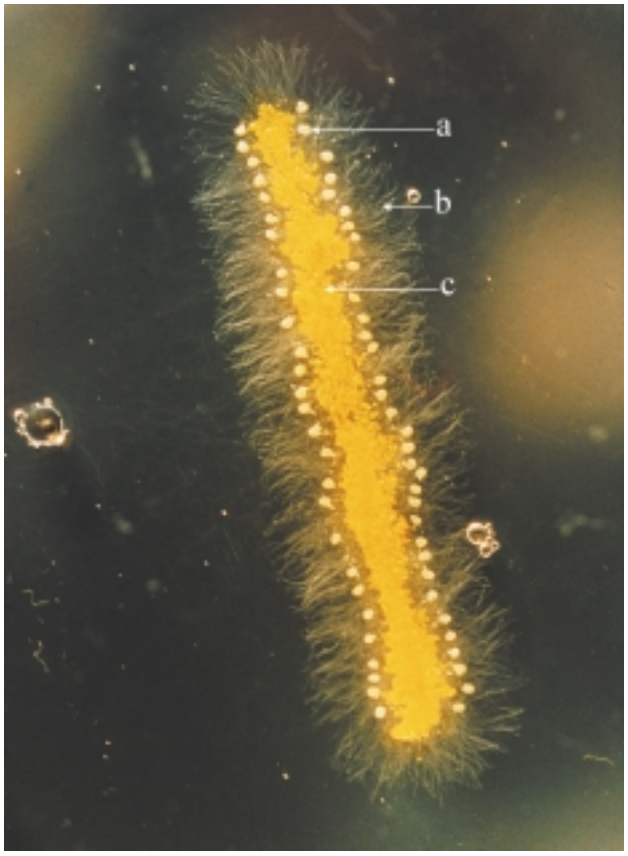

Figure 1. A set-up of the in vitro ovular pollination experiment. A row of pollen mass from Stargazer was spread on the BK medium in the middle of a petri dish, with the isolated ovules of Gelria being placed at 1 mm apart. The length and width of the pollen mass were approximately 4 and 0.5 cm, respectively. Picture was taken 24 h after pollination. a, ovule; b, pollen tubes; c, pollen mass.

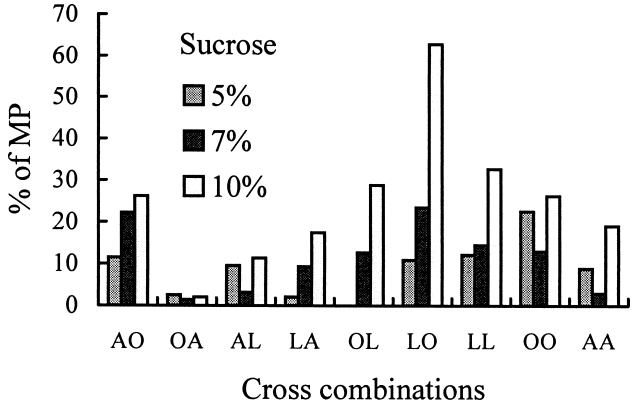

Figure 2. Percentage of micropylar penetration (MP) in vitro for various interspecific crosses at different sucrose concentrations. Flowers were randomly picked from each cultivar and used for these experiments. Fifty to one hundred ovules from one flower were treated in each set. Abbreviations: A, Asiatic hybrids; O, Oriental hybrids; L, *Lilium longiflorum*.

Timing of Pollen Tube Penetration in vitro

Microscopic examination was performed to follow growth of the Stargazer pollen tube tip towards the *longiflorum* micropyle, with the isolated ovules being placed ca. 1 mm apart from the pollens. The pollen was found to grow at speeds of 0.5-0.7 mm per h, depending on the speed of pollen tube growth. The penetration started at 3-5 HAG and finished at 8-12 HAG. After 24 HAG, the pollen tubes reached a length of 1.5-3.0 mm.

During these experiments, different distances between the ovules and the pollen grains were tested. A distance about 1 mm apart was found to be the best for penetration, and no penetration was observed with a distance longer than 2 mm. Multiple penetration occurred in some of the ovules, with as many as five pollen tubes penetrating into the same micropyles (Figure 5)

Here, it is important to note that the time needed to complete penetration is far less than what is required for generative cell division (17 HAG). In other words, the generative cell is not divided before completion of penetration. Therefore, to get ready for fertilization, an improved culture condition is apparently needed to complete generative cell division before micropylar penetration.

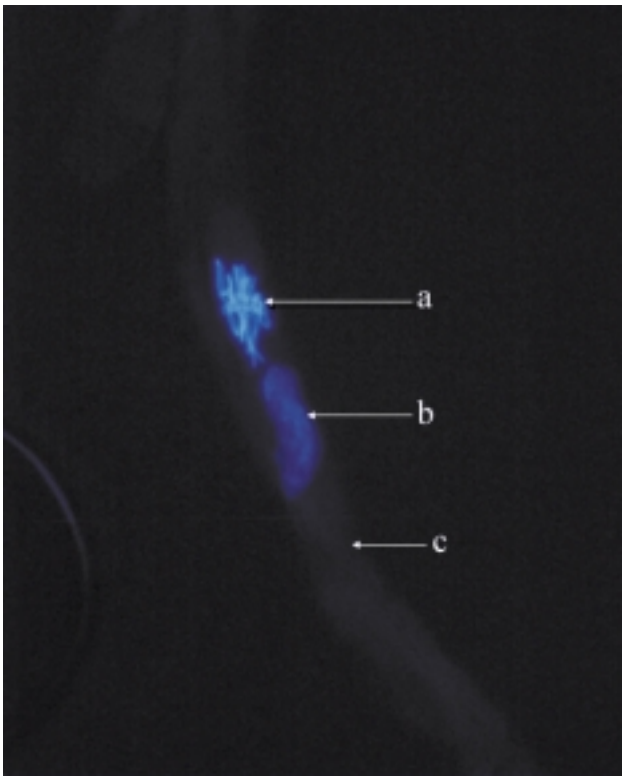

Figure 3. The division of generative cell. The Oriental hybrid Stargazer pollen were cultured and examined microscopically at intervals of 1 h after DAPI staining. Most of the pollen started to germinate after 1-2 h, and some of them started entering the metaphase at 16 h after germination. a, metaphase stage of generative cell division; b, vegetative cell; c, pollen tube. (40×)

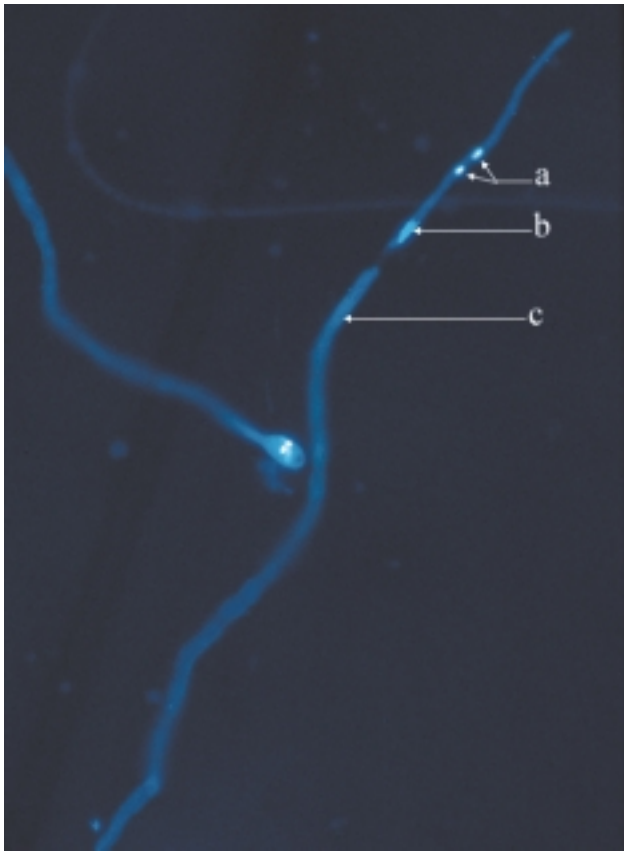

Figure 4. The sperm cell formation in pollen tube. The Oriental hybrid Stargazer pollen at 17 h after germination, sperm cells started to appear with the number reaching the maximum (3%) within the following 2 h. a, sperm cells; b, vegetative cell; c, pollen tube. (20×)

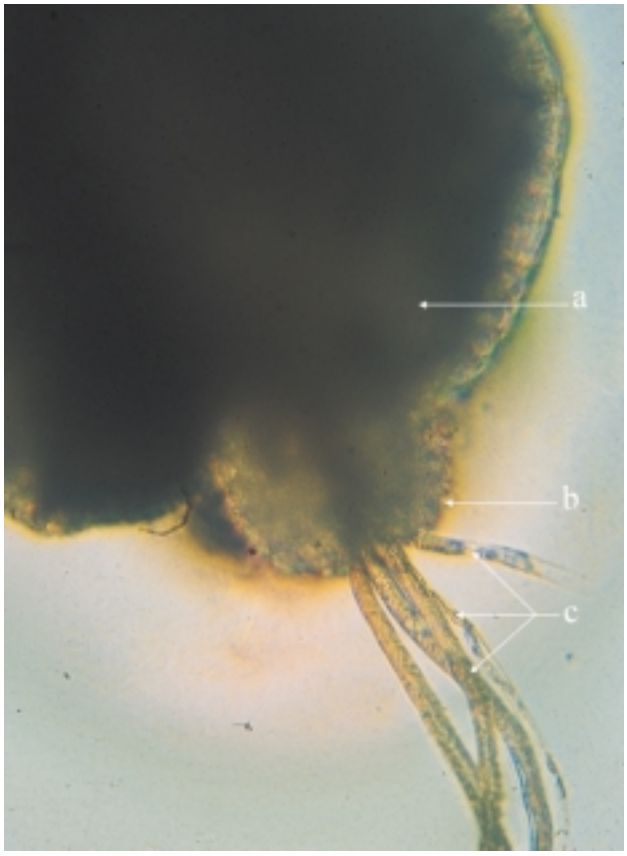

Figure 5. Multiple pollen tube penetration. Five pollen tubes penetrating into the same micropyle. Connecticut King x Gelria crosses (A x L cross) in containing BK media. a, ovule; b, micropyle; c, pollen tubes. (40×)

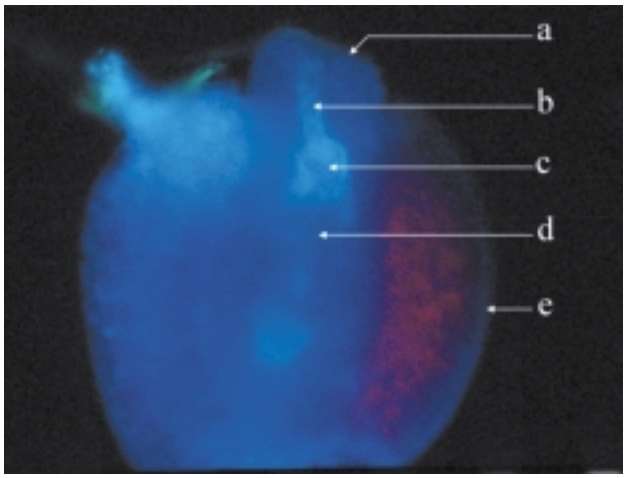

Figure 6. DIOC staining of the synergid cells containing pollen tube cytoplasm. Mont Blanc x Stargazer crosses (A x O cross) were carried out to monitor the transfer of pollen tube cytoplasm into the synergid cell. DIOC staining of the mitochondria and membranes was to mark the cytoplasm. The pollen tube contents were found to enter into the synergid after micropylar penetration. a, micropyle; b, pollen tube; c, pollen tube contents; d, embryo sac; e, ovule. (20×)

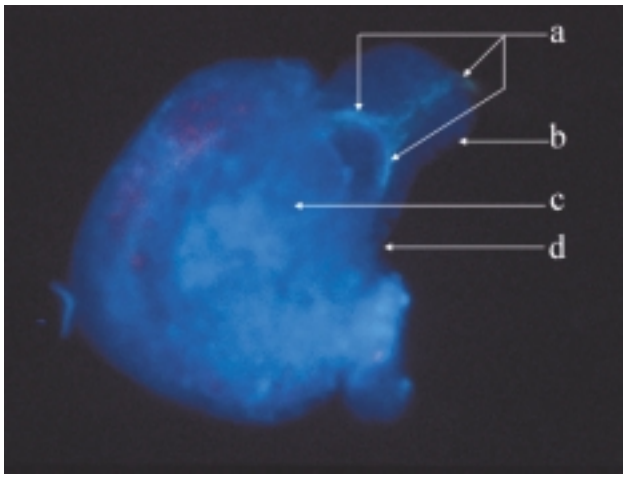

Figure 7. The DIOC staining of multiple penetration of pollen tubes. Mont Blanc x Stargazer crosses (A x O cross) had multiple penetration of pollen tubes. The DIOC signal shows that the pollen tubes only traveled between the inner integument and the nucellus without entering a synergid of embryo sac. a, pollen tubes; b, micropyle, c, embryo sac; d, ovule. (20×)

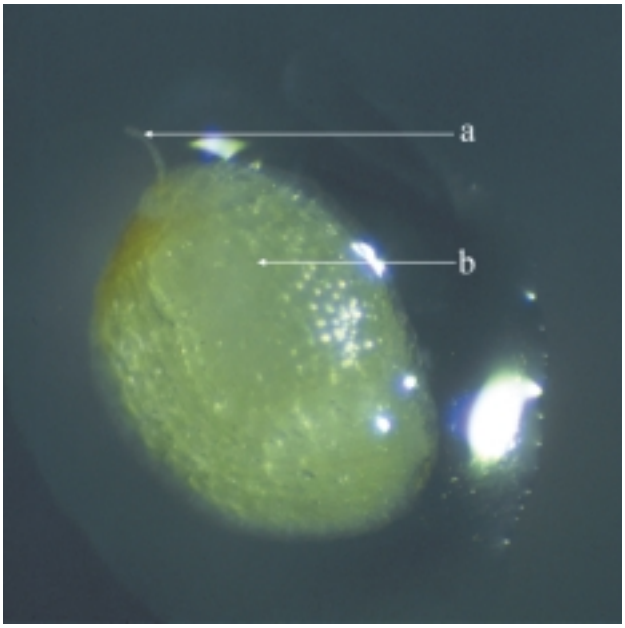

Figure 8. Ovule penetrated by pollen tube started to grow and enlarge, when cultured. At early stage, ovule became green and swollen slightly. Gelria x Connecticut King crosses (L x A cross). a: pollen tube; b: ovule became green and swollen.

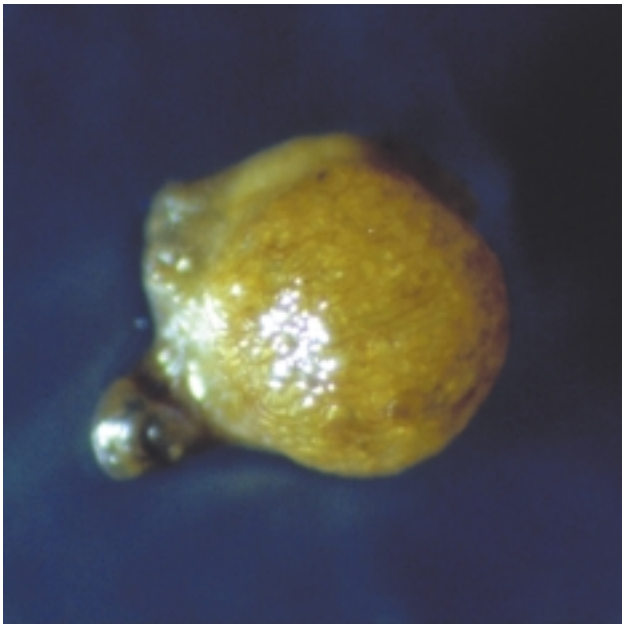

Figure 9. Ovule turned brown in culture. After 60 days in culture, the ovule started to turn brown and no ovule germination occurred. Stargazer x Connecticut King crosses (O x A cross).

Van Tuyl et al. (1982, 1986) employed cut-style pollination for crosses and got results of low seedset. Re-examining the phenomenon, Janson et al. (1993) found that: 1) pollen tubes need to take up substances from stylar exudate during germination, and 2) the ability of pollen tube penetration depends on the length of the style because the percentage of seedset did increase when a longer style part was left at the ovary. Therefore, it was suggested that the low seedset in cut-style pollination may be caused by the low rate of pollen tube penetration instead of sperm cell formation.

Transfer of Pollen Tube Cytoplasm into Synergid Cells

Mont Blanc x Stargazer crosses were carried out to monitor the transfer of pollen tube cytoplasm into the synergid cells, using DIOC to stain the mitochondria and membranes to mark the cytoplasm. The pollen tube contents were found to enter into the synergid cells after micropylar penetration (Figure 6). Among a total of 119 ovules treated, 16 were found to be penetrated, representing a 13% micropylar penetration. However, in only 4 ovules (3-4%) had pollen tubes entered a synergid; in 5 ovules (5-6%) the pollen tube tips stopped just before the synergid, and in 3 ovules (2-3%) the pollen tubes stopped inside the micropyle without entering a synergid. In the 4 ovules with pollen tubes that had entered into a synergid, the contents of the pollen tube were also transferred, but the sperm cells were not observed. Finally, 4 penetrated ovules had multiple penetrations of pollen tubes although they only traveled between the inner integument and the nucellus without entering a synergid (Figure 7). Since multiple penetration can occur in vitro, it should be investigated in vivo.

Since pollen tube penetration, generative cell division, and transfer of pollen tube cytoplasm into synergid cells have been demonstrated in vitro, a further analysis of sperm cell transfer into the synergids and fertilization process should be done to evaluate the impact of in the vitro ovular pollination method.

Culture of Penetrated Ovules

There was no ovule germination after ovule culture. Ovules cultured with pollen tubes started to grow and enlarge. After 6 weeks of culture, only the nucellus and the outer and inner integuments swelled. Under the culture conditions used, the viability of the ovules was prolonged but the embryo sacs degenerated in all the cases investigated. In other words, whether the ovules were penetrated or not, they stayed alive while the embryo sacs inside degenerated (Figures 8 and 9).

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藉胚珠體外授粉法進行百合種間之雜交

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由於受精前後之障礙，以亞洲型百合為母本，東方型百合或鐵炮百合為父本之組合的種間雜交後裔從未有成功的例子被報導過。本研究用體外胚珠受粉法使花粉管直接進入體外培養之胚珠內。獲得下列幾點結果：(1) 在體外培養中，胚珠與花粉間最適當之授粉距離為 1 公釐；(2) 種間雜交之障礙並不存在於花粉管穿入胚珠之珠孔處；(3) 以百合不同栽培種進行種內和種間之正反雜交，其花粉管都能正常的穿入胚珠之珠孔；(4) 在體外胚珠受粉法中發生多個花粉管進入同一胚珠珠孔之現象，而這些花粉管只經過胚囊外圍，並沒有進入胚囊之助細胞；(5) 以亞洲型百合為母本與東方型百合之雜交組合中，有少數花粉管進入胚囊之助細胞，並在進入胚囊之助細胞後釋放出花粉管之內含物；(6) 花粉管穿入胚珠之珠孔所需的時間 (花粉發芽後 8-12 小時) 早於花粉管內精子形成的時間 (花粉發芽後 17 小時)，此或許為體外胚珠授粉法受精失敗的可能原因。

關鍵詞：種間雜交；體外胚珠授粉；百合屬；穿入胚珠之珠孔；花粉管；助細胞；精細胞。