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Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses

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The use of a complete and integrated in vitro pollination, fertilization and embryo rescue system in lily was examined. By combining pollination techniques to overcome pre-fertilization barriers with in vitro methods to overcome post-fertilization barriers, interspecific lily crosses could be made more efficiently. In vitro cut-style pollination and in vitro grafted style technique were developed and applied on various interspecific crosses using *Lilium longiflorum*, *L. dauricum*, *L. henryi*, and both Asiatic and Oriental hybrids as the parents. In addition, methods for ovary culture, ovary-slice culture and ovule culture were generated. Ovule swelling score in ovary culture was used to evaluate media effects on ovule development. Using the integrated in vitro pollination and fertilization protocol it was not only possible to raise the total number of hybrid plantlets in a single interspecific cross, but also the number of successful interspecific combinations.

Key words: Lilium species; Asiatic hybrids; Oriental hybrids; interspecific hybridization; ovary culture; embryo rescue; in vitro grafted style pollination.

Introduction

The genus *Lilium* comprises about 85 species, classified into seven sections [1]. The majority of cultivated cultivars, i.e. the Asiatic hybrids, originate from interspecific crosses between species of the *Sinomartagon* section and a smaller group of cultivated varieties, the Oriental hybrids, from crosses in the *Archelirion* section. Most cultivars are grown for bulb and cut flower production and the total harvest represents an important economic value in the Netherlands. Whereas lily flowers are appreciated world-wide, the assortment could still be considerably improved by exploiting traits from *Lilium* species of other sections. In particular, introduction of resistances against viral diseases, bulb rot (caused by *Fusarium oxysporum*) and *Botrytis*, and tolerances for suboptimal culture conditions (e.g. low light and low temperature), together with special flower colors and forms are topics of current lily breeding programs [2].

Possibilities for cross combinations in *Lilium* are limited by incompatibility and incongruity. Sexual barriers preventing interspecific hybridization have been separated into pre- and post-fertilization barriers [3]. In lily, many studies have dealt with methods for overcoming the pre-fertilization barriers [4—6]. In a comparison of several pollination methods, it was concluded that pre-fertilization bar-

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Abbreviations: BA, 6-benzylaminopurine; CPO, Centre for Plant Breeding Research; CSM, cut style method; cvs, cultivars; DAP, days after pollination; GSM, grafted style method; IAA, indole-3-acetic acid; MS, Murashige and Skoog medium; N, normal pollination; NAA, naphthalene acetic acid; ovar, ovary; ovul, ovule; 2,4-D, 2,4-dichlorophenoxy acetic acid.

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riers can optimally be bypassed by using the cut-style technique [2]. This method comprises the deposition of pollen on the stylar surface after cutting the style with stigma, and allows pollen to circumvent stylar barriers which normally inhibit pollen tube growth [7—10]. A complication, associated with the cut-style method, however, is the low seedset, probably caused by the premature arrival of pollen tubes in the ovary [11].

Once fertilization has occurred, hybrid embryo growth is restricted by post-fertilization barriers. Part of these barriers may be overcome by using embryo rescue methods as have been described for lily by several authors [12—16]. In compatible and intraspecific combinations, embryos can be excised out of immature seeds, starting from about 40 days after pollination, and subsequently cultured and germinated in vitro.

The integrated use of cut-style pollination followed by embryo rescue has generated some new interspecific hybrids, but the number of plants retrieved is low and insufficient for an efficient application in lily breeding programs. The low number of hybrid plants recovered may be attributed to (i) the diminished fertilization rate after cut-style pollination, and/or (ii) the relatively long time interval between pollination and the moment embryo rescue can be started.

Various in vitro methods have been developed to overcome incongruity barriers in a number of plant species. Pre-fertilization barriers are bypassed using in vitro pollination and fertilization [17], while post-fertilization barriers may be overcome by the culture of ovaries immediately after pollination, or by ovule culture [18,19]. By combining in vitro pollination, placenta culture and in ovulo-embryo culture, incongruity barriers in interspecific crosses between *Nicotiana* species were bypassed [20]. High numbers of interspecific *Brassica* hybrids were obtained by consecutively employing ovary, ovule and embryo culture [21—23] and interspecific hybridization of *Trifolium* was achieved by consecutive use of ovule and embryo culture [24].

Some of the basic in vitro methods are already being exploited in *Lilium*: embryo rescue is used as a routine, while ovary slices may be cultured successfully starting 40 days after pollination [25]. The purpose of our work is to establish a complete and integrated in vitro pollination, fertilization and embryo rescue system in order to provide a way for overcoming both pre- and post-fertilization barriers between *Lilium* species.

Materials and Methods

Plant material

Lilium species, hybrids and cultivars were available in the CPO-species and cultivar collection (Table I). Species widely differing in the flowering period were simultaneously brought into flower by storing bulbs at low temperature (0°C) and planting them in pots from January to July in the years 1987—1989. Previous forcing trials proved that an early flowering species as *L. dauricum* takes 40—60 days to flower, while late ones (*L. henryi* and *L. longiflorum*) need a forcing period of about 100—120 days.

Greenhouse temperatures varied from 15°C at night to 20—25°C during the day with summer peaks of 30—35°C.

Pollination methods

Pollen was collected in the morning, and, whenever possible, directly used for pollinations; in certain cases pollen was stored in a desiccator with

Table I. The *Lilium* species, cultivars and hybrids used with the corresponding CPO-numbers.

Name	Type	CPO-number
L. dauricum	Species	73139*, 78190
L. concolor	Species	77543-6, 81185
L. henryi	Species	72122-2, 72202
L. longiflorum	Cultivars	771017**, 78372, 78917, 80392-5
'Whilito'	Asiatic hybrid	80287-1
'Enchantment'	Asiatic hybrid	89138
'Snowstar'	Asiatic hybrid	89137
'Connecticut King'	Asiatic hybrid	89102
'Esther'	Asiatic hybrid	89139
'Star Gazer'	Oriental hybrid	89144
unnamed	Oriental hybrid	783613

*Two different accessions were used with respective CPO-numbers.
**771017 = *L. longiflorum* 'Indian Summer selection'; 78372 = 'Gelria'; 78917 = 'White American'; 80392-5 = 'Albivetta'.

silica gel at room temperature (17—20°C). After storage, pollen was rehydrated at 100% RH for 2 h at 22°C. Flowers of the female parents were emasculated 1 day before anthesis. Pollinations were carried out when the stigma was receptive, about 2 days after emasculation. Following pollination, the stigma or cut stylar surface was covered with an aluminum foil cap.

For normal pollination (N), pollen was directly applied onto the stigma. For the cut-style method (CSM), the style was cut with a razor blade 0—2 mm above the ovary; subsequently, some stigmatic exudate of *L. longiflorum* was placed on the cut surface and the style was immediately saturated with pollen. For the grafted style method (GSM), compatible pollen was deposited on a compatible stigma and after 1 day, the style with germinated pollen was cut 1—2 mm above the ovary and attached to an ovary of another plant. Style and stigma were joined using a piece of a drinking straw filled with *L. longiflorum* stigmatic exudate or were stuck together with only the exudate. For all pollination methods, intraspecific compatible cross combinations (i.e. pollen and ovaries from plants of the same *Lilium* species but from different accessions) were used as controls, whereas for incongruent (interspecific) crosses, several combinations of unrelated lily species or hybrids were used. After pollination, pollen tube growth in pistils was observed, using aniline blue fluorescence [26].

Pollinations were carried out in the greenhouse, in a climate room or in a glass vial. In the greenhouse, ovaries matured on the plant and fruits were examined for presence of embryos starting from 35 DAP. Embryos were rescued on the medium of Asano and Myodo [14] (see 'In vitro methods' below). For pollinations in the climate room, plants were grown in the greenhouse and flowers were cut at anthesis with about 2 cm of the pedicel attached. The flowers were placed in Smithers Oasis (a commercial plastic) soaked in a 2% sugar water solution with 0.01% hydroxyquinoline citrate in the climate room at 25°C with a 16-h photoperiod and a light intensity of 20 W m^{-2}. At stigmatic receptivity, the flowers were pollinated. At 5—8 DAP, part of the flowers was used for ovary culture (see 'In vitro methods' below). Other flowers matured on the Oasis blocks, while embryo development was monitored and embryo rescue was started from 40 DAP. For pollinations in the glass vial (in vitro pollinations), see 'In vitro methods' below.

In vitro methods

In vitro pollination. Flowers of *Lilium longiflorum* cultivars and Asiatic hybrids (Table I) were cut from plants grown in the greenhouse at different stages before anthesis (18, 9, 7, 3, 2 days). The buds were sterilized in 70% ethanol (1 min), and commercial bleach containing 2% chlorine (15 min), and subsequently rinsed three times in sterile distilled water. After rinsing, petals and anthers were dissected with a scalpel knife, and flower buds with the complete pistil were placed vertically in a long test tube (20 cm) partly filled with medium. Two media were tested: MS medium [27], and B5 medium [28], with or without cytokinin (BA and zeatin) and auxin (IAA, 2,4-D and NAA), supplemented with sugar at different concentrations (2, 4, 6, 8 and 10%) and solidified with 0.7% agar. Vials were closed with cotton plugs and placed in a climate room (light intensity, 12 W m^{-2}; photoperiod, 16 h; temperature, 24°C). When exudate production indicated stigmatic receptivity, flowers were pollinated by pollen deposition on the stigma. Aseptic pollen was obtained by dehiscence of sterilized flowers under sanitary conditions. The viability of aseptic pollen was determined by in vitro germination tests. All three types of pollination (N, CSM and GSM) were applied in vitro.

Success of in vitro pollination was scored 30—45 DAP by slicing the ovaries and careful dissection of embryos from the ovules, or 60—90 DAP by counting the number of mature seeds with embryos and endosperm.

Ovary-ovule culture and embryo rescue. Ovary-ovule culture was applied with various *Lilium* species, hybrids and cultivars (Table I). Ovary culture was started 5—8 DAP, ovaries were sterilized as described for buds above. Subsequently, ovaries were transversely sectioned in about six to eight 3—4 mm thick sections and slices were polarly placed on the medium. Two different media were tested: (A) the B5 medium [28] as modified by Prakash and Giles [29] supplemented with 400 mg/l glutamine, 50 mg/l asparagine, 100 mg/l serine, 0.1

mg/l NAA, 0.1 mg/l 2,4-D, 10% sucrose and 0.8% agar at pH 5.8; and (B) the MS medium [27] as modified by Asano and Myodo [14] for lily embryo rescue and supplemented with 1 μg/l NAA, 2% sucrose and 0.7% agar at pH 5.0; in other experiments, sucrose concentrations were increased to 5 and 10%, and the NAA concentration was raised to 1 mg/l.

For the induction of gynogenic haploids, Prakash and Giles [29] described that the presence of young anthers did positively affect lily ovule swelling. Therefore, we tested the effects of anthers on in vitro ovary development by placing anthers of *L. longiflorum* next to ovary slices.

Ovary sections were incubated at 25°C in the dark. Forty-two DAP, the swelling of ovules was scored on a scale from 1 to 9 (1 = no swelling, 9 = very strong swelling) and the swollen ovules (scale size starting from 3) were excised and cultured. When B5 based media were used for ovary culture, ovules were incubated on the same medium, in which hormones were omitted and the sucrose concentration was reduced to 2%. Using MS based media for the preceding ovary culture, ovules were incubated on MS media with 5% sucrose, supplemented with 0.1 mg/l NAA (pH 5.5). No anthers were placed on these media and the ovules were incubated in the dark. Emerged plantlets were transferred to the embryo rescue medium [14] containing MS salts, 1 μg/l NAA, 2% sucrose and pH 5.0.

Results

Greenhouse pollinations

Success of lily crosses depended on several factors. In intraspecific combinations made in the greenhouse, the number of seeds varied between crosses: combinations between *L. longiflorum* cultivars produced 100—400 seeds per ovary, while crosses between Asiatic hybrids gave 50—300 seeds. Quantities of obtainable seeds were affected by external conditions; especially sub-optimal light and temperature conditions reduced seed numbers considerably. Additionally, in some combinations of maternal and paternal genotypes, incompatibility reactions negatively affected seed numbers.

The results of interspecific combinations largely depended on the cross partners and the pollination method. Using CSM, some combinations were relatively easy to make (e.g. *L. dauricum* × *L. concolor*) while others (e.g. Asiatic cvs × Oriental cvs) have so far not been reported. Sub-optimal growing conditions seriously reduced seed production in all interspecific cross combinations.

Optimization of in vitro pollination. ovary-ovule culture and embryo rescue

In vitro pollination. In vitro pollination (i.e. pollination of excised flower buds in vitro) was optimized for both Asiatic hybrids and *L. longiflorum* cvs (Fig. 1). The stage at which flower buds were excised did significantly affect the number of embryos (Table II). Most embryos were found in flowers excised 3 days before anthesis. Use of younger flowers (9 and 18 days before anthesis) did not result in seedset, while fertility of flowers cut 2 days before anthesis was often reduced during disinfection; the petals of these flowers were ajar and bleach could easily penetrate the pistil and impede stigmatic receptivity.

After in vitro deposition of aseptic pollen on the

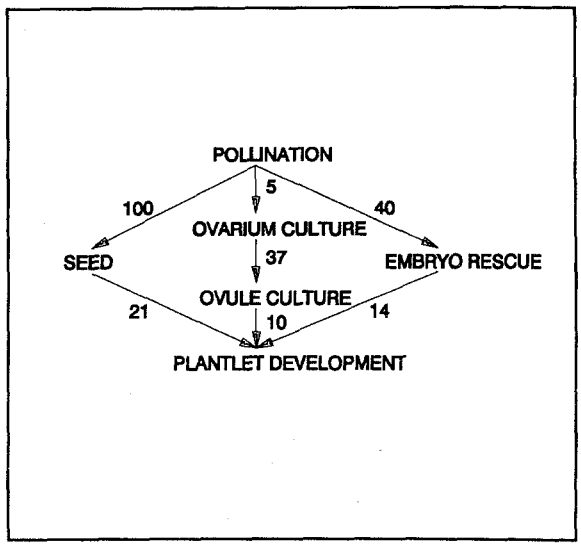


Fig. 1. Diagram of alternative routes for application of in vitro culture methods. Numbers are minimal days in a compatible intraspecific situation (crosses between different *L. longiflorum* cvs). Note that in the normal in vivo situation, seedlings are produced after 121 DAP, while in the embryo rescue and ovar-ovul route, plantlets emerge from 52 to 54 DAP.

Table II. Effect of flower bud stage on the mean number of seeds with embryos scored 90 DAP after compatible in vitro pollination of Asiatic hybrid 'Snowstar' with 'Connecticut King' pollen. 'Snowstar' flowers were cut at different stages before anthesis, incubated in vitro (MS medium, 7% sucrose, no hormones) and pollinated with 'Connecticut King' pollen at the moment of stigmatic receptivity. As a control, the same cross was made in the greenhouse.

Days before anthesis	No. of ovar	Embryos per ovar
18	10	0
9	10	0
7	10	5.0
3	10	18.7
2	10	11.6
Control	3	51.5

stigma, the medium composition influenced subsequent in vitro maturation of fruit capsules. Major effects were found for the sucrose concentration, and although swelling of capsules was largest at the highest sucrose concentration (10%) (Figs. 2—4), most embryos were found in ovaries cultured at 6—8% sucrose (Table III). Best results were obtained with an MS medium (pH 5.8), supplemented with 7% sucrose; other media components (i.e. auxins and cytokinins) or a completely different medium (B5) did not affect numbers of obtained embryos.

Ovary-ovule culture. Ovary-ovule culture comprised the culture of ovary slices and individual ovules. Five DAP in the greenhouse, 18 *L. longiflorum* 'Gelria' ovaries intraspecifically pollinated with *L. longiflorum* 'Indian Summer' were cut in ovary slices and placed on two media (MS and B5) supplemented with different sucrose and auxin concentrations in the presence (1 week or continuous) or absence of anthers. Inside the ovaries, volumes of individual ovules increased considerably (Fig. 5) and at 42 DAP, ovules were dissected and mean ovule swelling was scored. Highest swelling of ovules was recorded in ovaries cultured on B5 medium in the continuous presence of anthers, and swelling scores were positively related with the number of plantlets obtained. On B5 medium in total 19 and on MS 11 plantlets were recovered, of which 14 and 7 originated from the treatment with the continuous presence of anthers, respectively. On MS medium, ovule enlargement was certainly lower, but in a subsequent experiment (Table IV), sucrose and auxin concentrations of the MS medium were raised which positively affected swelling score and plantlet development. For ovary slice culture, an MS medium (pH 6.0), supplemented with 10% sucrose and 1 mg/l NAA proved to be successful.

Table III. Effect of different sucrose concentrations in the MS medium (without hormones) on the mean number of seeds with embryos scored 70 DAP after compatible in vitro pollination of *L. longiflorum* 'Gelria' buds with *L. longiflorum* 'White American' pollen. As a control the same cross combinations was made in the greenhouse.

Sucrose (%)	No. of ovar	Embryos per ovar
2	4	33.5
4	2	59.0
6	4	84.0
8	3	76.0
10	3	61.0
Control	41	143.0

Swelling of ovules was interpreted as a fertilization reaction, but not all swollen ovules contained embryos. Conversely, plantlets never germinated from ovules with a swelling score less than 2. In a compatible intraspecific combination, ovules started to germinate from 10 days after initiation of ovule culture (Fig. 1). Ovules germinated by producing a real seedling (Fig. 6), or regenerated via embryogenesis (Fig. 7) or first formed a root system (Fig. 8) which could develop callus from which regeneration was initiated (Fig. 9). Similar amounts of plantlets originated from a seedling, embryo, root or callus interface. Of all 400 ovules in a typical *L. longiflorum* ovary, approximately 200—300 may be excised for ovule culture from which 50—80 plantlets could be germinated. The optimal medium for ovule culture contained MS salts (pH 5.5), supplemented with 5% sucrose and 0.1 mg/l NAA. After a cold treatment of 8—10 weeks 5°C, to induce a rest period, plantlets (Fig. 10) were transferred to soil and grew into normal, vital, flowering plants.

Combinations of different pollination methods and culture techniques

The success of interspecific crosses depended on

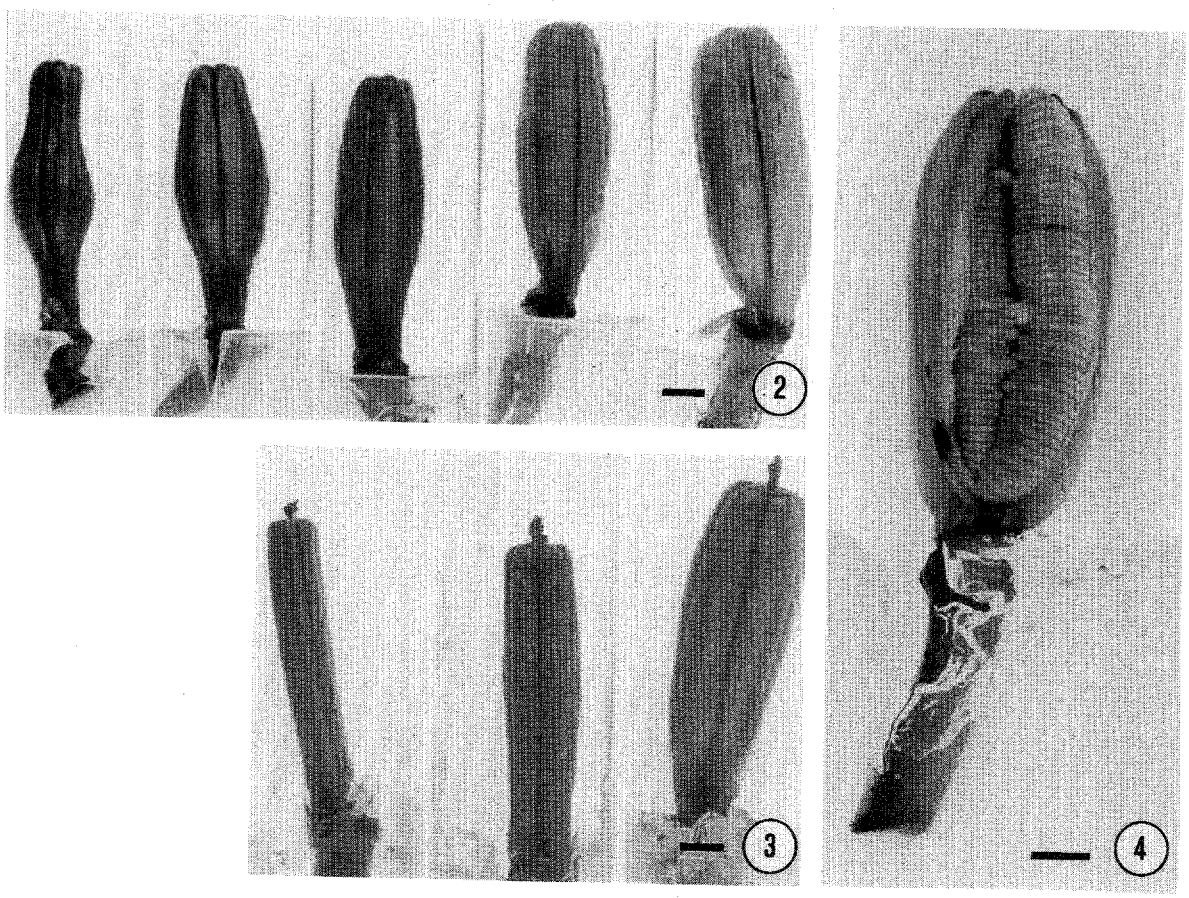


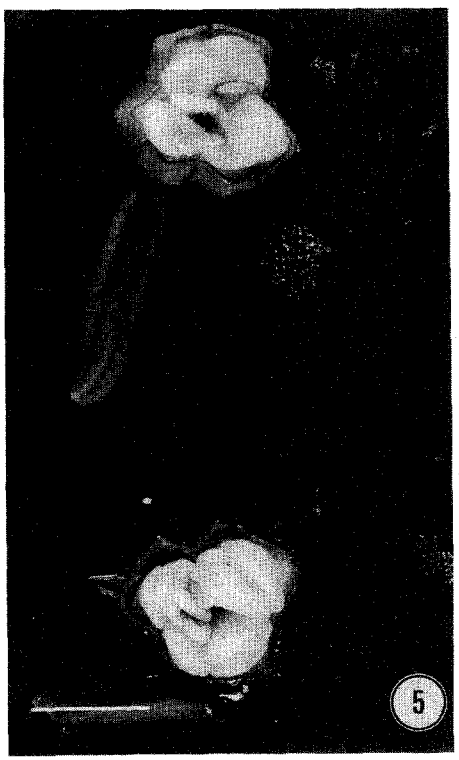
Fig. 2. In vitro pollinated ovaries from *L. longiflorum* 'Gelria', 4 weeks after compatible pollination, on media supplemented with 2, 4, 6, 8, or 10% sucrose (from left to right). Bar = 1 cm.

Fig. 3. In vitro pollinated ovaries from *L. longiflorum* 'Gelria', 1, 2 and 4 weeks (from left to right) after compatible pollination, on medium supplemented with 7% sucrose. Bar = 1 cm.

Fig. 4. In vitro pollinated ovary from the Asiatic hybrid 'Whilito', 6 weeks after compatible pollination, on medium supplemented with 7% sucrose. Note the swollen ovules inside the capsule. Bar = 1 cm.

the feasibility of alternative pollination methods. Table V shows the results of an intraspecific control combination made in the climate room. Compared to N, CSM greatly reduced seedset, while GSM reduced seedset about 60% but gave about 2—3 times more seeds per set ovary than using CSM. In this experiment, the number of successful grafts was low (17%), which was probably due to the long period (up to 70 DAP) ovaries had to mature on Oasis blocks in the climate room and with the contamination hazards associated with GSM.

Potentials of alternative pollination methods for interspecific crosses, could be improved by combining GSM and CSM with ovary-ovule culture (Table VI). Whereas the interspecific combinations (i.e. *L. longiflorum* × *L. henryi* and *L. longiflorum* × *L. dauricum*) proved impossible after N, incongruity barriers could be overcome by using CSM and GSM in the climate room followed by ovar-ovul culture. The number of ovules retrieved was different for the two pollination methods; CSM resulted in a higher mean ovule swelling score (5.3 vs. 2.9) and a larger number of cultured ovules than GSM, but not



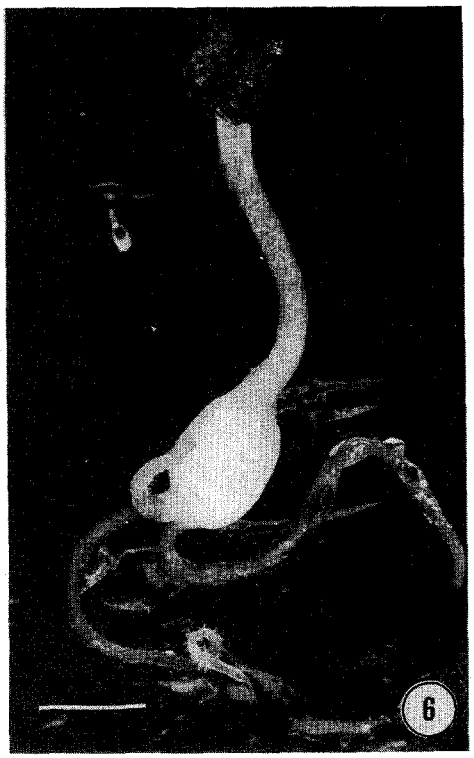


Fig. 5. Asiatic hybrid 'Enchantment' ovary slices, 4 weeks after compatible pollination, on MS medium supplemented with 9% sucrose and 1 mg/l NAA, in continuous presence of anthers. Note the swollen ovules inside the ovary slices. Bar = 1 cm.

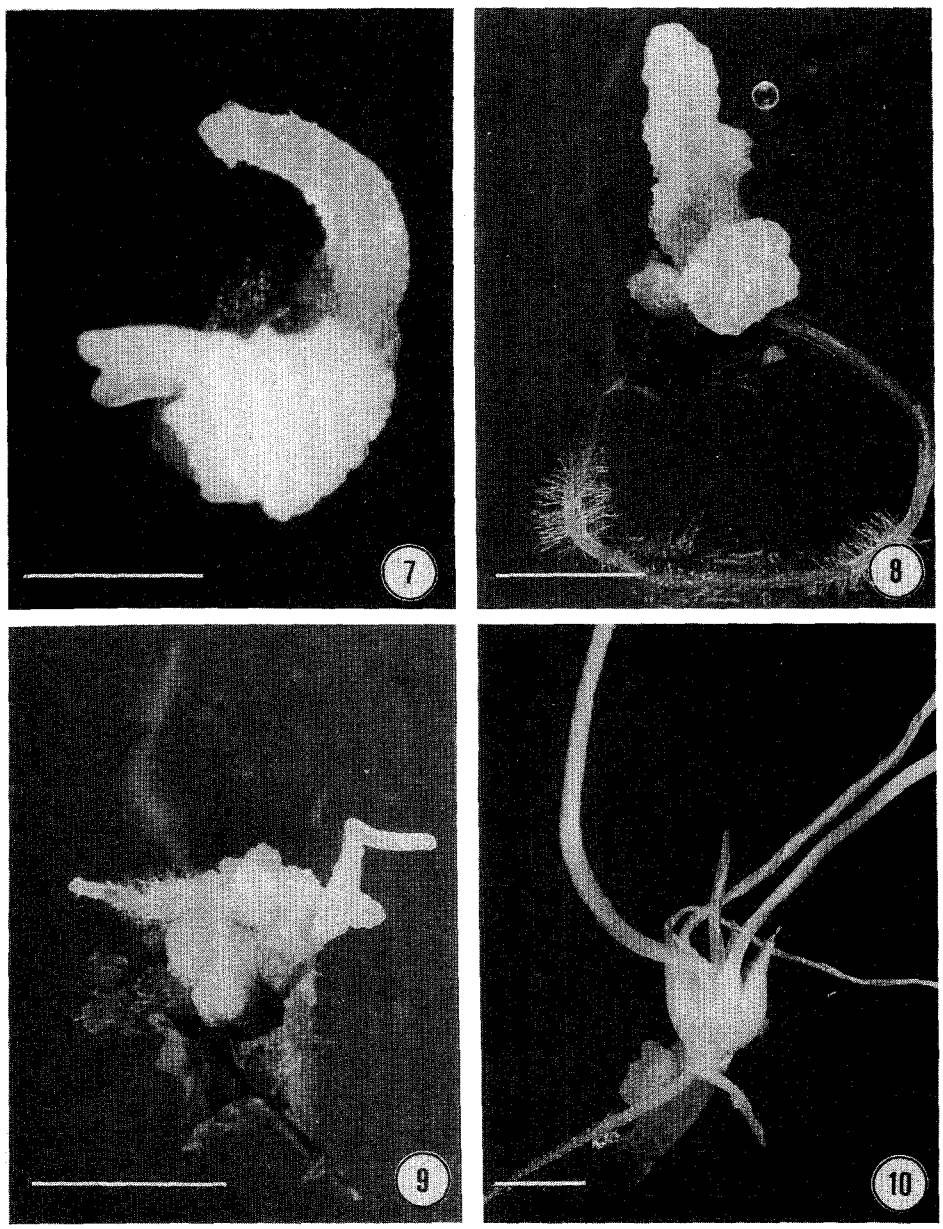
Fig. 6. Seedling of a directly germinated ovule of the Asiatic hybrid 'Enchantment', 12 weeks after compatible pollination, on MS medium supplemented with 5% sucrose and 0.1 mg/l NAA. Bar = 0.5 cm.

Table IV. Effect of sucrose concentration on ovary-ovule development after compatible intraspecific crosses (*L. longiflorum* 'Gelria' × 'White American'). Eight DAP days after N (normal pollination), ovaries were cultured on MS medium supplemented with different sucrose concentrations (2, 5 and 10%) and 1 mg/l NAA. Anthers wre continuously placed on the medium. Forty two DAP, ovule swelling was scored on a scale from 1 to 9, and swollen ovules (minimum score 3) were incubated on MS medium with 5% sucrose and 0.1 mg/l NAA; between 52 and 90 DAP ovule germination was determined.

Medium sucrose (%)	No. of ovar	Swelling score	No. of ovul	Plantlets per ovar
2	10	2.4	150	0
5	10	4.4	94	1.2
10	10	4.8	646	6.4

always in a higher number of plantlets. Retained ovules germinated between 10 days and 40 weeks after culture initiation, the process of ovule germination and plantlet development being similar to that found after intraspecific crosses. The total number of retrieved plantlets after both CSM and GSM was low. The hybrid character of several of these plants was confirmed by iso-electric focussing [32].

Integrated in vitro GSM and CSM pollination followed by ovar-ovul culture was applied for incongruent combinations between *L. longiflorum* × 'Whilito' and between Asiatic and Oriental hybrids (Figs 11 and 12) (Table VII). Especially in the latter combination, GSM was successful; 13 possible hybrid plantlets were recovered after in vitro style grafting and subsequent ovar-ovul culture and em-



Figs. 7—9. Regeneration via embryogenesis (Fig. 7), regeneration via callus with root phase (Fig. 8) and regeneration via callus stage (Fig. 9) from ovules obtained after the interspecific cross *L. longiflorum* × *L. dauricum*, 32 weeks after pollination, on the same medium as indicated in Fig. 6. Bar = 0.5 cm.

Fig. 10. Hybrid plantlet with bulb formation, from an ovule obtained after the interspecific cross *L. longiflorum* × *L. dauricum*, 40 weeks after pollination, ready to be transferred to soil. Bar = 1 cm.

Table V. Effect of pollination technique on the number of seeds. A comparison between three types of GSM (grafted style method see text), CSM (cut style method) and N (normal pollination) in a compatible cross between *L. longiflorum* 'Gelria' and *L. longiflorum* 'Albivetta'. Pollinations were made in the climate room, numbers represent pollinated flowers (ovar), set pods (ovar) and matured seeds (70 DAP).

Pollination method	No. of ovar	No. of set ovar	No. of seeds	Seeds per set ovar
N (clim)	25	19	451	23.7
CSM	25	19	85	4.5
GSM exudate	26	1	15	15.0
GSM straw exudate	26	7	96	13.7
GSM straw medium	26	5	36	7.2

bryo rescue. These plantlets didn't flower yet but, visually the characters are intermediate between the parents. While in the control experiments, both in the greenhouse and in the climate room, using either N or CSM pollination, no embryo development was found.

Table VI. Comparison of the number of obtained plantlets per ovary after the incongruent combinations *L. longiflorum* × *L. henryi* and *L. longiflorum* × *L. dauricum* using three pollination methods. As a control, *L. longiflorum* 'Gelria' was intraspecifically crossed with 'White American' pollen both in the climate room and in the greenhouse. Interspecific pollinations were carried out in the climate room; 8 DAP, ovaries were cultured on MS medium (10% sucrose, 1 mg/l NAA); 42 DAP, ovules were excised and the swelling score was determined. Subsequently, swollen ovules (number ovul) were cultured on MS medium (5% sucrose, 0.1 mg/l NAA), plantlet development was scored from 10 to more than 40 weeks after start of ovary culture (nd = not determined).

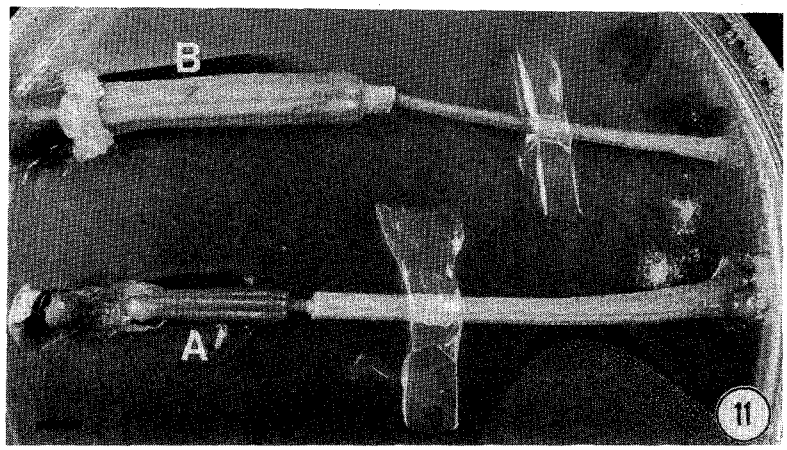
Cross	Pollination method	No. of ovar	Swelling score	No. of ovul	Plantlet per ovar
Intra	N (greenhouse)	41	nd	nd	143
Intra	N (clim)	24	nd	nd	19
L. henryi	N	10	4.8	2206	0
L. henryi	CSM	10	5.6	2622	2.1
L. henryi	GSM	10	3.2	1195	0.1
L. dauricum	N	13	3.9	3475	0
L. dauricum	CSM	14	5.1	4568	3.5
L. dauricum	GSM	14	2.7	2446	4.8

Discussion

Application of in vitro techniques for overcoming incongruity barriers in interspecific *Lilium* crosses was for a long time restricted to the use of embryo rescue [12—16]. Only recently, Hayashi et al. [30] and Straathof et al. [31] have exploited possibilities for ovule and ovary culture. In the present work, these methods have been optimized and attuned to integrated in vitro pollination, fertilization and embryo rescue. With the currently developed methods, time intervals between pollination and in vitro culture have been reduced from 40 DAP with embryo rescue, to 7 DAP with ovary slice culture and -7 DAP using in vitro pollination.

Several important benefits from the developed in vitro methods can be distinguished. Firstly, application of more controlled conditions during the processes of pollination, fertilization and embryo development ensures that experiments can be repeated, almost independent of the season. Hence, conditions for each process can be optimized and crossing barriers can be studied more systematically. Secondly, the improved methods stimulate the application of new pollination procedures. For instance, GSM appears to be an important improvement for overcoming pre-fertilization barriers. Thirdly, the in vitro methods make it possible to develop an integrated procedure for culture conditions from fertilization till embryo development and seedling germination. This enables the overcoming of post-fertilization barriers which normally impede interspecific combinations.

With respect to the possible routes for application of in vitro culture methods, as exemplified in Fig. 1, it is of importance to establish at what time interval each culture step can optimally be initiated. The occurrence of post-fertilization barriers is associated with the particular cross combination and may result in deviations at different stages of embryo development. Correspondingly, the developmental stage at which ovules have to be transferred from e.g. ovary culture to ovule culture will be different for each cross. Evidently, a premature transfer will result in aberrations in embryo development, while at a delayed shift of culture conditions, embryos may abort by post-fertilization barriers. As yet, it is unknown to what extent the



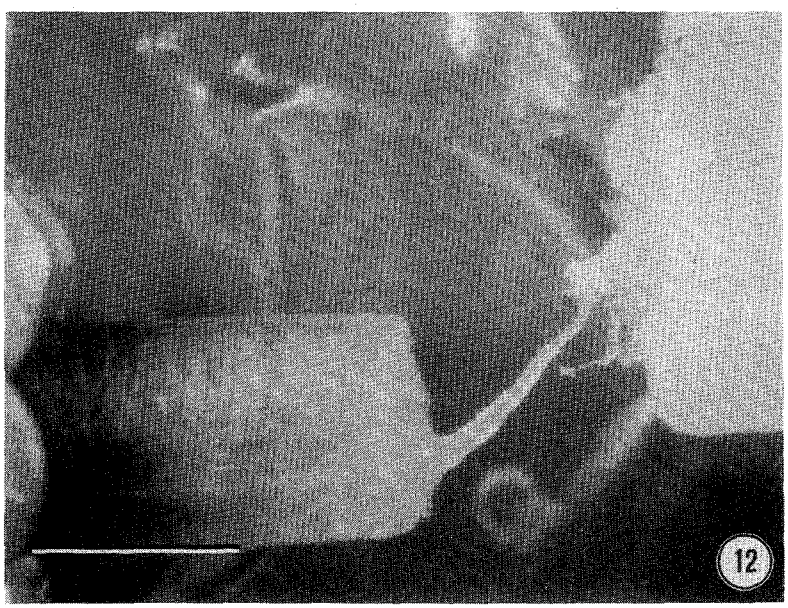


Fig. 11. GSM (grafted style method of pollination), (A) style of Asiatic hybrid 'Esther' compatibly pollinated with 'Connecticut King' pollen and attached to an ovary of the Oriental hybrid 'Star Gazer', and, (B) style of 'Star Gazer', compatibly pollinated with Oriental no. 783613 pollen and attached to an 'Esther' ovary.

Fig. 12. Detail of graft end from situation (A) in Fig. 11. Both style ends are slightly separated to show the bundle of pollen tubes transferring the stylar surfaces. Bar = 0.2 cm.

ovary slice method is more suitable for sustaining embryo development in comparison to the situation in which the whole ovary is cultured. This may also depend on the specific post-fertilization barrier to overcome and may be different for each interspecific cross combination.

Under normal greenhouse conditions, seed of an intraspecific lily cross requires about 100 days to mature, whereas under in vitro conditions, ovules can germinate from 50 to 280 DAP. Apparently, some ovules maintain dormancy for a long period. Ovules have been incubated at 25°C, and it is known that high temperatures can bring lily seeds or bulbs in a rest condition [33]. Currently, we are trying to

Table VII. Combination of different in vitro pollination methods with ovary-ovule culture techniques. Incongruent combinations were made between *L. longiflorum* and the Asiatic hybrid 'Whilito'; and reciprocally between Oriental hybrids (OR) (i.e. 'Star Gazer' and CPO-number 783613) and Asiatic hybrids (AS) (i.e. 'Connecticut King' and 'Esther'). As a control, *L. longiflorum* was intraspecifically pollinated. Three methods of pollination (N, CSM, GSM) were applied in vitro, pistils were incubated on the MS medium (7% sucrose, without hormones); between 7 and 30 DAP, ovaries were sectioned and placed on MS medium with 9% sucrose; from 42 DAP, swollen ovules were excised and scored (number ovul) and subsequently cultured on MS medium with 5% sucrose. All ovules and seeds were carefully examined for embryo development, obtained embryos were rescued on the Asano and Myodo medium [14] (nd = not determined).

Cross	Pollination method	No. of ovar	No. of ovul	Plantlets per ovar
Intra (greenhouse)	N	9	nd	125.4
Intra	CSM	9	nd	8.2
L. longiflorum × 'Whilito'	N	9	nd	0
L. longiflorum × 'Whilito'	CSM	9	nd	0.78
OR × AS	N	10	0	0
OR × AS	CSM	10	1058	0
OR × AS	GSM	10	868	0.1
AS × OR	N	10	0	0
AS × OR	GSM	10	1336	1.2

break dormancy by decreasing the temperature after 2 months of ovule culture to 17°C.

As indicated by Hayashi et al. [30], who applied ovary culture 40 days after self-pollination of *L. formosanum*, a high concentration of sucrose and a high pH are favourable for ovule development. Also in our experiments, high sucrose concentrations and high pHs had a favourable effect on ovule development at the early stages of in vitro culture. Although not definitely proved, the presence of auxins also seems to be of importance, especially in early stages using the ovary slice method. Additions of auxins were, however, not necessary when whole ovaries were cultured after in vitro pollination. Ovary slice development appeared to be positively affected by the presence of young anthers. Possibly, the anthers excrete substances (hormones) which stimulate ovule or ovary growth and are not available in the culture medium.

Ovule swelling score in ovary culture is used to evaluate media effects on ovule development. It is, however, evident that a high swelling score is not a guarantee for having a high number of embryos in the cultures. Further research will be necessary to distinguish ovules with and without embryos in an early stage of in vitro culture, before transferring them to a new medium.

Using the newly developed culture and pollination methods, the program of diallel crosses with six lily species could be carried out successfully, especially, when compared with the outcome of earlier work using more conventional embryo rescue techniques [2]. Not only the total number of plantlets in the various interspecific crosses, but also the number of successfully obtained hybrids was much higher. At present, we are analyzing the hybrid characteristics of the progeny.

A powerful application of in vitro pollination techniques is the possibility to combine in vitro culture methods with new pollination methods which are not possible under normal in vivo conditions. Particularly, the combination of in vitro pollination and GSM seems to open new possibilities for overcoming crossing barriers between several incongruent lily species.

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