

APPLICATION OF FOUR POLLINATION TECHNIQUES AND OF HORMONE TREATMENT FOR BYPASSING INTERSPECIFIC CROSSING BARRIERS IN *LILIUM* L.

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Abstract

Crosses between genotypes of the 3 commercially important groups of lilies, i.e. *Lilium longiflorum* Thunb., the Asiatic hybrids and the Oriental hybrids, are hampered by interspecific crossing barriers. Several methods have been developed to bypass pre-fertilization barriers or post-fertilization barriers in lily, e.g. the cut-style method and embryo rescue techniques.

Five techniques were studied comparatively for their capacity to bypass barriers between different types of lilies: the grafted-ovary method, placental pollination, warm-water treatment, the mentor pollen technique and hormone treatment. For the grafted-ovary method, a compatible pollinated pistil was grafted one day after pollination on the ovary of another species. It appeared that the pollen tubes were not able to penetrate from one ovary into another ovary. After placental pollination, no embryo germination was found, even not when pre-pollinated styles were placed near the ovules. After a warm-water treatment (6 minutes of 50 °C) of pistils, many seeds were harvested of self-pollinated flowers of *L. longiflorum*. However, interspecific crossing barriers could not be bypassed. The percentage of ovules with pollen tube penetration did not change by mixing pollen with radiated mentor pollen. It appeared that the mentor pollen were able to penetrate the ovules. After treating ovaries with 0.1% BAP, on average 6 embryos per flower of the cross *L. longiflorum* 'Gelria' x Asiatic hybrid 'Mont Blanc' could be rescued when ovule culture was started around 40 days after pollination.

Key words: lily, interspecific hybridization, hormone treatment, grafted-ovary method, placental pollination, mentor pollen, warm-water treatment

1. Introduction

The genus *Lilium* comprises about 85 species, which are classified in seven sections (De Jong 1974). Commercially important lily cultivars are mainly cultivars belonging to the group of Asiatic hybrids or to the group of Oriental hybrids and cultivars of *L. longiflorum* from the section *Leucolirion*. The Asiatic hybrids and the Oriental hybrids have resulted from interspecific crosses within the section *Sinomartagon* and within the section *Archelirion*, respectively. The present-day assortment of lily can still be largely improved by making crosses between the three important groups of lily cultivars and also by exploiting traits from *Lilium* species of the four other sections. Especially, introduction of resistances against viral diseases, bulb rot (caused by *Fusarium oxysporum*) and *Botrytis*, but also improvement of flower longevity and tolerance for suboptimal culture conditions (e.g. low light and low temperature), together with special flower colours and flower shapes are important targets for lily breeding programmes.

Crosses between species of different sections of *Lilium* are often hampered by crossing barriers (Van Creijl et al. 1993). Pollen tube inhibition in the style was reported for many interspecific lily crosses (Ascher and Peloquin 1968, Asano 1980). Once embryos have been formed, the production of hybrids is often hindered by embryo abortion (Dowrick and Brandram 1970, Asano and Myodo 1977). A wide range of techniques has been developed to bypass crossing barriers in *Lilium*. Pollen tube inhibition in the style can be bypassed successfully by using the cut-style method (Asano and Myodo 1977, Van Tuyl et al. 1991, Van Creijl et al. 1993). However, normal pollen tube growth is hindered after cut-style pollination which has been shown by the low seed set in compatible crosses

(Van Tuyl et al. 1988). This low seed set might be caused by the premature arrival of pollen tubes in the ovary (Janson et al. 1993). To permit the pollen tubes to attain their normal lengths, the grafted-style method has been developed (Van Tuyl et al. 1991). For this method, compatible pollen is deposited on a compatible stigma and after one day attached to the ovary of an incongruent partner. This method has as disadvantage that it is difficult to get a durable connection between the two styles. For executing pollination methods under standard and optimal environmental conditions, an in vitro pollination procedure has been developed (Van Tuyl et al. 1991). Three techniques have been developed to prevent embryo abortion in interspecific lily crosses: ovary-slice culture, ovule culture and embryo culture (Kano et al. 1988, Van Tuyl et al. 1991, Okazaki et al. 1994).

Hybrids have been obtained of many interspecific lily crosses, by combining pollination techniques with embryo rescue methods (Van Creijl et al. 1993, Okazaki et al. 1994, Niimi et al. 1996, Van Tuyl and Van Holsteijn 1996). However, hybrids have not been obtained of a range of crosses and the production of hybrids of several crosses is rather low. Several techniques have been successful in bypassing pre-fertilization barriers in other crops, like hexane treatment of the stigmatic surface (Whitecross and Willing 1975), mentor pollen and pioneer pollen (Whitecross and Willing 1975, Stettler 1968), placental pollination (Zenkteler et al. 1987) and pollination of isolated ovules (Kameya and Hinata 1970, Stewart 1981). Hormone treatments have been suggested as a method to bypass both pre-fertilization barriers and post-fertilization barriers (Khanna et al. 1994, Ahmad et al. 1995). The development of new pollination methods and embryo rescue techniques might be useful for the production of new unique lily hybrids or for increasing the rate of hybrid production of several interspecific lily crosses.

The aim of this research was to examine the potentials of the grafted-ovary method, of placental pollination, of warm-water treatment and of the mentor pollen technique for bypassing pre-fertilization barriers and to determine the effect of hormone treatment on the number of seeds or emerged plantlets which can be produced from interspecific *Lilium* crosses.

2. Materials and Methods

2.1 Plant material

Lilium longiflorum 'Gelria' was used in all crosses as maternal genotype. Compatible pollinations were carried out with *L. longiflorum* 'Indian Summer' and 'White American'. The Asiatic hybrid 'Mont Blanc' and the Oriental hybrid 'Star Gazer' were used as paternal genotype for interspecific crosses. Pistils of *L. longiflorum* 'Gelria', *L. longiflorum* 'Romero', Asiatic hybrids 'Connecticut King' and 'Orlito' and Oriental hybrid 'Laura Lee' were used as donor pistils in experiments with the grafted-ovary method. Pollinations were carried out in September and October 1991 and 1992. Twelve flowers were used per cross, per experiment for each year. Plants were grown in pots in a greenhouse at a minimum temperature of 15 °C at night and a day temperature of 20-24 °C, with peaks of 30-35 °C in the summer. From two days before anthesis, the plant material used for pollinations on the plant was placed in a climate room at a constant temperature of 24 °C.

2.2 Methods

Flowers were placed in vitro prior to the application of the grafted-ovary method and placental pollination. For in vitro pollination, flower-buds were collected 2 (*L. longiflorum*) or 4 (Asiatic hybrids and Oriental hybrids) days before anthesis. The petals and anthers were excised and the remaining parts of the flower (henceforth called 'flower') were placed in test-tubes at a temperature of 24 °C (Van Tuyl et al. 1991).

The warm-water treatment, the mentor pollen technique and the hormone treatment were executed on flowers pollinated on the plant. The pollination methods used for pollinations on the plant and the methods for ovary-slice culture and ovule culture are described by Van Tuyl et al. (1991). The pollen tube growth in the pistil and the penetration of the pollen tubes in the ovules were studied 7-14 days

after pollination (DAP) in 2 pistils per cross, per experiment per year. The methods used for microscopical observations are according to those described for tulip by Van Creij et al. (1997a).

2.3 Experiments

2.3.1 Grafted-ovary method:

Pistils of *L. longiflorum* 'Gelria' (henceforth called 'mother pistil') and the pistils used as donor pistils (see 2.1) were both compatible pollinated two days after the stigma was receptive. One day after pollination, the style and the upper part (1/4) of the ovary of *L. longiflorum*, of the Asiatic hybrid or of the Oriental hybrid were grafted on the lower part (3/4) of the ovary of the mother pistil of *L. longiflorum* 'Gelria', as shown in Van Creij et al. (1997b). The grafted pistils were placed upright in test-tubes with in vitro pollination medium. The pollen tube growth was studied at 1 DAP in the styles of the mother pistils used in 1992. Ovules from the mother pistils were placed separately in vitro at 28-45 DAP in 1991. In 1992, mother ovaries were used for ovary-slice culture started at 7-8 DAP, followed by ovule culture at 38-41 DAP.

2.3.2 Placental pollination:

The ovaries were cut longitudinally into six sectors on the day of stigmatic receptivity (1991) or 1-2 days after the stigma was receptive (1992). Each sector contained a placenta with a row of ovules without (1991) or with (1992) the ovary wall. Pollen was abundantly applied on the placenta. In 1991, also compatible pollinated styles (henceforth called 'donor styles') were placed with the basal cut end nearby two rows of ovules. One or two styles were used per flower. The Petri dishes with the explants remained 10 days in the light (12 Wm^{-2}) (1991) or were placed directly in the dark (1992). The pollen tube growth in the donor pistils was analyzed at 10 DAP in three styles per cross. The pollen tube penetration in the ovules was studied in 1991 in 4 rows of ovules per cross. Ovule culture was carried out around 5 (1991) or 7 (1992) weeks after pollination. Only the swollen ovules were placed in vitro.

2.3.3 Warm-water treatment:

Styles of intact flowers were immersed in water of $50 \text{ }^{\circ}\text{C}$ during 6 minutes prior to pollination. The length and the width of the pods were measured two months after pollination. The pods were collected between 60-80 DAP and the number of seeds per pod was determined.

2.3.4 Mentor pollen technique:

Mentor pollen were obtained by irradiation of pollen of *L. longiflorum* 'Indian Summer' with 100 Krad. One part of mentor pollen was mixed with one part of the paternal genotype and the mixture was used for the pollinations. Twelve flowers were pollinated with the mentor pollen only. Stigmatic pollinations were executed in crosses with the mentor pollen only and in crosses with a mixture of mentor pollen with pollen of *L. longiflorum* 'Indian Summer'. The cut-style method was applied when pollinations were carried out with mixtures of mentor pollen with pollen from the Asiatic hybrid 'Mont Blanc' or with pollen from the Oriental hybrid 'Star Gazer'. For the cut-style method, the style of *L. longiflorum* 'Gelria' was cut 1-2 mm above the ovary one day after stigmatic receptivity and pollen was applied immediately on to the cut surface. As control, the cut-style method was also applied in the compatible cross with pollen of *L. longiflorum* 'Indian Summer'. The length and the width of the pods were measured two months after pollination. The pods were collected between 60-80 DAP and the number of seeds per pod was determined.

2.3.5 Hormone treatment:

Flowers were pollinated on the stigma in crosses with pollen of *L. longiflorum* 'White American'. The cut-style method (see 2.3.4) was applied in interspecific crosses with the Asiatic hybrid 'Mont Blanc' and the Oriental hybrid 'Star Gazer'. The hormone 6-benzylaminopurine (BAP) was dissolved in a mixture of lanolin (3 parts) with water (1 part) to a final concentration of 0.1% BAP. The mixture

was applied at 4 DAP at the base of the ovary. As control, 11 flowers pollinated with *L. longiflorum* 'White American' were not treated with BAP. The length and the width of the pods were measured one and two months after pollination. Swollen ovules of the cross with the Asiatic hybrid 'Mont Blanc' as pollen parent were placed separately on ovule culture medium at 36-43 DAP. Pods of crosses with *L. longiflorum* 'White American' matured on the plant and the seeds were harvested and counted.

3. Results

The donor pistil (style + part of ovary) was placed at 1 DAP on the mother ovary for the grafted-ovary method. The mother pistil was pre-pollinated, but at 1 DAP the pollen tubes had passed only 12-14% of the style length. After grafting, the pollen tubes had grown through the whole donor style and into the donor part of the ovary. Finally, 20% of the ovules of the donor ovaries contained an embryo in the compatible cross. In the incongruent crosses with the Asiatic hybrid 'Mont Blanc' and the Oriental hybrid 'Star Gazer' as parental genotypes, in 2% and 8% of the ovules of the donor ovaries, respectively, an embryo was found. Finally, a maximum of 2% of the ovules of the mother ovaries was penetrated by a pollen tube, independent of the cross made. After ovule culture with over 2000 ovules, no embryo germination was observed.

Compatible pollinated styles were placed nearby rows of ovules of *L. longiflorum* 'Gelria' for the placental pollination technique or ovules of this cultivar were pollinated directly. In the cross with pollen of *L. longiflorum* 'Indian Summer', pollen tubes passed on average 24% of the total length of the compatible donor styles. Pollen tubes protruding from the basal cut end of the styles of the donor pistils was not observed in this cross. In crosses with pollen from the Asiatic hybrid 'Mont Blanc' or the Oriental hybrid 'Star Gazer', the pollen tubes penetrated on average 90% and 99% of the total length of the style of the compatible donor styles, respectively. In 30% and 78% of the donor styles compatible pollinated with the Asiatic hybrid 'Mont Blanc' and the Oriental hybrid 'Star Gazer', respectively, pollen tubes protruded. Both after direct pollination of the ovules and after pollination of a donor style placed nearby the ovules, no (in 83% of the rows of ovules analyzed) or only few ovules were penetrated by a pollen tube. The highest percentage ovules with pollen tube penetration was 3.3%, which was found in one of the four rows of ovules analyzed in the cross *L. longiflorum* 'Gelria' x Oriental hybrid 'Star Gazer'. No difference in the percentage of pollen tube penetration in the ovules was found between direct pollination of the ovules and the use of donor styles. After the application of ovule culture with over 2500 ovules, no germinated embryo was found in both years.

The results obtained after warm-water treatment of styles of *L. longiflorum* 'Gelria' are shown in Table 1. The pollen tubes of *L. longiflorum* 'White American' grew through the style of 'Gelria', into the ovary and finally penetrated on average 64% of the ovules. In this cross and after self-pollination of *L. longiflorum* 'Gelria', the ovaries were well swollen after two months and finally over 100 seeds per pod were harvested. In crosses with pollen of the Asiatic hybrid 'Mont Blanc' and of the Oriental hybrid 'Star Gazer', the pollen tubes penetrated about half the style length. No pollen tubes were observed in the ovules and remaining parts of the ovaries. No seeds were harvested of both these crosses.

The results obtained after pollination with mixtures of mentor pollen with compatible pollen or incongruent pollen are presented in Table 2. After normal pollination of *L. longiflorum* 'Gelria' with mentor pollen only, pollen tubes grew into the ovary and penetrated 12% of the ovules. No seeds were harvested. Pollen tubes penetrated the ovules after pollination with a mixture of mentor pollen and pollen of *L. longiflorum* 'Indian Summer'. However, less pollen tube penetration was found after the application of the cut-style method than after normal stigmatic pollination. Ovaries reached smaller lengths and widths and finally lower numbers of seeds were harvested after cut-style pollination compared to normal stigmatic pollination. Low percentages of pollen tubes penetrated the

ovules after pollination with a mixture of mentor pollen and pollen of the incongruent pollen parents. Of these crosses, all ovaries were dead within two months after pollination.

The results obtained after treating ovaries with 0.1% BAP in lanolin at 4 DAP are shown in Table 3. Both treated and non-treated flowers of *L. longiflorum* 'Gelria', pollinated with *L. longiflorum* 'White American' formed well developed ovaries and comparable numbers of seeds could be harvested. In the cross with the Asiatic hybrid 'Mont Blanc', the ovaries appeared to be somewhat smaller in size as compared to the ovaries obtained from the compatible pollinations. However, on average 6 embryos per ovary germinated after the application of ovule culture. Ovaries from the cross *L. longiflorum* 'Gelria' x Oriental hybrid 'Star Gazer' all died within one month after pollination. In this cross, pollen tube growth was only observed in the upper part of the ovary.

4. Discussion

The grafted-ovary method, placental pollination, warm-water treatment, the mentor pollen technique and hormone treatment have been examined for their prospects in bypassing interspecific crossing barriers in the genus *Lilium*.

The grafted-ovary method was studied for obtaining a more durable connection between the donor pistil and the mother pistil as compared to the grafted-style method. Van Tuyl et al. (1991) reported a low number of successful grafts (17%) after using the grafted-style method. In the grafted-ovary method, ovaries remained well attached during several days. Although the pollen tubes grew to the bottom of the donor ovary, they mostly failed to pass the graft and to grow into the mother ovary. To enhance the pollen tube passage from the donor ovary into the mother ovary in the grafted-ovary method seems to be more difficult to realise than to develop a more durable connection for the grafted-style method. Therefore, the grafted-ovary method seems not to be a more suitable method compared to the grafted-style method.

A maximum of 3.3% of ovules was penetrated by a pollen tube after direct placental pollination, which was lower compared to the average penetration percentage of 5% obtained by Janson (1993) for compatible *L. longiflorum* crosses. She found a significant increase in ovule penetration after culturing the explants on filterpaper and after removing the ovary wall. Optimization of the method used in our experiments for direct pollination of ovules seems therefore to be possible. Pollen tubes protruded from only 36% styles, averaged for all styles used, after placing compatible pollinated styles nearby the explants. Janson (1993) observed also problems with the passage of the pollen tubes from the style to the placenta after grafting styles at the placentas. However, after successful grafting on average 25% of the ovules was penetrated by a pollen tube. Therefore, it seems to be possible to optimize placental pollination for lily resulting in the production of viable hybrid embryos. However, a time-consuming study seems to be needed, focusing on the pollination conditions, culture conditions and culture methods.

Warm-water treatment of styles of the self-incompatible species *L. longiflorum* resulted in the production of many seeds after self-pollination, as was also found by Hopper et al. (1967). Influence of the warm-water treatment on pollen tube growth was not found after pollination of *L. longiflorum* 'Gelria' with Asiatic hybrid 'Mont Blanc' or with Oriental hybrid 'Star Gazer'. Pollen tubes reached lengths in these crosses comparable with those found in other lily experiments.

Stigmatic pollination with a mixture of mentor pollen and pollen of an incongruent pollen donor has resulted in the production of a number of hybrid embryos (Van Tuyl et al. 1982). In our experiments, the combination of the cut-style method with the mentor pollen technique did not result in the production of pods containing viable seeds of the interspecific crosses. In fact, the percentages of ovules with pollen tube penetration were not higher than obtained after cut-style pollination with pollen of an incongruent donor only, as found in other experiments. The percentages of ovules with penetration of pollen tubes of the incongruent partner might even be overestimated in Table 2, since mentor pollen also enter the ovules (see Table 2, cross with mentor pollen only). Therefore, the

combination of the cut-style method with the mentor pollen technique is not suitable for bypassing pre-fertilization barriers, at least in these lily crosses.

The seed set did not differ significantly in the compatible cross between the untreated ovaries and the ovaries treated with 0.1% BAP. The ovaries from the cross *L. longiflorum* 'Gelria' x Oriental hybrid 'Star Gazer', all died within one month. A probable positive effect of the hormone treatment can only be found in this cross when embryo rescue techniques are started within one month after pollination and compared with the embryo production in non-treated ovaries. The high number of germinated embryos obtained from the cross *L. longiflorum* 'Gelria' x Asiatic hybrid 'Mont Blanc' has not been reported previously (Straathof et al. 1987, Van Tuyl et al. 1991, Van Tuyl et al. 1988). The treatment of the ovaries with BAP might, therefore, have a positive influence in this cross. Emsweller and Stuart (1948) found that treatment of *Lilium* ovaries with 1% naphthalene acetamide reduced seed set in several interspecific crosses, while in other crosses seeds were only obtained after application of this hormone. Further studies are needed before conclusions can be drawn on the effect of the treatment with 0.1% BAP in bypassing crossing barriers in interspecific lily crosses.

Five methods have been examined for their ability to bypass pre-fertilization barriers or post-fertilization barriers. The grafted-ovary method proved not to be better applicable than the grafted-style technique. Warm-water treatment and the mentor pollen techniques appeared not to bypass crossing barriers. Placental pollination and hormone treatment offer prospects, however, more research is needed.

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Table 1: The percentage of total style length traversed by pollen tubes (% PT), the percentage ovules with pollen tube penetration (% PE), the lengths and widths of ovaries two months after pollination and the number of seeds per pod (Seeds) after warm-water treatment of styles (6 minutes 50 °C) of *L. longiflorum* 'Gelria', followed by pollination with four lily cultivars.

Pollen donor	% PT	% PE	Length/Width	Seeds
<i>L. longiflorum</i> 'Gelria'	nd	nd	55/21	160
<i>L. longiflorum</i> 'White American'	99	64	55/21	114
Asiatic hybrid 'Mont Blanc'	55	0	28/2	0
Oriental hybrid 'Star Gazer'	56	0	30/3	0

nd: not determined

Table 2: The percentage of total ovary length traversed by pollen tubes (% PO), the percentage ovules with pollen tube penetration (% PE), the lengths and widths of ovaries two months after pollination and the number of seeds per pod (Seeds) after pollination of *L. longiflorum* 'Gelria' with mentor pollen only or mixtures of mentor pollen with three lily cultivars. Flowers were normal pollinated on the stigma (NP) or the cut-style method was applied (CSM).

Pollen donor	method	% PO	% PE	Length/Width	Seeds
mentor pollen only	NP	49	12	42/7	0
<i>L. longiflorum</i> 'Indian Summer'	NP	74	46	55/22	137
<i>L. longiflorum</i> 'Indian Summer'	CSM	51	0.9	45/18	8
Asiatic hybrid 'Mont Blanc'	CSM	23	4.5	nd	0
Oriental hybrid 'Star Gazer'	CSM	40	0.5	nd	0

nd: not determined

Table 3: The percentage of total ovary length traversed by pollen tubes (% PO) and the lengths and widths of ovaries one and two months after pollination in crosses between *L. longiflorum* 'Gelria' and three other lily cultivars, treated (+) or not treated (-) with 0.1% BAP in lanolin at 4 DAP. The number of seeds per pod harvested on the plant after compatible pollination (*L. longiflorum* 'White American') and the number of germinated embryos per ovary obtained after ovule culture of incongruent crosses are also presented.

Pollen donor	BAP	% PO	One month Length/Width	Two months Length/Width	Seeds or Embryos
<i>L. longiflorum</i> 'White American'	-	nd	50/14	55/20	194
<i>L. longiflorum</i> 'White American'	+	78	50/14	53/21	220
Asiatic hybrid 'Mont Blanc'	+	72	48/11	iv	6.2
Oriental hybrid 'Star Gazer'	+	32	40/5	nd	0

iv: ovaries were used for ovule culture

nd: not determined