APPLICATION OF THREE POLLINATION TECHNIQUES AND OF HORMONE TREATMENTS FOR OVERCOMING INTERSPECIFIC CROSSING BARRIERS IN *Tulipa*

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Abstract

In tulip, interspecific crossing is restricted by both pre-fertilization and post-fertilization barriers. In order to introduce traits from wild species into the cultivar assortment these barriers must be bypassed. By application of embryo rescue techniques, unique hybrids have been obtained of several interspecific tulip crosses. Recently, four techniques have been tested on their capacity to overcome interspecific crossing barriers in tulip, namely hormone treatments of ovaries, cut-style method, grafted-ovary method and placental pollination. After treating ovaries with 0.1% BAP at 12 days after pollination, seeds were obtained on the plant from the cross *T. gesneriana* x *T. agenensis*. This cross has never been given seeds *in vivo* before. Treating ovaries with 1% BAP seemed to have a negative effect on seedset in compatible crosses, 1% NAA did not give any effect. After the application of the cut-style method, the percentage pollen tube penetration did not increase in crosses between *T. gesneriana* and 5 other *Tulipa* species. Apparently, crossing barriers were not overcome by using the cut-style method, nor after using the grafted-ovary method. After placental pollination, pollen tube penetration percentages were not increased compared to stigmatic pollination, however, most of the penetrated ovules with pollen tube penetration showed embryo germination.

Key words: interspecific hybridization, hormone treatments, cut-style method, grafted-ovary method, placental pollination, tulip

1. Introduction

The commercial assortment of tulips consists mainly of cultivars *T. gesneriana* and of Darwin hybrids, obtained by interspecific hybridization between *T. gesneriana* and *T. fosteriana*. The genus *Tulipa* L. comprises between 40 (Stork, 1984) and more than 100 (Hall, 1940, Botschantzeva, 1962) species, depending on the species concept used. These species represent an enormous gene pool from which traits can be used for the improvement of the present-days assortment. For example, genes for disease resistance, for a short forcing period, for new flower colours and for new flower shapes could enrich the assortment. From most interspecific tulip crosses, through the use of conventional breeding methods, seeds are not obtained or only a few seeds per ovary are formed (Van Eijk *et al.*, 1991, Van Raamsdonk *et al.*, 1995b).

Both pre-fertilization barriers and post-fertilization barriers are found in interspecific tulip crosses. Pre-fertilization barriers have been identified for crosses

between *T. gesneriana* and thirteen other tulip species (Van Creij *et al.*, 1996a). Depending on the cross, the pollen tubes stopped growing already in the stigmatic tissue or they grew to a certain extent into the ovarian cavity, penetrating between 0 - 76% of the ovules (Van Creij *et al.*, 1996a). From the cross *T. gesneriana* x *T. agenensis* (formerly name *T. oculus-solis* (Van Raamsdonk and De Vries, 1995a)), seeds have never been harvested on the plant (Van Eijk *et al.*, 1991). Although, in this cross embryos were formed, they died prematurely or showed a retarded development. The endosperm showed an aberrant development or was degenerated (Van Creij *et al.*, 1996b).

Like in many other crops, in several interspecific tulip combinations, post-fertilization barriers can be bypassed by using embryo-rescue techniques (Van Creij *et al.*, 1996c). In several crops, pre-fertilization barriers can be bypassed by using methods as the mentorpollen technique (Stettler *et al.*, 1968, Van Tuyl *et al.*, 1988), gamma-ray irradiation to pollen or to egg cells (Yamakawa, 1971, Shintaku *et al.*, 1988), a combination of bud pollination with treatment of stigmas with an artificial medium (Gradziel and Robinson, 1991), treatment of stigmas with hexane or ether (Whitecross and Willing, 1975), the cut-style method (Van Tuyl *et al.*, 1991, Wietsma *et al.*, 1994), the grafted-style method (Van Tuyl *et al.*, 1991), placental pollination (Marubashi and Nakajima, 1985, De Verna *et al.*, 1987, Zenkteler, 1990) and *in vitro* fertilization with isolated gametes (Faure *et al.*, 1994). Hormone treatments have been suggested as method to overcome both pre-fertilization barriers and post-fertilization barriers (Emsweller and Stuart, 1948, Khanna *et al.*, 1994).

The aim of this research was to examine the potentials of the cut-style method, of grafting ovaries and of placental pollination for circumventing pre-fertilization barriers and to determine the effect of hormone treatments on the number of seeds or emerged plantlets which can be produced in interspecific *Tulipa* crosses.

2. Materials and Methods

2.1 Plant material

In all crosses, *T. gesneriana* 'Christmas Marvel' was used as mother. Compatible pollinations were carried out between 'Leen van der Mark' and 'Prominence'. For interspecific crosses *T. praestans* 'Zwanenburg', *T. kaufmanniana* (CPRO-DLO number 65252-1), *T. agenensis* (75145) (formerly name *T. oculus-solis* (Van Raamsdonk and De Vries, 1995a)), *T. praecox* (83209), *T. altaica* (68596) (formerly name *T. kolpakowskiana* (Van Raamsdonk and De Vries, 1995a)) and *T. turkestanica* (70650) were used. *T. gesneriana* 'Cassini' was used as donor pistil in experiments with the grafted-ovary method. Pollinations were carried out in February-March 1992 and March 1993. For all experiments, between 7-19 flowers were used per cross. The origin of the plant material, culture conditions and pollination methods are according to Van Creij *et al.* (1996a).

2.2 Methods

Hormone treatments and the cut-style method were executed on flowers pollinated on the plant. Before the application of the grafted-ovary method and placental pollination, flowers were placed *in vitro*. For *in vitro* pollination flower-buds were collected 5-7 days before anthesis and disinfected by alcohol (96%) flaming. The petals and anthers were dissected and the remaining parts of the flower (henceforth called 'flower') were placed in test-tubes. Additional information on *in vitro* pollination is described in Van Creij *et al.* (1992), the methods for ovary-slice culture, ovule culture and verification of hybrids are presented in Van Creij *et al.* (1996c) and the microscopical techniques are according to Van Creij *et al.* (1996a).

2.3 Experiments

2.3.1 Hormone treatments:

The hormones 6-benzylamino purine (BAP) and Ó-naphthalenacetic acid (NAA) were dissolved in a mixture of lanolin (3 parts) with water (1 part) to final concentrations of 0.1% BAP (exp. I, 1992) and 1.0% BAP or 1.0% NAA (exp. II, 1993). Each mixture was applied 12 days after pollination (DAP) at the base of the ovary. In experiment II, the hormone mixtures were replaced 2 weeks later by fresh mixtures. Ovary-slice culture was applied to swollen ovaries of incongruent combinations, 8 (exp. I) or 6 (exp. II) weeks after pollination. The ovules were dissected from the ovary-slices at 9 weeks after pollination and placed individually on ovule culture medium. Two flowers per cross were used for the analysis of pollen tube growth. The results were statistically analyzed by means of the t-test.

2.3.2 Cut-style method:

For this method (CSM), the (short) style was cut just above the ovary and pollen was applied immediately on the cut surface. As control, flowers were pollinated on the stigma. In all flowers, the pollen tube growth in the pistil and pollen tube penetration into the ovules was studied.

2.3.3 Grafted-ovary method:

On the day the stigmas were receptive, the upper part (1/4) of the pistil of *T. gesneriana* 'Cassini' (henceforth called 'donor') was grafted on the lower part (3/4) of the pistil of *T. gesneriana* 'Christmas Marvel' (mother), as shown in Figure 1. The grafted pistils were placed upright in test tubes. Seven weeks after pollination, the ovules of swollen ovaries were placed on ovule culture medium. In 2 flowers per cross, the pollen tube growth was analyzed.

2.3.4 Placental pollination:

One day after the stigma was receptive, the ovaries were cut longitudinally into six sectors, each containing a placenta with a row of ovules and the ovary wall. Pollen (exp. I, 1992) or pre-germinated pollen (exp. II, 1993), showing pollen tube tips protruding from the pollen grains, was applied on the placenta (the number of pollen grains being

three times the number of ovules). After pollination, the explants were transferred to medium for ovary-slice culture. Per cross, 10 rows of ovules were analyzed on pollen tube penetration into the ovules. The explants remained 3 weeks (exp. I) or 2 days (exp. II) in the light, before being placed into the dark. Ovule culture was executed between 8 and 9 weeks after pollination. In exp. II, only the swollen ovules were placed in vitro. In exp. I, 5 flowers per combination were used for *in vitro* normal stigmatic pollination.

3. Results

In Table 1, the numbers of seeds or the numbers of germinated ovules are presented from untreated and hormone treated (at 12 DAP) ovaries. In the crosses with 'Prominence' and *T. kaufmanniana* (exp. I), the seedset of untreated ovaries and ovaries treated with 0.1% BAP was comparable. After pollination with *T. kaufmanniana*, 0.4 seeds per flower (SD=0.5) germinated from the 2.4 seeds produced per flower from untreated ovaries. From ovaries treated with 0.1% BAP, 1.5 seeds per flower (SD=1.1) germinated of the 2.5 seeds obtained per flower. In this cross, 49% of the ovules was penetrated by a pollen tube. In the cross with *T. agenensis* (exp. I), each of the 3 ovaries treated with 0.1% BAP produced 1 seed after maturation on the plant. From this cross, 2233 ovules from in total 5 flowers were placed *in vitro*, of which 6% germinated. In 13% of the ovules, a pollen tube had penetrated. In the crosses with 'Leen van der Mark' (exp. II), the pollen tube penetration percentage of the ovules varied between 54% and 70% for the individual flowers. The number of seeds did not differ significantly between the untreated ovaries, the ovaries treated with 1% NAA and the ovaries treated with 1% BAP. However, after the treatment with 1% BAP, 3 of the 7 ovaries died prematurely. When *T. praestans* was used as pollen donor (exp. II), none of the in total 3447 ovules placed in vitro germinated. The percentage pollen tube penetration varied between 6% and 8% for the individual flowers.

Table 1: The number of seeds per ovary obtained after maturation on the plant (pl) or the number of germinated ovules per ovary after embryo-rescue *in vitro* (iv) for ovaries treated 12 DAP with no hormones or with NAA or BAP of crosses between *T. gesneriana* 'Christmas Marvel' and different pollen donors of two experiments (exp.). In brackets the standard deviation is given.

Exp.	Pollen donor	pl/iv	No hormones	BAP 0.1%	BAP 1%	NAA 1%
I	Prominence	pl	196 (8)	192 (28)	--	--
	T. kaufmanniana	pl	2.4 (1.4)	2.5 (1.1)	--	--
	T. agenensis	pl	0.0	1.0 (0.0)	--	--
		iv	--	27 (6)	--	--
II	Leen vd Mark	pl	179 (45)	--	109 (75)	129 (63)
	T. praestans	iv	0.0	--	0.0	0.0

--: not done

with *T. kaufmanniana*. From both crosses, 0.6 germinated ovules per pollinated flower were obtained.

The results obtained after placental pollination are shown in Fig. 2, Table 4. For all cross combinations, at most 1.2% of the ovules showed germination. In the compatible crosses with 'Prominence' and 'Leen van der Mark' as pollen sources, both the penetration percentage (1.8%-2.3%) as the number of seeds per ovary (0.20-3.0) was lower after placental pollination, compared to stigmatic pollination on the plant and *in vitro*. In the cross with *T. kaufmanniana*, also less germinated ovules were obtained after placental pollination compared to the number of seeds harvested after stigmatic pollination on the plant. In the cross with *T. altaica*, one ovule showed germination. In the cross with *T. turkestanica*, no pollen tube penetration was found.

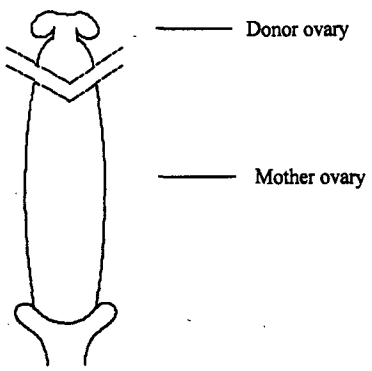

Fig. 1: The grafted-ovary method

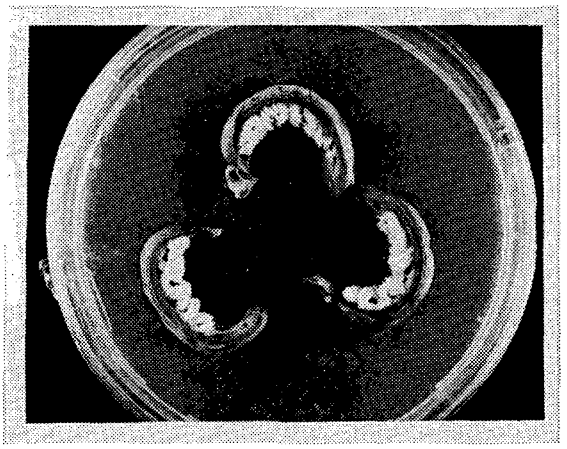

Fig. 2: Swollen ovules after placental pollination (31 DAP)

Table 3: Germination of ovules after the grafted-ovary method followed by ovule culture (about 7 weeks after pollination) in the crosses between *T. gesneriana* 'Christmas Marvel' (mother) and *T. gesneriana* 'Prominence' or *T. kaufmanniana* (T. kauf). Presented are the number of pollinated flowers (flowers), the number of ovaries used for ovule culture (ovaries), the number of cultured (ovules) and germinated ovules, the percentage of germinated ovules (%_ovules) and the number of germinated ovules per pollinated flower (%_flower).

Cross	Flowers (A)	Ovaries	Ovules (B)	Germinated (C)	%_ovules (C/B)	N_flower (C/A)
Prominence	11	3	1125	7	0.62	0.64
T. kauf.	8	1	326	5	1.53	0.63

Table 4: The percentages of ovules with pollen tube penetration (%_PE), the percentage of germinated ovules (%_ovules) and the number of germinated ovules per pollinated flower (N_flower) after placental pollination, pollination and maturation of intact flowers *in vitro* and on the plant in crosses with *T. gesneriana* 'Christmas Marvel' (mother) and several cultivars and *Tulipa* species. (flowers = number of pollinated flowers)

Exp	Cross	Methods	Flowers	% PE	% ovules	N flower
I	Prominence	placental	10	1.8	1.2	3.0
		in vitro	5	66	--	29
		plant	5	--	--	196
	T. kaufmanniana	placental	11	0.23	0.17	0.36
		in vitro	5	--	--	0.80
		plant	5	--	--	2.0
	T. turkestanica	placental	10	0.00	0.00	0.00
		plant	5	--	--	0.00
II	Leen van der Mark	placental	10	2.3	0.30	0.20
		plant	5	55	--	179
	T. altaica	placental	10	0.43	0.42	0.10
		plant	5	--	--	0.00

--: not done

4. Discussion

Hormone treatments, the cut-style method, the grafted-ovary method and placental pollination have been examined for their prospects in bypassing crossing barriers in interspecific tulip crosses.

In the cross *T. gesneriana* x *T. agenensis*, seed set after pod maturation on the plant was obtained after treatment of the ovaries at 12 DAP with 0.1% BAP. The production of seeds of this cross has never been reported before. The seed set in the compatible crosses was not affected by this hormone treatment. After *in vitro* culture (started 8 weeks after pollination) of the cross *T. gesneriana* x *T. agenensis*, 6% of the ovules showed germination, while in 13% a pollen tube had penetrated. These are better results than are described in a following article (Van Creij *et al.*, 1996c), where only 3% of the ovules germinated when cultured from 7 weeks after pollination, while 21% showed pollen tube penetration. Application of 0.1% BAP is, therefore, potentially a useful method to realize interspecific tulip crosses.

In the compatible cross, the seed set did not differ significantly between the untreated ovaries and the ovaries treated with 1% BAP or 1% NAA. Three of the 7 ovaries treated with 1% BAP died, however, prematurely. Compared to the use of 0.1% BAP, these poorer results can be caused by the higher concentration or through the

double application of the hormones (12 and 26 DAP). In *Lilium*, however, treatment of the ovaries with 1% naphthalene acetamide reduced the seed set in several crosses, while in others seeds were obtained only after hormone application (Emsweller and Stuart, 1948). In both treated and untreated ovaries of the cross with *T. praestans*, no hybrids were produced. In a another experiment (Van Creij *et al.*, 1996c), 0.2 ovules per flower showed germination in vitro of this cross (embryo-rescue started 3 and 5 weeks after pollination), after pollen tube penetration in 21% of the ovules. The poor results in the present crosses with *T. praestans*, reported here, could originate from the low penetration percentage (6-8%) and/or the later date (6 weeks after pollination) at which embryo-rescue methods were started. More research is needed before conclusions can be drawn on the effect of the treatments with 1% NAA and 1% BAP.

The cut-style method did not affect pollen tube growth nor did it have a negetative effect, in the cross with *T. praecox*,. The desired effect of bypassing barriers in the stigma or style, as found in crosses with *T. turkestanica*, has not been achieved. Apparently, pre-fertilization barriers can not be circumvented in tulip by this method, like in *Lilium* and *Fritillaria* (Van Tuyl *et al.*, 1988, Wietsma *et al.*, 1994). In these latter crops, pollen tube growth is mostly inhibited in the style in incongruent crosses (Ascher and Peloquin, 1968, Wietsma *et al.*, 1994).

After the application of the grafted-ovary method in tulip, in some of the ovaries none of the pollen tubes grew to the bottom of the donor part. This could be improved by grafting the ovaries 1 or 2 days later when more pollen tubes had entered the style and had penetrated the ovules in the upper part of the donor ovary (Van Creij *et al.*, 1996a). The passage of pollen tubes from the donor ovary into the mother ovary, was a critical point which was also observed for the grafted-style method in *Lilium* (Van Tuyl *et al.*, 1991). Like the grafted-style method used for lily, in tulip, the grafted-ovary method was applied for permitting pollen tubes to reach their normal lengths obtained after normal stigmatic pollinations before pollen tubes have to penetrate into the ovules. In contrast with *Lilium* (Janson *et al.* 1993), the pollen tube penetration percentage was not reduced after cut-style method in compatible tulip crosses. In tulip, the application of the grafted-ovary method gives, therefore, only disadvantages compared with the cut-style method.

After placental pollination, pollen tubes had penetrated not more than 2.3% of the ovules. A high percentage of penetrated ovules showed embryo germination. In the cross with *T. altaica*, one ovule showed germination, however this embryo was not verified for hybrid character. Crosses between *T. gesneriana* and *T. altaica* have never succeeded, not even after the application of embryo rescue techniques (Van Creij *et al.*, 1996c). Apparently, a high percentage of tulip embryos can be rescued by using the studied method. After placental pollination, the penetration percentages were not higher than in stigmatic pollination (Van Creij *et al.*, 1996a). Pre-fertilization barriers seem, therefore, not to be bypassed. After optimizing the pollination procedure, penetration percentages might be increased. Zenkteler (1990) observed that pollen germinated only on ovules which were not wet and Janson (1993) reported higher penetration percentages when the ovules were placed on filterpaper. In experiment II, the ovules were covered with fluid by

the use of pregerminated pollen in BK-medium. Several factors can be investigated to optimize the procedure of placental pollination, for example the age of the ovaries (Balatkova and Tupy, 1972), the culture media (Kameya and Hinata, 1970) and the type of placental pollination (Zenkteler, 1990, Slusarkiewicz-Jarzina and Zenkteler, 1983, Marubashi and Nakajima, 1985). Further research needs to be done on the transfer of seedlings into the soil, since many germinated embryos showed abnormalities, as also found by Custers *et al.* (1992).

Four methods have been examined for their ability to circumvent crossing barriers. The cut-style method and the grafted-ovary method did not give positive results in bypassing pre-fertilization barriers. Placental pollination offers prospects, however, more research is needed. Treating the ovaries with 0.1% BAP in lanolin, resulted for the first time in seed set on the plant in the cross *T. gesneriana* x *T. agenensis*. It is supposed that in this cross post-fertilization barriers must have been weakened. The use of other hormones and different concentrations, on a broader range of interspecific tulip crosses, could give a better idea on the practical use of hormone treatments for tulip breeding.

Acknowledgements

We wish to thank H.M.C. van Holsteijn and J.L. van Went for critical reading this paper, W. Eikelboom, P. Veldkamp and B. de Haas for their technical assistance and W.A. van Dijk and P. van Empel for their constant care of the plant material. Part of the work was supported by the Urgency Programme for Research on Diseases and Breeding of Flower Bulbs.

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