Interspecific hybridization in the genus *Tulipa* L.

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Interspecific hybridization in the genus *Tulipa* L.

M.G.M. van Creij

Proefschrift

ter verkrijging van de graad van doctor,
op gezag van de rector magnificus
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dr. C.M. Karssen,
in het openbaar te verdedigen
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Stellingen behorende bij het proefschrift getiteld:

Interspecific hybridization in the genus *Tulipa* L.

1. De variatie gevonden in de mate van optreden van kruisingsbarrières binnen een combinatie moet toegeschreven worden aan moeilijk controleerbare fysiologische effecten. *Dit proefschrift.*

2. Iedere embryo-reddingstechniek, toegepast op hetzelfde gewas, stelt zijn eigen eisen ten aanzien van de samenstelling van het medium. *Dit proefschrift.*

3. De efficiëntie van het verkrijgen van gekiemde embryo's van tulp kan aanzienlijk verbeterd worden door strenge selectie in het uitgangsmateriaal in combinatie met toepassing van verschillende bestuivings- en embryo-reddingstechnieken. *Dit proefschrift.*

4. Bolvorming in vitro van tulp en de uitplantbaarheid van in vitro gevormde tulpenbolletjes verdient een hoge prioriteit binnen het huidige tulpenonderzoek. *Dit proefschrift.*

5. Zolang de mechanismen die kruisingsbarrières in interspecifieke combinaties veroorzaken niet gedefinieërd zijn, is het duidelijker om één term voor deze barrières te gebruiken.

6. Alleen door tulpenonderzoek kan de tulp voor Nederland een levend, promotioneel effectief symbool blijven.

7. Het begeleiden van promovendi is in 1 aspect vergelijkbaar met het opvoeden van kinderen: om het beste contact met ze te behouden moeten ze op een gegeven moment losgelaten worden.

8. Materialisme van het individu leidt tot milieuvervuiling.

9. Voor een optimale uitvoering van human resource management zouden mannen het taalgebruik van vrouwen gedeeltelijk over moeten nemen.
   Mensink, J.C.M., 1991. Dynamiek in human resource management. Lemma b.v., Utrecht, 228 pp.
   Tannen, D., 1994. Woorden aan het werk: Hoe vrouwen en mannen op het werk met elkaar praten. Prometheus, Amsterdam, 328 pp.

10. Gezien de duur van het zwangerschapsverlof wordt de zwaarte van een zwangerschap en de post-partum periode in Nederland, ten opzichte van andere europese landen, onderschat.
    Kleiverda, G., 1990. Transition to parenthood: women's experiences of "labour". Thesis, University of Amsterdam, Amsterdam, pp 1-30.

11. Degenen die hard roepen dat ze het druk hebben, hebben tijd over.

Marjan G.M. van Creij
Dordrecht, 7 januari 1997

voor mijn ouders

Voorwoord

In juli 1990 ben ik gestart met het onderzoek getiteld "Bloembiologische technieken in soortkruisingsonderzoek bij lelie, tulp en gladiool". Dit project was één van de projecten uit het Urgentieprogramma Bollenziekte- en veredelingsonderzoek, dat voor 50% gefinancierd werd door het Nederlandse bollenbedrijfsleven via het Produktschap voor Siergewassen en voor 50% door het Ministerie van Landbouw, Natuurbeheer en Visserij.

Tijdens de sollicitatiegesprekken is mij gevraagd of ik graag wilde promoveren. Mijn antwoord daarop was: Nee. Dit bleek een goed antwoord te zijn, want het zag er niet naar uit dat een promotie op dit project mogelijk zou zijn. Nu 6½ jaar later ligt er dan toch een proefschrift over soortkruisingsonderzoek bij tulp. Dit is gelukt door de verlenging van het 3-jarige project met 9 maanden, doordat tulp toch niet zoveel problemen bleek te geven bij in vitro cultures als gedacht en door de hulp en steun van heel veel mensen. Een ieder die bijgedragen heeft aan dit onderzoek en al degenen die voor een leuke werksfeer gezorgd hebben wil ik daarvoor hartelijk danken. Enkelen van hen wil ik hierbij nogmaals noemen.

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Ook in onze naaste omgeving is het niet onopgemerkt gebleven dat ik een proefschrift aan het schrijven was. Tegen vrienden wil ik dan ook zeggen: we hebben weer tijd! (Alhoewel een nieuwe wereldburger zal ons de komende tijd weer aardig bezighouden.) Rigarda, Peter, Carlijn, Daan en Loes bedankt voor de steun de afgelopen jaren en voor het regelen van zaken de ik dreigde te vergeten. Het is fijn te zien dat Martijn zich zo goed bij jullie thuis voelt. Tegen mijn ouders wil ik zeggen: dit proefschrift is niet voor niets aan jullie opgedragen, zonder jullie steun, moreel en praktisch, had dit proefschrift er nog lang niet gelegen. En natuurlijk wil Martijn nog steeds graag met oma Mieke en opa Wim spelen! Aat, samen hebben we het gered, ...eindelijk weer vrije tijd. Tegen onze Martijn, nu al weer ruim 2 jaar oud, willen we dan ook samen zeggen: We gaan voortaan gezellig met z'n vieren op stap!

Contents

Abbreviations

BA	- 6-benzyladenine
BAP	- 6-benzylaminopurine
CH	- casein hydrolysate
CSM	- cut-style method
CM	- *Tulipa gesneriana* 'Christmas Marvel'
DAP	- days after pollination
GA	- gibberellic acid
IAA	- indoleacetic acid
LSD	- least significant difference
LvdM	- *Tulipa gesneriana* 'Leen van der Mark'
MES	- 2-morpholinoethanesulfonicacidmonohydraat
MS	- medium of Murashige and Skoog (1962)
NAA	- α-naphthalenacetic acid
Tpr	- *Tulipa praestans* 'Zwanenburg'
WAP	- weeks after pollination
2,4-D	- 2,4-dichlorophenoxyacetic acid

General introduction

1

Tulips

The tulip is a monocotyledonous plant and a member of the family of *Liliaceae*. The primary gene centre of the genus *Tulipa* L. is located in the Pamir Alai and Tien Shan mountain ranges in Central Asia (Hoog 1973). Diversification took place from this region, resulting in a distribution area from Morocco to western Europe and to western China. A secondary gene centre is found in the Caucasus.

The number of species of the genus *Tulipa* ranges from about 40 (Stork 1984) to more than 100 (Hall 1940, Botschantzeva 1962). Van Raamsdonk and De Vries (1992, 1995) have revised the genus *Tulipa* recently. They subdivided the genus in two subgenera, *Tulipa* and *Eriostemones* (Boissier) Van Raamsdonk, and described 49 species. The total number of tulip species is supposed to be about 55 (Van Raamsdonk, personal communications). The taxonomical classification of the species, as described by Van Raamsdonk and De Vries (1992, 1995), is presented in Table 1.

Tulips were introduced from Turkey into Europe. In 1594, more than 400 years ago, tulips flowered for the first time in The Netherlands. The introduced tulips have been grown and bred for a long time, which resulted in a diversity in flowering earliness, growth, vigour and flower shape. These tulips, whose original species have not been determined, are grouped together and are called *T. gesneriana* L. The present-day commercial assortment of tulips still consists mainly of cultivars from *T. gesneriana* (Fig. 1). The second group of cultivars, the Darwin hybrids, have been obtained from interspecific crosses between cultivars of *T. gesneriana* and genotypes of *T. fosteriana* Hoog ex W. Irving.

The tulip is the most important ornamental bulb crop in The Netherlands. In 1996, 9363 hectare of tulips were cultivated. The *T. gesneriana* and Darwin hybrids assortment consists of more than 1100 cultivars. The 10 most popular cultivars, however, occupy more than 35% of the area. On only 7% of the total tulip area (643 ha), other tulip species are grown, of which *T. fosteriana* (149 ha), *T. greigii* Regel (282 ha) and *T. kaufmanniana* Regel (108 ha) are the most cultivated species. Tulips are grown both for bulb production and for cut

Fig 1. *T. gesneriana* 'Prominence'

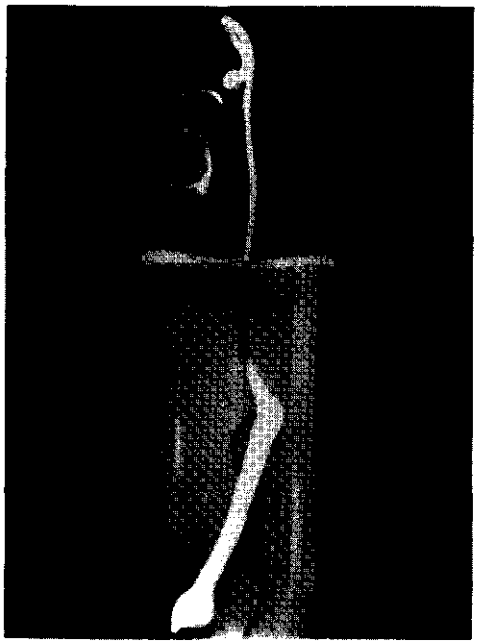


Fig 2. Seedling development of *T. gesneriana* in vitro.

flower production. In 1995, the turnover for bulb production was about 600 million Dutch guilders and for cut flower production about 274 million Dutch guilders (PVS/BKD 1996). More than 70% of the tulip flowers and flower bulbs produced in The Netherlands are exported. The Dutch flower bulb production is accountable for about 85% of the world bulb production (Le Nard and De Hertogh 1993).

Growing tulips

Tulips are vegetatively propagated. Only for breeding purposes, tulips are crossed and seeds are harvested. Seeds of tulip require a period of low temperature after sowing to induce germination and to initiate a bulb primordium (Niimi 1978). The embryo produces one cotyledonary leaf, the primary root and a hollow diverticulum called a "dropper' (Fig. 2). The bulb primordium is positioned at the tip of the dropper. The dropper grows further into the soil and at the end of the dropper a bulblet is produced (Taillandier and Riviere 1981). This small tulip bulb needs four to five years of additional growth before it has reached a

Table 1. The taxonomical classification of the species of the genus *Tulipa* in the sections (bold names) of the two subgenera, *Tulipa* and *Eriostemones*, according to Van Raamsdonk and De Vries (1992, 1995).

subgenus *Tulipa*		
Tulipa	***Eichleres*** (Hall) Van Raamsdonk	***Tulipanum*** de Reboul
T. gesneriana L.	*T. ingens* Hoog	*T. agenensis* DC.
T. armena Boiss.	*T. lanata* Regel	*T. systola* Stapf
T. hungarica Borbas	*T. tubergeniana* Hoog	*T. kuschkensis* B. Fedtschenko
T. suaveolens Roth	*T. eichleri* Regel	*T. julia* C. Koch
T. didieri Jord.	*T. fosteriana* Hoog ex W. Irving	*T. aleppensis* Boiss. ex Regel
	T. greigii Regel	*T. praecox* Tenore
	T. albertii Regel	
	T. sosnovskyi Akhverdov et Mirzojeva	
	T. praestans Hoog	
	T. kaufmanniana Regel	
	T. tschimganica Bochantzeva	
	T. dubia Vvedensky	
	T. subpraestans Vvedensky	
Kolpakowskianae (Hall) Van Raamsdonk	***Clusianae*** Baker	
T. altaica Pall. ex Sprengel	*T. clusiana* DC.	
T. lehmanniana Mercklin	*T. montana* Lindley	
T. tetraphylla Regel	*T. linifolia* Regel	
subgenus *Eriostemones* (Boissier) Van Raamsdonk		
Australes sensu Hall	***Saxatiles*** sensu Hall	***Biflores*** sensu Hall
T. australis Link	*T. humilis* Herb.	*T. turkestanica* Regel
T. primulina Baker	*T. pulchella* Fenzl.	*T. polychroma* Stapf
T. biebersteiniana Schultes	*T. saxatilis* Sieb. ex Sprengel	*T. biflora* Pallas
T. sylvestris L.	*T. bakeri* A.D. Hall	*T. sogdiana* Bunge
T. whittallii (Dykes) A.D. Hall	*T. aucheriana* Baker	*T. neustruevae* Pob.
T. ophanidea Boiss. ex Heldr.		*T. tarda Stapf*
T. hageri Heldr.		*T. dasystemon* Regel

critical minimal size for flowering. This minimal size depends on the genotype, but in general a bulb of *T. gesneriana* must have reached a circumference between 6 and 8 cm (Le Nard and De Hertogh 1993).

Tulips grown for bulb production or cut flower production are vegetatively propagated. Daughter bulbs develop from the buds which are located in the axil of the bulb scales. The average propagation rate of most tulip cultivars is between two and three bulbs each year (Le Nard and De Hertogh 1993).

The tulip bulb has an annual replacement cycle, which can be divided into three main phases (Le Nard and De Hertogh 1993):

(1) Mother-bulbs are planted in autumn, when the soil temperature decreases. The roots of the mother-bulb grow rapidly until November-December. The already differentiated shoot elongates slowly and the daughter-bulbs show a slight growth. The scales of the mother-bulbs senescence slowly.

(2) During early spring, when the temperature increases, plant growth becomes very active. Rapid shoot and floral bud elongation leads to flowering. Flowering tulips form two or more leaves. The growth rate of the daughter-bulbs increases and is maximal after flowering. The mother-bulb scales shrivel and progressively disappear.

(3) At the end of the spring, the aerial organs (stem, leaves and flower(s)) of the mother-bulbs senescence and the growth of the daughter-bulbs ceases. The daughter-bulbs are in an apparent state of dormancy. However, during this period, an active differentiation of floral and vegetative buds takes place and root primordia are formed. All these organs are present in the daughter bulbs by the end of the summer.

An important factor affecting the growth and development of tulip is temperature. For flower initiation (phase 3), a relatively high temperature is needed (17-20 °C or higher). Thereafter (phase 1), a cold period is required (2-9 °C). Physiological changes occur at this low temperature needed for root growth and for the preparation of the shoot for adequate floral stalk elongation and flower development at the subsequent higher temperatures (14-20 °C) (phase 2). For the commercial flower production, flowering is often programmed by simulating the temperature conditions required in nature (forcing). The cold period can be given partially by storing the bulbs in cooled ventilated chambers, prior to planting the bulbs. The optimal length of the total cold treatment varies with the genotype. An overview concerning the physiology of tulips has been published by Le Nard and De Hertogh (1993).

Improvement of tulips

The quality of flower bulbs and cut flowers of tulip is affected greatly by environmental factors, such as temperature and light (Le Nard and De Hertogh 1993). The production of cut flowers and flower bulbs of an optimal quality is seriously threatened by many pathogens. The most important pathogens in The Netherlands are the fungi *Fusarium oxysporum* (bulb-rot) and *Botrytis tulipae* and Tulip Breaking Virus (TBV). Also other fungi (*Pythium* spp., *Rhizoctonia tuliparum/solani*), viruses (Tobacco Necrosis Virus (TNV) and Tobacco Rattle Virus (TRV)), mites and nematodes (*Trichodoridae*, *Pratylenchus penetrans* and *Ditylenchus dipsaci*) can cause great losses. Resistance against Fusarium and TBV is present in the cultivar assortment. High levels of resistance for Fusarium are found in *T. gesneriana* (Van Eijk et al. 1985). This resistance is not absolute. All cultivars of *T. gesneriana* are susceptible for TBV, while some cultivars of *T. fosteriana* show high levels of resistance for this virus. Introduction of genes for resistance could considerably improve the present-day tulip assortment, resulting in the use of less chemicals needed for disease control. Beside the introduction of resistances in the assortment, a shorter cold requirement and forcing period, an improved flower longevity and new flower shapes and flower colours are important targets for tulip breeding.

The fast introduction of new cultivars enriched with desirable traits for the present-day tulip production is hampered by several factors. The main hindrance for a fast introduction is the long period needed for the development of a new cultivar. After crossing, 5 to 6 years are needed to obtain a flowering bulb. Subsequently, it takes another 10 to 20 years to screen the tulips on desirable characters and to propagate the bulbs for commercial release. The production of large numbers of bulbs needed for the introduction of a new cultivar could be fastened when the multiplication rate could be increased. Despite many efforts to develop a fast multiplication system in vitro (Baker et al. 1990, Hulscher et al. 1992, Hulscher and Krijgsheld 1995, Chanteloube et al. 1995), the production of tulip bulbs still occurs almost exclusively by propagation in the soil.

The most applicable method for the improvement of the cultivar assortment of tulip is the exploitation of the genetic variation of other tulip species through interspecific hybridization. *T. gesneriana* has been crossed successfully with only 12 out of the approximately 55 tulip species by using conventional breeding methods (Van Eijk et al. 1991, Van Raamsdonk et al. 1995). *T. gesneriana* proved to be compatible with other species of the same section. Crosses between *T. gesneriana* and representatives of the section *Eichleres* produced hybrids in several cases (Fig. 3). Crosses between *T. gesneriana* and species of the other sections produced no verified hybrids, except for *T. systola* Stapf (former name *T. stapfii* Turrill (Van Raamsdonk and De Vries 1995)) from the section *Tulipanum*. Crosses between the two

subgenera have never been successful (Van Eijk et al. 1991; Van Raamsdonk et al. 1995). Beside cultivars of *T. gesneriana*, the assortment consists of the Darwin hybrids (crosses between *T. gesneriana* and *T. fosteriana*), which are mostly triploid. The use of these triploid cultivars for further breeding is impossible due to F1-sterility.

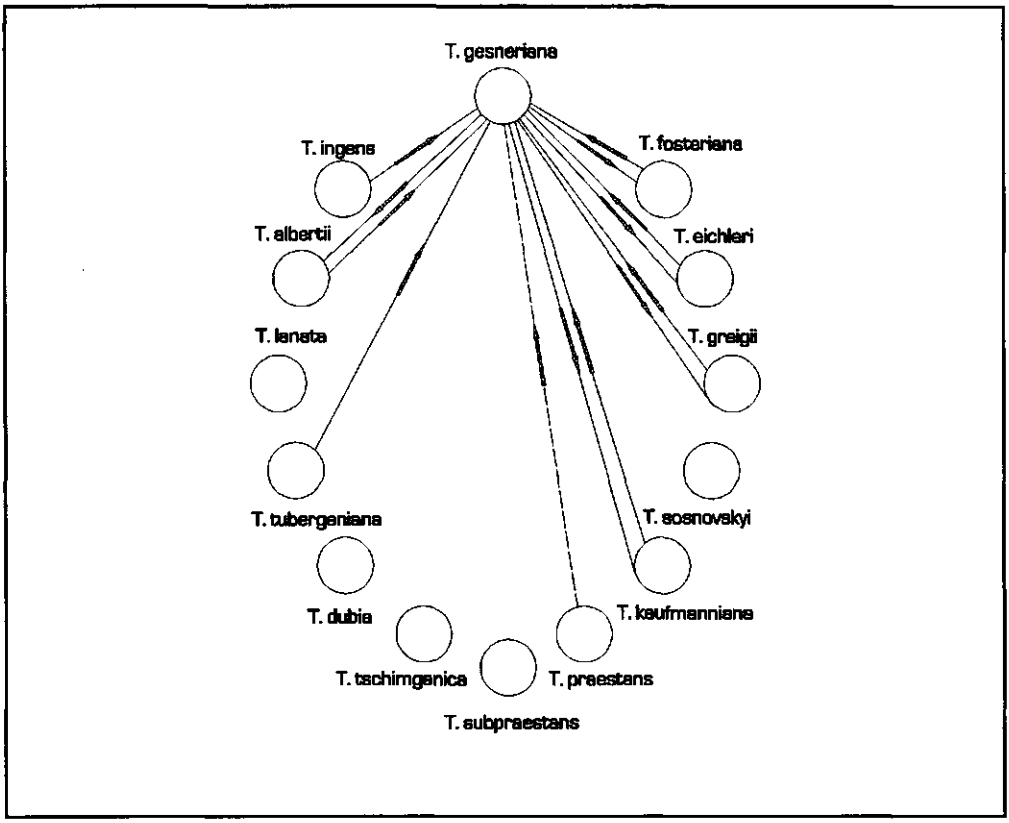


Fig. 3. Crossing polygon of crosses between *T. gesneriana* from the section *Tulipa* and species from the section *Eichleres*. The direction of the arrow indicates pollen flow, absence of lines indicate the cross has not been made, dotted lines indicate an unsuccessful cross, normal lines illustrate a successful cross.

Crossing barriers

Two types of crossing barriers can impede sexual reproduction in flowering plants after pollen deposition on the stigma: incompatibility and incongruity. Incompatibility originates of the activity of S-alleles resulting in a disturbed progamic phase. Incompatibility operates in intraspecific crosses. Incongruity occurs in interspecific crosses and is caused by a lack

of genetic information of one partner about the other (Hogenboom 1973). During all processes from pollen germination to seed maturation and plant formation, incongruity barriers can prevent interspecific hybridization in whole or in part. This introduction focuses on incongruity in interspecific crosses. Reviews concerning sexual reproduction and crossing barriers were written by Shivanna (1982) and Liedl and Anderson (1993).

Pre-fertilization barriers

Different types of incongruity barriers have been found to operate prior to fertilization, e.g. failure of pollen to germinate, arrest of pollen tube growth in the stigma or style, failure of pollen tubes to penetrate the ovules and in-ovular pollen tube growth arrest. Absence of pollen germination (Knox et al. 1972, Busmann-Loock et al. 1992) or inhibition of stigmatic penetration (Sitch and Romero 1990, Lelivelt 1993, De Jeu and Jacobsen 1995) have been observed in several interspecific crosses. In interspecific crosses of numerous crops, pollen tube growth is inhibited in the pistil, for example in *Nicotiana* (Kuboyama et al. 1994), *Oryza* (Sitch and Romero 1990), *Lycopersicon* (Chen and Adachi 1992), *Capsicum* (Zijlstra et al. 1991, Bermawie and Pickersgill 1992), *Aphelandra* (Henny 1978), *Fagopyrum* (Hirose et al. 1994), *Prunus* (Perez and Moore 1985), *Salix* (Mosseler 1989), *Alstroemeria* (De Jeu and Jacobsen 1995) and *Lilium* (Ascher and Peloquin 1968). Pollen tubes often showed abnormalities such as irregular callose deposition, branching and swollen tips. Once pollen tubes have entered the ovary, pollen tubes might fail to penetrate the ovules (Chichiricco and Grilli Caiola 1986, Zijlstra et al. 1991, Chichiricco 1996).

Fertilization barriers

Fertilization can be blocked after pollen tube penetration into the ovule. The failure of the pollen tube to discharge in the synergid region, with subsequent pollen tube overgrowth into the central cell, was observed in crosses between *Solanum melongena* L. with *S. sisymbriifolium* Lam. and between *Lycopersicon esculentum* Mill. with *L. peruvianum* Mill. (Gradziel et al. 1993). In-ovular pollen tube arrest has been reported by Williams et al. (1986) for interspecific crosses between *Rhododendron* species. In hexaploid wheat *Triticum aestivum* L. (2n=42) x diploid *Zea mays* L. (2n=20) crosses, pollen tubes penetrated in about 80% of the ovules. Sperm nuclei were found in the embryo sac of only 28% of the ovules. The egg cell and central cell were both fertilized in 4% of the ovules, whereas only the egg cell was fertilized in 18% and only the central cell in 6% of the ovules (Laurie and Bennett 1990). The failure of gametes to fuse is suggested to be the barrier preventing embryo development after bud pollination of *Fagopyrum tataricum* (L.) Gaertn. with pollen from *F. esculentum* Moench. (both 2n=16) (Samimy 1991). Failure of gametes to fuse occurred also in intergeneric crosses of *Saccharum officinarum* L. with *Zea mays* (Vijendra

Das 1970).

Post-fertilization barriers

Crossing barriers can also hinder the production of hybrids after successful fertilization. Malfunctioning of the embryo and/or endosperm and/or maternal tissue can hinder the formation of functional seeds. Embryo abortion is often observed in interspecific crosses. In the cross *Saccharum officinarum* x *Zea mays* (Vijendra Das 1970), only few divisions of the zygote and endosperm occurred before degeneration. Embryo abortion was preceded by the absence or the retarded or abnormal development of the endosperm in interspecific crosses between species of crops such as *Brassica* (Meng and Liu 1993), *Hordeum* (Thörn 1993), *Lycopersicon* (Barbano and Topoleski 1984), *Pisum* (Conicella and Errico 1993), *Trifolium* (White and Williams 1976, Przywara et al. 1989), *Cyclamen* (Ishizaka and Uematsu 1992), *Nicotiana* (Weaver 1958), *Allium* (Dolezel et al. 1980, Nomura and Oosawa 1990) and *Lilium* (Dowrick and Brandram 1970). Choudhurry (1959) observed the collapse of cells of the internal zone of the inner integument prior to endosperm degeneration in interspecific *Lycopersicon* crosses. The endothelium proliferated after degeneration of the endosperm in crosses between *Lycopersicon esculentum* and members of the '*peruvianum*-complex' (Chen and Adachi 1992). However, in the cross *Phaseolus vulgaris* L. x *P. coccineus* L., the endosperm did not degenerate, but a thick layer of endosperm tissue was formed which surrounded the underdeveloped embryos. Such a thick layer of endosperm was not observed in normal seeds. So-called 'shrunken' seeds were obtained in this cross, containing embryos with malformed cotyledons and compressed embryonic axis (Shii et al. 1982). In the cross *Gossypium hirsutum* L. x *G. arboreum* L., normal endosperm development was found in embryo-less seeds, whereas the endosperm aborted in ovules with embryo (Weaver 1957). Abnormalities in (pro-)embryo formation were found in other crops. Endosperm degeneration was preceeded by degeneration of the suspensor in interspecific *Lupinus* crosses (Busmann-Loock et al. 1992). Beside endosperm degeneration, Lancaster Johansen and Smith (1956) observed in interspecific *Arachis* crosses that the suspensor and the embryo were not fully delimited and that organs of the embryo did not differentiate.

Barriers occurring after the obtention of hybrid embryos can still hamper breeding. Hybrids can die before they reach maturity (Shintaku et al. 1985), which can be caused by chlorosis (Plourde et al. 1990) or hybrid albinism (Przywara et al. 1989, Yao et al. 1995). Hybrids which reach maturity may not flower (Singh and Singh 1989) or the pistils and/or anthers might be greatly reduced (Phillips et al. 1982, Gradziel and Robinson 1989). A reduced fertility or complete sterility of F1-hybrids has been found in interspecific hybrids of numerous crops. Even when F1-hybrids produce functional gametes, crossing barriers can still occur during backcrossing of these hybrids (Asano 1980a, Kuboyama et al. 1994, Filler

et al. 1994).

Techniques to bypass incongruity barriers

A wide range of techniques has been developed to bypass crossing barriers. Manipulation of the fertilization process is rather difficult. Most techniques focus, therefore, on bypassing crossing barriers prior to fertilization or post-fertilization.

Bypassing pre-fertilization barriers

Bud-pollination: Bud-pollination of flowers of *Petunia parodii* W.C.S. with pollen of *P. inflata* Fries resulted in hybrid seed set, whereas pollen tubes stopped growing in the style after normal pollination (Sink et al. 1978).

Artificial medium on stigmas: This method comprises the treatment of the stigmas with an artificial medium analogous to stigmatic exudate. The combination of this method with bud pollination allowed pollen germination and growth in the cross *Lycopersicon peruvianum* x *L. esculentum* (Gradziel and Robinson 1991).

Hormone treatment: Spraying 75 mg/l of gibberellic acid (GA_3) on *Hordeum vulgare* L. pistils at 5 or 90 minutes after pollination with pollen of *Triticum aestivum*, followed by a second treatment at 24 hours after pollination resulted in an improved pollen tube growth. The increase of pollen tube length reached 120 minutes after pollination was correlated with an increase in seed set (Khanna et al. 1994). In the cross *Triticum aestivum* x *Zea mays*, a treatment with 2,4-D at 1 day after pollination significantly increased pollen tube arrival at the micropyle and the rate of successful egg cell and/or central cell fertilization (Wedzony and Van Lammeren 1996). The treatment of pistils of *Hordeum vulgare* pollinated with *Secale cereale* L. with gibberellic acid or the auxin indoleacetic acid (IAA) promoted pollen tube growth. However, although an increase in frequency and degree of ovary development was observed, embryos did not grow past the pro-embryo stage (Larter and Chaubey 1965). In several other crops, the hormone treatments applied were not effective for bypassing pre-fertilization barriers. Application of 75 mg/l GA_3 at 10 minutes after pollination did not affect pollen tube growth in interspecific *Oryza* crosses (Sitch and Romero 1990), nor did the application of a spray of 0.01 or 0.1% GA_3 or 0.001-0.1% α-naphtylacetate at several times after pollination influence pollen tube growth in interspecific *Populus* crosses (Stettler et al. 1980). Treatments with auxins, the cytokinin 6-benzylaminopurine or gibberellic acid at various concentrations did not accelerate pollen tube growth in the cross *Lilium longiflorum* Thunb. x *L. elegans* Thunb. (Asano 1981).

Hexane treatment: The treatment in which the stigmatic surface was carefully wiped of with

a brush moistened with hexane before pollination resulted in the production of high numbers of hybrids of the crosses (*Populus deltoides* Bartr. x *P. fremontii* S. Watson) x *P. alba* L. and *P. deltoides* x *P. tremuloides* Michx. (Whitecross and Willing 1975). However, hybrids of the cross *Populus tremuloides* x *P. trichocarpa* T. were not obtained by using this method (Stettler et al. 1980). According to the authors, this might be due to having applied the treatment too lightly.

Irradiation: Irradiation of the pollen or pistils prior to pollination in vitro, followed by ovule culture resulted in the production of viable hybrid plants of the cross *Nicotiana repanda* Willd. x *N. tabacum* L.. Hybrid seedlings were also obtained in vitro without the application of irradiation. However, these latter seedlings developed chlorosis and died (Shintaku et al. 1988).

Pioneer pollen and mentor pollen: These methods are based on the use of compatible pollen which is genetically inactivated by irradiation, but still capable of pollen tube growth. This pollen is used for pollination prior to the pollination with incongruous pollen (pioneer pollen) or mixed with incongruous pollen (mentor pollen) and pollinations are carried out with this pollen mixture. Hybrids were obtained from interspecific *Populus* crosses after the application of the pioneer pollen technique. However, less hybrids were produced than after the hexane treatment (mentioned above) (Whitecross and Willing 1975). Stettler (1968) reported the production of unique hybrids of the crosses *Populus trichocarpa* x *P. grandidentata* Michx. and *P. trichocarpa* x *P. tremuloides* by using the mentor pollen technique. However, hybrids were not obtained from the latter reciprocal cross after application of this technique (Stettler et al. 1980). The mentor pollen technique was found not to be effective in interspecific *Cucumis* crosses (Den Nijs and Oost 1980).

Cut-style method: Pollen tube inhibition in the pistil can be bypassed by cutting the style above the ovary and subsequent pollination at the cut surface of the remaining portion of the style. Following this method, hybrids have been obtained of interspecific crosses of *Solanum* (Swaminathan 1955), *Nicotiana* (Swaminathan and Rabhakrishna Murty 1957), *Lilium* (Asano and Myodo 1977, Van Tuyl et al. 1991, Van Creij et al. 1993, Okazaki et al. 1995) and the cross *Fritillaria imperialis* L. x *F. raddeana* Sweet (Wietsma et al. 1994). Style-cuttings might give intrinsic problems for normal pollen tube growth, resulting in a lower seed set in comparison with stigmatic pollination, as is found for compatible intraspecific *Lilium longiflorum* 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).

Grafted-style method: Pollen is deposited on the stigma of a compatible donor flower. One day after pollination, the style of the donor flower is cut several millimetres above the ovary and grafted on the ovary of the selected incongruent flower. The advantage of this method above the cut-style method is the opportunity it offers for pollen tubes to attain their normal

lengths before they reach the ovules. Hybrids of interspecific *Lilium* crosses have been obtained successfully by using this method (Van Tuyl et al. 1991).

Placental pollination and pollination of isolated ovules: Pollen can be applied directly to ovules attached to the placenta (placental pollination) or to isolated ovules to bypass crossing barriers in the pistil. Placental pollination can be executed in various ways: intra-ovarian pollination, pollination after removal of the ovary wall and pollination of ovules attached to the placenta. Immature embryos have been obtained from interspecific crosses in crops of various families such as *Caryophyllaceae*, *Cruciferea*, *Graminae*, *Solanaceae* and *Liliaceae* by using these methods. Results of these crosses are presented in reviews on in vitro pollination by Bhojwani and Razdan (1983) and Zenkteler (1990a, 1992). In the following part, experiments with artificial pollination will be presented which resulted in the production of viable hybrid embryos.

Placental pollination:

* Intra-ovarian pollination: This method comprises the injection of a pollen suspension into the intact ovary or the pollination of ovules through an opening made in the ovary wall. By using this method, hybrids have been obtained from crosses between *Argemone mexicana* L. and *A. ochroleuca* Sweet (Kanta and Maheshwari 1963) and of the cross *Brassica napus* L. x *B. campestris* L. (Zenkteler et al. 1987). Globular embryos have been produced in this way from several intergeneric crosses within the *Brassicaceae* (Zenkteler 1990b).
* ovary wall peeled off: Pollination of exposed ovules after removal of the pistil and ovary wall resulted in the production of hybrids from the cross *Nicotiana tabacum* x *N. knightiana* Goodsp. (Slusarkiewicz-Jarzina and Zenkteler 1983) and from the cross *Petunia parodii* x *P. inflata* (Sink et al. 1978).
* ovules attached to the placenta: In vitro pollination of ovules attached to dissected placenta was effective in obtaining hybrids of interspecific crosses of *Nicotiana* (Marubashi and Nakajima 1985, DeVerna et al. 1987) of an interspecific cross of *Melandrium* and of the intergeneric cross *Melandrium album* Mill. x *Silene schafta* L. (Zenkteler 1967).

Pollination of isolated ovules: Hybrids have been produced by pollinating isolated ovules, which were placed on a culture medium, of interspecific *Gossypium* crosses (Stewart 1981) and of an interspecific *Brassica* cross (Kameya and Hinata 1970).

Bypassing fertilization barriers

In vitro fusion of isolated gametes: Isolated single sperm and egg cells can be fused through electrofusion or chemofusion. Plants could be regenerated after fusion of *Zea mays* gametes (Kranz and Lörz 1993, Faure et al. 1994). This method offers great potentials for obtaining hybrids between different species or genera.

Bypassing post-fertilization barriers

Second pollination: Hybrids were obtained from the cross *Solanum tuberosum* Linn. x *S. acaule* Bitter by combining a second compatible pollination with embryo rescue techniques. Pollination of *S. tuberosum* with pollen of *S. acaule* was followed by a second pollination with compatible pollen. The double pollinations promoted fruit development, because more embryos were formed, thus preventing premature fruit dropping. Hybrid ovules/seeds could be recognized by the absence of a dominant embryo-spot marker and these ovules or only the embryos of these seeds were cultured (Iwanaga et al. 1991).

Amino acids: The injection of 1.0 mg/ml of either ϵ-amino-*n*-caproic acid or L-lysine-HCl improved the number of seeds which contained normal embryos from the cross *Triticum turgidum* L. x *Secale cereale* and x *S. montanum* L, as observed 15 days after pollination. At this day, embryo culture was started (Taira and Larter 1977).

Hormone treatments: Treatment of ovaries of *Lilium* with 1% naphthalene acetamide reduced the seed set in several interspecific crosses, while in others seeds were only obtained after hormone application (Emsweller and Stuart 1948). In an interspecific *Anthurium* cross, berry abortion was prevented and seed development strongly promoted by dipping the entire spadix during 10 minutes in a solution of 6-benzyladenine (BA) 6 and 12 weeks after pollination (Geier 1990). Oettler (1983) and Comeau et al. (1992) used post-pollination gibberellic acid (GA) sprays to enhance embryo development and seed setting. Gosal and Bajaj (1983) used a mixture of GA with naphthalenacetic acid (NAA) and kinetin, and Williams et al. (1987) smeared 1% NAA on the pedicel and calyces of pollinated flowers for the same purpose. Hybrids were obtained from interspecific *Zinnia* crosses after treating the ovaries with indoleacetic acid (IAA), whereas no hybrid embryos developed without IAA treatment (Shahin et al. 1971). Only after a treatment with GA_3, interspecific hybrids were produced in the genus *Lens* (Ahmad et al. 1995). Hybrid plant inviability of crosses between *Nicotiana tabacum* and *N. repanda* was overcome by potting hybrid plants with several leaves in vermiculite and supplying these plants with ¼MS containing 2 mg/l IAA (Zhou et al. 1991).

Embryo rescue techniques: Embryos often abort after interspecific crosses (see crossing barriers). Various methods have been developed to enable these hybrid embryos to survive in vitro: ovary culture, ovule culture and embryo culture. The use of these techniques has been reviewed by several authors (Raghavan and Srivastava 1982, Rangan 1982, 1984, Collins and Grosser 1984, Williams 1987, Williams et al. 1987, Sharma et al. 1996). In *Lilium*, a fourth technique, ovary-slice culture, has been applied successfully for the production of interspecific hybrids (Kanoh et al. 1988, Van Tuyl et al. 1991). For this method, ovaries are cut transversely into 2 mm thick discs and placed on medium. Raquin et al. (1993) halved the ovaries prior to culturing and obtained interspecific *Populus* hybrids. By using these techniques, interspecific and intergeneric hybrids have been produced of

numerous crops. Hybrid production published in literature till 1987 has been summarized by Williams et al. (1987). In Table 2, hybrids produced after 1987 are presented.

Embryos could be rescued in *Lilium* (Asano 1980b) at an earlier developmental stage in comparison with normal embryo culture, through placing the embryo on nurse endosperm. This method was also used for the production of interspecific hybrids in crops such as *Glycine* (Broué et al. 1982), *Trifolium*, *Lotus* and *Ornithopus* (Williams and De Lautour 1980). Macerated nurse endosperm had a beneficial effect on embryo growth in the cross *Triticum durum* L. x *Secale cereale* (Bajaj 1980). Nurse endosperm is derived from a compatible cross. For ovule culture, a positive effect of culturing ovules attached to the placenta as compared to culture of isolated ovules has been reported by Munoz and Lyrene (1985) and Lagriffol and Monnier (1985). A higher percentage of developing embryos was obtained in *Ipomoea* when the ovules were sliced prior to culture (Kobayashi et al. 1993). Sliced ovules are mostly cultured on liquid media. This improves the nutrient exchange from the culture medium to the developing embryo (Mathias et al. 1990, Buitendijk et al. 1995).

The different embryo rescue techniques are often combined e.g. ovule culture has been followed by embryo culture (Ladizinsky et al. 1985, Ohsumi et al. 1993, Kishi et al. 1994) or ovary(-slice) culture has been executed prior to ovule culture (Mathias and Boyd 1988, Van Tuyl et al. 1991) or embryo culture (Raquin et al. 1993). Hybrids have been obtained in several crops after regeneration of callus formed on cultured embryos. In interspecific *Vigna* crosses, hybrid embryos did not germinate. However, hybrid plants could be regenerated from callus formed on the embryos (Chen et al. 1989). Wojcieschowska and Pudelska (1992) obtained intergeneric hybrids from the cross *Hordeum vulgare* x *Secale cereale* after embryo callus culture. In the cross *Moricandia arvensis* (L.) DC. x *Brassica oleracea* L. embryos failed to develop into plantlets. However, hypocotyls could be cut and cultured to induce regeneration (Takahata 1990).

Chromosome doubling by colchicine or oryzalin treatment: Sterility of F1-hybrids can be caused by the lack of chromosome pairing during meiosis. The fertility of these hybrids might be restored by chromosome doubling. Colchicine was used successfully for chromosome doubling in crops such as *Actinidia* (Harvey et al. 1995), *Nicotiana* (Marubashi and Nakajima 1985) and *Lilium* (Van Tuyl 1989). In the latter two crops, it was reported that the fertility of the F1-hybrids was restored. As an alternative for colchicine, oryzalin, a herbicide with anti-mitotic activity, was used successfully for polyploidization in *Lilium* and *Nerine* (Van Tuyl et al. 1992a).

Integrated system of techniques for bypassing incongruity barriers

Various techniques have in most cases to be applied for the production of viable hybrid plants of a specific cross. If pre-fertilization barriers hinder interspecific or intergeneric hybridization, these barriers must first be bypassed. However, once pre-fertilization barriers are bypassed, often embryo rescue techniques must be used to save the hybrid embryos from a premature death. Finally, sterility of the F1-hybrids must often be overcome.

In vitro pollination offers the prospects to perform an integrated system of pollination, fertilization and embryo rescue techniques under optimal environmental conditions. In vitro stigmatic pollination proved to be successful for the production of seeds from *Nicotiana rustica* L. (Rao 1965), *Petunia violacea* Lindl. (Shivanna 1965), *Antirrhinum majus* L. (Usha 1965), *Trifolium* spp. (Leduc et al. 1990), *Zea mays* (Gengenbach 1984, Higgins and Petolino 1988), *Brassica napus* (Lardon et al. 1993), *Lilium* spp. (Van Tuyl et al. 1991) and *Nerine bowdenii* DC. (Van Tuyl et al. 1992b). Thus far, in vitro stigmatic pollination itself has not been reported to be effective in bypassing incongruity barriers. However, instead of normal stigmatic pollination, also other pollination methods can be used in vitro, such as cut-style pollination and intra-ovarian pollination. After in vitro pollination, the plant material needs no sterilization in case embryo rescue techniques are to be used. This is especially important when (a part of) the ovary wall has been removed before pollination. Once an in vitro pollination procedure has been developed, it can also be applied for the post-fertilization culture of whole ovaries. Such a combination of in vitro pollination with embryo rescue techniques has resulted in the production of interspecific hybrids in crops such as *Gossypium* (Refaat et al. 1984) and *Lilium* (Van Tuyl et al. 1991).

Factors affecting the rate of success in bypassing crossing barriers

Information concerning the developmental stage at which a crossing barrier(s) occur in a specific cross is essential to select the method(s) needed for bypassing this (these) barrier(s). The efficiency, however, of hybrid production of a specific cross between two species is also determined by many other factors.

Beside reciprocal differences in the crossing barriers found in crosses between two species, the genotypes of the species can influence the number of hybrids produced. Effects of the female parent (Espinasse 1985, Mujeeb-Kazi et al. 1986, Imanishi 1988, Chung and Kim 1990, Chen and Imanishi 1991, Kapila and Sethi 1993, Mont et al. 1993), the male parent (Takahata et al. 1993) or both parents (Oettler 1984) on the number of hybrids obtained after embryo rescue have been reported.

Environmental conditions, such as light and temperature, can influence the progamic

phase, fertilization and embryogenesis. Liu et al. (1992) found an optimum relative humidity and an optimum temperature range for pollen tube growth in vitro, for in vitro fertilization and ovule growth in the cross *Gossypium hirsutum* x *G. arboreum*. Dupuis and Dumas (1990) observed after in vitro pollination of *Zea mays*, mature pollen to be much more sensitive to temperature stress (both low (4 °C) and high (40 °C) temperatures) than the female gametophytes. Optimum temperatures were found for kernel growth in vitro in *Zea mays* (Jones et al. 1981), for the growth of isolated embryos in vitro of *Hordeum vulgare* (Dunwell 1981) and *Glycine max* (L.) Merr. (Lippmann and Lippmann 1993) and for the development of embryos grown in ovules of *Prunus* spp. (Ramming 1985). However, Obendorf et al. (1983) reported that the temperature during ovary culture of *Glycine max* did not affect the mature weight of seeds nor the number of seeds reaching maturity. They did observe a requirement for light during ovary culture for seed growth, maturation and germinability. A positive effect of light was reported on the accumulation of dry matter in grains cultured in detached ears of *Triticum aestivum* (Singh and Jenner 1983) and on the growth of early heart-shaped embryos of *Capsella bursa-pastoris* Medic. (Raghavan and Torrey 1963) and of *Glycine max* embryos (Raper et al. 1984). Dolezel et al. (1980) reported the influence of light on the growth of embryos of *Allium cepa* L. in vitro to depend on the developmental stage of the embryos. Balatková and Tupý (1972) did not find any differences between cultures in dark or in light of ovules cultured on excised placentae of *Nicotiana tabacum*.

The age of the explant at the start of the culture affect the rate of success after in vitro pollination and/or embryo rescue. The age of the flowers used for in vitro pollination influenced the seed set in *Lilium* (Van Tuyl et al. 1991). The developmental stage at which embryos are placed in vitro often affects the percentage of embryos which can be recovered. In many crops, the embryos must have a minimum age before they can be rescued (Mukherjee et al. 1991, Kishi et al. 1994). In *Helianthus*, this minimum age was found to be depending on the medium used (Espinasse et al. 1985). The percentages of recovered embryos increased in many crops with increasing embryo age, for example in *Ipomoea* spp. (Kobayashi et al. 1993), *Petunia hybrida* Vilm. (Wakizuka and Nakajima 1975), *Pelargonium X hortorum* Bailey (Scemama and Raquin 1990), *Lupinus* spp. (Kasten and Kunert 1991) and *Ornithogalum dubium* Houtt. (Niederwieser et al. 1990). Custers and Bergervoet (1990) observed, however, a higher frequency of plant formation for early heart-stage embryos of *Cucumis* spp. in comparison with more advanced stages. Interspecific crosses which are hampered by post-fertilization barriers often show an optimum stage for embryo recovery (Iwai et al. 1985, Kapila and Sethi 1993). The number of embryos which can survive in vitro often increases with the developmental stage of the embryo, while the number of viable embryos of interspecific crosses often decreases in time.

The type of embryo rescue technique applied also affects the number of embryos which can be recovered at a certain developmental stage. Ovule culture permitted the recovery of younger embryos in *Capsella* (Monnier and Lagriffol 1985), *Helianthus* (Espinasse et al. 1991), *Allium* (Nomura and Oosawa 1990) and *Trifolium* (Przywara et al. 1989) as compared to embryo culture. A significantly higher germination percentage was obtained after ovule culture than after embryo culture in *Brassica* (Diederichsen and Sacristan 1994). In *Lilium* (Hayashi et al. 1986, Van Tuyl et al. 1991), embryos could be rescued from an earlier stage using ovary-slice culture as compared to embryo culture. Younger embryos could be rescued after the application of ovary culture than after the use of ovule culture in *Pisum sativum* L. (Srivastava et al. 1980) and in *Vicia* species (Lazaridou et al. 1993). McCoy and Smith (1986), however, only obtained hybrids from interspecific *Medicago* crosses after the application of ovule culture followed by embryo culture. Embryo culture or ovule culture applied separately appeared to be unsuccessful. For in vitro pollination, an 'open in vitro system' was used in several crops, instead of the culture of the whole pistil in vitro. This method implies the insertion of the peduncle through an incision in a membrane in the medium (Donovan and Lee 1977, Barratt 1986). Seed set improved in several crops by using this 'open in vitro system' (Varga et al. 1988, Lardon et al. 1993).

The presence of accessory flower organs to cultured explants improved the seed set in several cases. Seed set after in vitro pollination was improved when accessory flower organs remained attached to the pistils in crops such as *Zea mays* (Higgins and Petolino 1988) and *Trifolium* spp. (Richards and Rupert 1980, Leduc et al. 1992). In *Triticum aestivum*, embryos were obtained from cultures started at the day of fertilization when the ovaries were cultured as complex explants. Only a few embryos could be recovered in cultures started 3 days post-anthesis if the ovaries with palea were grown, whereas detached ovaries could even not be cultured successfully when collected at 7 days post-anthesis (Mathias and Boyd 1988). In ovary culture of *Allium cepa*, the growth of excised ovaries was markedly retarded and the seeds were abortive, whereas fruits with viable seeds developed when the perianth was left intact (Guha and Johri 1966).

The media used for in vitro pollination influence the efficiency with which viable seeds or embryos can be obtained (Richards and Rupert 1980, Gengenbach 1984, Trione and Stockwell 1989, Van Tuyl et al. 1991, Lardon et al. 1993). The media required for embryo rescue also affect the number of embryos recovered. Media described for embryo rescue of a range of crops differ in composition, as can be seen in Table 2. Media used for embryo rescue are well summarized in the different reviews (mentioned above) and this subject will therefore only be briefly discussed here. The media used for embryo rescue usually contain a mixture of macronutrients and micronutrients (see for composition Williams et al. 1987) and sucrose. Sucrose functions as energy source, but can also be needed for the

establishment of the osmolarity. A vitamin mixture, hormones and agar are often added. Other components like amino acids, individually adjusted or in the form of casein hydrolysate, activated charcoal and picloram have been used in media for embryo rescue. Also, other complex components are added to media, for example coconut milk, cucumber juice (Przywara et al. 1989), juice of immature white clover seeds (Yamada and Fukuoka 1986), potato extract (Sharma et al. 1995), extracts of cotton ovules (Joshi and Johri 1972) and yeast extracts (Inomata 1977).

Research on crossing barriers in tulip

Little tulip research has focused on identifying and/or bypassing crossing barriers, although only 12 out of the approximately 55 tulip species can be crossed with *T. gesneriana* by using conventional breeding methods (see 'improvement of tulips' and Fig. 3). Kho and Baër (1971) analyzed pollen tube growth in interspecific tulip crosses between cultivars of *T. gesneriana* (mother) and species of the section *Eichleres*: *T. fosteriana*, *T. greigii* and a hybrid of *T. kaufmanniana* x *T. greigii*. Only a few seeds were obtained from these crosses. They observed in most cases pollen germination and a normal way of pollen tube growth through the short style into the ovarian cavity. However, after the pollen tubes penetrated the ovules, the majority of pollen tubes started to coil on reaching the embryo sac cavity.

Fertilization has been studied in several tulip species by Pecenicyn (1972). Embryo and endosperm development in compatible intraspecific *T. gesneriana* crosses has been described by Ernst (1901). Haccius and Hausner (1972) have analyzed embryo development within *T. gesneriana*, *T. altaica* Pall. ex Sprengel (former name *T. kolpakowskiana* Regel (Van Raamsdonk and De Vries 1995)) and *T. tarda* Stapf. Embryo development in *T. clusiana* DC. has been described by Wafai and Koul (1982). These authors only described the regular embryo development in tulip. The only report concerning abberations during embryogenesis of compatible crosses within *T. gesneriana* and within *T. fosteriana* is from Sayama et al. (1982). They observed several seeds with endosperm but without embryo. Analysis of embryogenesis in incongruent interspecific tulip crosses has only been reported for the cross *T. gesneriana* x *T. kaufmanniana* (Custers et al. 1995), from which seeds can be produced on the plant (Van Raamsdonk et al. 1995). Only 10%-25% of the embryos of the swollen ovules from this cross continued to grow, but the sizes reached by these embryos were smaller (0.5-2.8 mm) than after self pollinations of *T. gesneriana* (2.7-3.5 mm).

Techniques reported to be used for bypassing pre-fertilization barriers in tulip always concerned the circumvention of self incompatibility. After removal of the stigma and subsequent pollination on the cut surface of the style, self incompatibility was partially

overcome in *T. gesneriana*. Immersion of the stigma in water of 50 °C for 1 to 3 minutes slightly overcame self incompatibility in this species (Keichii and Murakami 1992). After self pollination of *T. gesneriana* ovules attached to the placenta, only seeds were obtained which did not germinate (Zubkova and Sladky 1975).

The application of embryo rescue techniques in tulip breeding has been reported by Van Tuyl et al. (1990) and Custers et al. (1992, 1995). Compatible *T. gesneriana* crosses were used as model system. With ovule culture, more embryos could be rescued from an earlier developmental stage (4 weeks after pollination) as compared to embryo culture. Also, more embryos could be rescued at each culture date with ovule culture in comparison with embryo culture. Preliminary research showed that embryos at 1 week after pollination could already be rescued by using the ovary-slice culture technique (Van Tuyl et al. 1990). After ovule culture, hybrids were recovered from the cross *T. gesneriana* x *T. kaufmanniana*, which can also be obtained after pollination and pod maturation on the plant (Custers et al. 1995).

To stimulate research on flower bulbs, the Royal General Bulbgrowers' Association (KAVB), the Dutch Bulb Exporters Association (BVB) and the Commodity Board for Ornamental Plants (PVS) together with the Dutch government decided in 1989 to finance the Urgency Programme for Research on Diseases and Breeding of Flower Bulbs. Additional research on flower bulbs was necessary because the use of chemicals for the protection of especially bulb crops against diseases had to be diminished to respect the natural environment. Thirteen research projects were financed with the general goals to study soil pathogens, to enlarge the genetic variation of the bulb assortments or to analyze the prospects for conventional breeding. The research presented in this thesis is part of the project titled: "The development of flower biological techniques to overcome crossing barriers in *Lilium* and *Tulipa*".

Outline of the thesis

The main goal of the research on circumventing crossing barriers in tulip was to bypass crossing barriers between cultivars of the present-day assortment and the other tulip species. The present-day assortment consists mainly of cultivars from *T. gesneriana* and from Darwin hybrids. Since the latter are mostly triploid hybrids, we decided to use only cultivars of *T. gesneriana* as model. Four cultivars of *T. gesneriana* were used in the experiments: Christmas Marvel, Leen van der Mark, Prominence and Cassini. These cultivars belong to the 10 most cultivated cultivars. 'Christmas Marvel' was also selected, because it has proved to be a good parent in interspecific crosses (Van Eijk et al. 1991).

The research was divided in two subgoals: (1) the identification of crossing barriers in crosses between *T. gesneriana* and a number of representative tulip species and (2) the development of techniques to bypass these barriers.

Pre-fertilization development and post-fertilization development have both been studied to identify incongruity barriers in tulip. The results of the analysis of pre-fertilization development in crosses between *T. gesneriana* and species from all eight sections of both subgenera are presented in Chapter 2. For studying post-fertilization development we first had to study embryogenesis in an intraspecific *T. gesneriana* cross to detect the possible abberations which occur in compatible crosses (Chapter 3). Thereafter we analyzed the cross *T. gesneriana* x *T. agenensis* DC. (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)) (Chapter 3). This cross was selected because already unique hybrids of this cross were obtained in our embryo rescue experiments (Chapter 5).

A range of techniques were tested for their ability to bypass crossing barriers in tulip. Three techniques have been studied on their ability to bypass pre-fertilization barriers: the cut-style method, the grafted-ovary method and placental pollination (Chapter 4). Many experiments concerned the development of techniques to bypass post-fertilization barriers, because it became obvious that in many of the used interspecific crosses the pollen tubes did penetrate ovules. The prospects for hormone applications for bypassing post-fertilization barriers in interspecific tulip crosses have been studied (Chapter 4).

Two embryo rescue methods were studied: ovule culture and ovary-slice culture. Embryo culture had proved to be a less efficient method as compared to ovule culture (Custers et al. 1992) and we therefore did not use this method. The promising results of preliminary research on ovary-slice culture in *T. gesneriana* (Van Tuyl et al. 1990), together with the good results obtained in *Lilium* after using this technique (Van Tuyl et al. 1991) made us decide to study this technique. Bulblet formation in vitro and the transfer of the bulbs grown in vitro into the soil is very difficult, both after ovule culture (Custers et al. 1992) and after in vitro propagation of tulip bulbs (Le Nard and Chanteloube 1992, Hulscher et al. 1992). Since improving bulb formation in vitro and the transfer of these bulbs into the soil seemed to be already a full-time research, we focused in the embryo rescue experiments on the percentage of ovules showing germination. Results of the analysis of the efficiency of ovule culture and ovary-slice culture started at different dates after pollination in compatible *T. gesneriana* crosses and several interspecific crosses are presented in Chapter 5. As media components are an important factor in determining the efficiency of an embryo rescue technique, several components were tested in media for ovary-slice culture and ovule culture (Chapter 6). To get some insight in the changes in the media which occur during culturing, the pH of the media were measured in time and the carbohydrate concentration of the media were analyzed (Chapter 6).

An in vitro pollination procedure has been developed (Chapter 7) to be able to perform an integrated system of pollination, fertilization and embryo rescue techniques under optimal environmental conditions. Once an in vitro pollination procedure has been developed, it can also be used for the post-fertilization culture of whole ovaries. Much attention has been paid to the optimization of the medium, which proved to be an important factor in determining the rate of success. Also, in media used for in vitro pollination, the pH and the carbohydrate concentration was studied in time.

The main lines of the results of our study on interspecific hybridization in the genus *Tulipa* will be discussed in Chapter 8. Directions for further research will presented in this chapter.

Table 2. Applications of embryo, ovule and ovary culture for the production of hybrids in interspecific and intergeneric crosses. When more than 5 crosses within a genus have resulted in hybrid production, the genus name together with 'spp.' is only presented.

Species	Culture+	Media#	References
interspecific:			
Actinidia chinensis Planch. x *A. melanandra* Franch.		LS + suc 3% ± mannitol 8% + CH 400 ± z 0.1/1.0 ± GA_3 0.5/10.0 ± IAA 0.01, agar, pH=5.8	Mu et al. 1990, Harvey et al. 1995
Allium chinense Maxim. x *A. cepa* L. x *A. tuberosum* Rottl. ex Spreng x *A. ampeloprasum* L. x *A. fistulosum* Linn. x *A. thunbergii* G. Don	EC OC-EC OVC-EC	various media tested: MS + 3%(EC)/5%(OVC-OC), agar, pH=5.8	Nomura and Oosawa 1990, Nomura and Makara 1993, Nomura et al. 1994
Allium L. spp.	OVC	BDS + suc 10%, glutamine 500, IBA 2, BAP 1.2 - BDS + IBA 2, BAP 0.12	Keller et al. 1996
Alstroemeria L. spp.	OC	various media: ¼MS macronutrients + MS micronutrients and vitamins, suc 2%-12% (6% or 9% standard), CH 400, pH=5.8	Buitendijk et al. 1995, De Jeu and Jacobsen 1995
	OC	MS + suc 3%, glutamine 146, pH=5.5	Lu and Bridgen 1996
Arachis hypogaea L. x *A. stenosperma* greg. et Greg. nom. nud.	EC	MS + suc 10%, charcoal 0.5% - MS + suc 3%, GA 1	Ozias-Akins et al. 1992
Arginine chinensis Planch. x *A. melandra* Franch.	EC	various media: ½LS/1LS + suc 3% ± mannitol 8% ± IAA 0.01 ± z 0.1/1.0 ± GA_3 0.5/10, CH 400, agar, pH=5.8	Kin et al. 1990

+ EC embryo culture, OC ovule culture, OVC ovary culture; - One type of embryo rescue followed by another type of embryo rescue; / Both types of embryo rescue were separately executed

For composition of the different media described see Williams et al. 1987 or the individual references (B5, B5G1, B5S, BDS, Heller, HLH, Jensen, KM, L2, LS, MS, White, Nitsch, Nitsch and Nitsch, Norstog (II), SH, U2.5); - One type of medium followed by another type of medium; / Both types of media used

The carbohydrate concentrations are presented in percentages, if not stated otherwise in the text the other concentrations are give in mg/l

Abbrevations: suc sucrose, ml myo-inositol, CH casein hydrolysate, CM coconut milk, YE yeast extract, CJ cucumber juice, CS immature white clover seeds, IAA indoleacetic acid, NAA naphthalene acetic acid, 2,4-D 2,4-dichlorophenoxy acetic acid, IBA 3-indolebutyric acid, k kinetin, z zeatin, BAP 6-benzylamino-purine, BA 6-benzyladenine, GA gibberellic acid, 2-iP N-isopenylamino purine

Table 2. (Continued)

Species	Culture+	Media#	References
Brassica juncea (L.) Czern. x *B. campestris* L.	OVC	Various media tested: MS/White ± k 0.5/2.5 ± IAA 2 ± NAA 0.5 ± CH 300/500	Mohapatra and Bajaj 1988
Brassica oleracea L. x *B. campestris*	OC	modified MS + suc 3%, NAA 0.1, k 0.1, CM 10%, CH 300, agar, pH=5.8	Hossain et al. 1988
Brassica napus L. x *B. oleracea*	EC	MS salts + Jensen C17 amino acids and vitamins + suc 6%, citric acid, tri-potassium citrate 300 ± agar, pH=5.5	Quazi 1988
Brassica spinescens Pomel x *B. campestris*	OC-EC	MS + k 0.05, NAA 0.005, GA 0.5, CH 50	Agnihotri et al. 1990
Brassica oleracea x *B. campestris* & reciprocal	OC	various media, best: MS + suc 1%, gelrite	Plümper and Odenbach 1991
Brassicoraphanus Sagezet x *Brassica x napus* x *B. oleracea* x *B. campestris*	OVC/EC	various media tested:White/MS/½MS/¼MS + suc 5%, agar, pH=5.8 / White + suc 3%, agar, pH=5.8	Long et al. 1992
Brassica oleracea x *B. rapa* Linn. & reciprocal	OC	MS salts + Jensen C17 amino acids and vitamins + suc 6%, citric acid, pH=5.5/ Ripley and Arnison (B5G1) - B5G1 + agar	Ozminkowski and Jourdan 1994
Brassica oleracea x *B. campestris* & reciprocal	OC	MS ± agar	Diederichsen and Sacristan 1994
Cajanus platycarpus (Benth.) L.J.G. van der Maesen x *C. cajan* (L.) Millspaugh	OC-EC	MS + NAA 0.5, BAP 0.5 - MS + NAA 0.1, BAP 1.0	Mallikarjuna and Moss 1995
Camellia japonica L. x *C. chrysantha* (Hu) Tuyama	EC	various media: MS + suc 3%-8%, NAA 0.5-1.0, k 0.1-0.5, YE 0-1g/l, agar	Yamaguchi et al. 1987
Cucumis metuliferus Naud. x *C. anguria* L.	EC	shaken water culture - MS + suc 3%, mI 100, CH 1g/l, BAP 1.0 μM, agar, pH=5.7	Fassuliotis and Nelson 1988
Cuphea paucipetala greenwoodii S.A. Graham x *C. laminuligera* Koehne	OC-EC	Nitsch and Nitsch macronutrients and vitamins + MS micronutients, suc 6%, FeNaEDTA 40, amino acids, NAA 0.5, thidiazurone 0.01, liquid and solid phase (gelrite and activated charcoal 0.5%)	Mathias et al. 1990

Table 2. (Continued)

Species	Culture[+]	Media[#]	References
Cyclamen persicum Mill. x *C. hederifolium* Aiton.	OC	various media, best: MS + suc 3% ± CM 10%, agar, pH=5.8	Ishizaka and Uematsu 1992
Glycine max (L.) Merr. x *G. tomentella* Hayata	EC	B5 + suc 2%, mI 100, vitamins, IBA 0.1, agar, pH=5.5 - same medium + 2-iP 0.25	Chung and Kim. 1990
Glycine max x *G. tomentella*	EC	various media, best: B5 nutrient salts + suc 3%, vitamins	Coble and Schapaugh 1990
Gypsophila paniculata L. x *G. manginii* Hort.	OC-EC	MS + suc 3%, GA_3 2.9 μM, agar, pH=5.8	Kishi et al. 1994
Helianthus annuus L. x *H. maximiliani* Schrad. x *H. niveus* (Benth) Branbegee x *H. ciliaris* DC. x *H. tuberosus* L.	OC-EC	various media tested: B5/B5S/MS/Nitsch and Nitsch + suc 3%/6%/9%/12%, NAA 0.1, agar, pH=5.85±0.05	Espinasse et al. 1991, 1995
Ipomoea triloba Linn. x *I. trifida* G. Don	OC	½MS + suc 8%, agar	Kobayashi et al. 1993
(*I. triloba* x *I. lacunosa* Linn.) x *I. batatas* Poir.			Kobayashi et al. 1994
(*I. ramoni* Choisy x *I. lacunosa*) x *I. littoralis* Blume			
Lilium L. spp.	EC	MS + suc 2%, NAA 0.001, agar, pH=5.8	Van Tuyl et al. 1988
Lilium spp.	OVC*-OC	MS + suc 9%/10%, NAA 1, agar, pH=6.0 - MS + suc 5%, NAA 0.1, agar, pH=5.5	Van Tuyl et al. 1991
Lilium spp.	EC	MS + suc 2%-4%, NAA 0.0001-0.01, agar, pH=5.0	Okazaki 1990, Okazaki et al. 1992, 1995
L. 'Casablanca' (Oriental hybrid) x *L.* 'Connecticut King' (Asiatic hyb.)	EC	various media, good: MS + suc 6%, auxins, gelrite	Okazaki et al. 1994
Lilium regale E.H. Wilson x *L. rubellum* Baker	OC	B5 macronutrients + ½MS micronutrients, suc 5%, amino acids, NAA 0.1, agar, pH=5.7	Niimi et al. 1996

* ovaries were sliced longitudinally in 3-4 mm thick slices

Table 2. (Continued)

Species	Culture+	Media#	References
Limonium perigrinum Bergius x *L. purpuratum* L.	EC	modified KM/modified B5 - MS macro/micronutrients + B5 vitamins, suc 1%, NAA 0.3, BA 0.6, agar, pH=5.8	Morgan et al. 1995
Lycopersicon esculentum Mill. x *L. glandulosum* C.H. Muller x *L. peruvianum* Mill. x *L. chilense* Dun.	OC	MS without phytohormones	Imanishi 1988, Chen and Imanishi 1991
Medicago sativa L. x *M. rugosa* Desr. x *M. arborea* Linn.	OC	modified SH medium (macronutrients, micronutrients, vitamins, inositol 1g/l, maltose 3%, proline 11g/l)	Piccirilli and Arcioni 1992
Nicotiana trigonophylla Dun. x *N. tabacum* L.	OC	various media, best: Nitsch + suc 4%, mannitol, vitamins, glycine, agar	Chung et al. 1988, 1996
Nicotiana africana Merxm. x *N. tabacum*	EC	MS + suc 3%, inositol 100, GA 0.1, k 0.05, YE 500, CH 500, NAA 0.05, agar	Nikova and Zagorska 1990
Pelargonium L' Hér. ex Aiton spp.	EC	White + CM 10%, agar - Nitsch + CM 15%	Bentvelsen et al. 1990
Phaseolus vulgaris L. x *P. acutifolius* A. Gray	EC	MS/B5 + suc 3%, agar	Andrade-Aguilar and Jackson 1988
Phaseolus vulgaris x *P. acutifolius*	OVC	Modified MS + suc 2%, ml 100, vitamins, amino acids, CH 1g/l	Sabja et al. 1990
Populus L. spp.	OVC*	½MS + sucrose 0.06 M, FeEDTA 10^{-4}M, agarose, pH=5.8	Raquin et al. 1993
Solanum tuberosum Linn. x *S. acaule* Bitter	OC/EC	MS + suc 4%, vitamins, ml 100, malic acid 100, hydrolyzed casein 1g/l, agar, pH=5.6	Iwanaga et al. 1991
Solanum stoloniferum Schlecht. & Bouche x *S. tuberosum* *S. chancayense* Ochoa x *S. chacoense* Bitter	EC	MS + suc 2%, vitamins, ml 100, IAA 0.1, k 0.001, adenine sulfate 0.001, agar	Singsit and Hanneman 1991

* ovaries were sliced vertically in 2 equal parts

Table 2. (Continued)

Species	Culture[+]	Media[#]	References
Solanum nigrum Linn. x *S. tuberosum* *S. villosum* Miller x *S. demissum* Lindl.	OVC-OC	HLH + suc 5% - MS + suc 3%	Eijlander and Stiekema 1994
Solanum L. spp. x *S. brevidens* Phil. x *S. etuberosum* Lindl. x *S. fernandezianum* Phil.	OC,EC	MS + suc 4%, vitamins, malic acid 100, hydrolyzed casein 1g/l, ml 100, agar, pH=5.6	Watanabe et al. 1995
Trifolium repens L. x *T. hybridum* L.	OC-EC	Nitsch + CJ 15% - EG (macronutrients, micronutrients, iron, glucose 3%, agar, pH=5.8)	Przywara et al. 1989
Trifolium ambiguum Bieb. x *T. montanum* Linn. x *T. occidentale* Coombe *Trifolium isthmocarpum* Brot. x *T. repens* x *T. nigrescens* Viv.	EC	various media: MS + suc 3%/4%, hormones, agar, pH=5.85	Ferguson et al. 1990
Trifolium alpestre L. x *T. pratense* L.	EC	L2 basal salts and vitamins + suc 12.5%, adenine 15 μM, picloram 25 nM, agar - L2 + suc 2.5%, picloram 4 nM, BA 0.66 μM, agar	Phillips et al. 1992
Vigna radiata (L.) Wilczek x *V. glabrescens* Marechal, Mascherpa & Stainier & reciprocal	EC	MS + suc 3%, ml 100, vitamins, glutamine 100, agar, pH=5.7	Chen et al. 1989
Zantedeschia Spreng. spp.	EC	MS + suc 3%, ml 100, thiamine-HCl 0.4, BA 1, agar, pH=5.8	Yao et al. 1995
intergeneric:			
Brassica oleracea x *Raphanus sativus* Linn.	OC	modified MS + suc 3%, NAA 0.1, k 0.1, CM 10%, CH 300, agar, pH=5.8	Hossain et al. 1988

Table 2 (Continued)

Species	Culture+	Media#	References
Brassica oleracea x *Raphanus sativus* & reciprocal	OC	MS + NAA 0.1, k 0.1, CM 10%, CH 300,	Hossain et al. 1990
Brassica napus x *Raphano Brassica*	OVC-EC	MS + NAA 0.1, k 1, GA 1, CH 10 - MS + NAA 0.01, k 0.1, GA 0.1, CH 10	Agnihotri et al. 1991
Brassica napus x *Sinapis pubescens* Linn.	OVC	various media tested: Nitsch and Nitsch/MS/Heller/White/ Nitsch + suc 5%, CH 300, agar	Inomata 1994
Eutrema wasabi Maxim. x *Armoracia rusticana* ph. Gaertn., B. Mey. et Scherb.	OC	MS + suc 3%, agar, pH=5.8	Ohi et al. 1994
Diplotaxis erucoides (L.) DC. x *Brassica campestris* x *B. juncea* x *B. napus* x *B. oleracea*	OVC/ OVC-OC	White + CH 500 - MS	Vyas et al. 1995
Hordeum L. spp. x *Secale* L. spp.	EC	C17/C21 (Jensen 1977) + suc 6%/4.5%, pH=5.5	Petersen 1991
Hordeum vulgare L. x *Secale cereale* L.	EC	B5 - B5 + 2,4-D 2 - MS + k 2	Wojciechowska and Pudelska 1992
Lycopersicon esculentum Mill. x *Solanum lycopersicoides* Dun.	EC	HLH / HLH + IAA 10^{-9} M, k 10^{-7} M	Gradziel and Robinson 1989
Moricandia arvensis (L.) DC. x *Brassica oleracea* x *B. campestris* & reciprocal x *B. nigra* Koch.	OVC-EC	MS ± CH 500, agar - MS/B5	Takahata 1990, Takahata and Takeda 1990
Moricandia arvensis x *Brassica napus* x *Brassica juncea*	OVC-EC	MS + without hormones, agar - MS + agar	Takahata et al. 1993
Nerine bowdenii DC. x *Amaryllis belladonna* L.	OVC	MS + suc 9%, NAA 1, agar, pH=6.0	Van Tuyl et al. 1992b

Table 2. (Continued)

Species	Culture[+]	Media[#]	References
Oryza punctata Katzchy, ex Steud x *Leersia tisseranti* A. Chev. x *L. perrieri* (A. Camus) Launert *Oryza latifolia* Desv. x *Leersia tisseranti* x *L. perrieri*	OC	MS	Katayama 1995
Raphanus sativus L. x *Sinapis pubescens* L. x *S. alba* L. & reciprocal x *S. arvensis* L. & reciprocal x *S. turgida* (Pers.) Del. & reciprocal	OVC-EC /EC	OVC: MS + suc 3%, CH 500, agar; EC: White + suc 3%, CH 500, agar	Bang et al. 1996
Sinapis alba x *Brassica napus* *S. arvensis* x *B. napus*	OC	Nitsch and Nitsch + suc 0.3-0.4 M, glutathione 30, L-glutamine 800, L-serine 100, NAA 0.5, BAP 0.05, activated charcoal 0.5%, agar - MS + suc 2%, agar	Mathias 1991
Sinapis alba x *Brassica napus*	OVC-OC	MS + IAA 1, k 0.2 - MS + IAA 2, k 0.5	Lelivelt 1993
Triticum aestivum L. x *Leymus innovatus* (Beal) Pilger x *Psathyrostachys juncea* (Fisch.) Nevski x *Elymus scabrus* (R.Br.) A. Love x *Agropyron fragile* (Roth) Nevski	OC	Various media, best: U2.5 (GA_3 0.02, 2-isopentenyl adenosine 0.25, polyamines, amino acids, gelrite, pH=5.5) - Norstog II/B5/MS ± IAA 0.5 ± GA_3 0.5	Plourde et al. 1989, 1990, Ahmad and Comeau 1991a,b, Comeau et al. 1992
Triticum aestivum x (x *Triticosecale* Wittmack)	EC	MS + suc 3%, CH 400, glutamine 50, agar, pH=5.8	Kapila and Sethi 1993
Triticum aestivum x *Hordeum vulgare* & reciprocal	EC	various media, best: MS + 2,4-D 1, k 0.5	Khanna et al. 1994
Triticum turgidum L. x *Psathyrostachys juncea*	EC	modified Norstog's medium	Mujeeb-Kazi et al. 1987

Interspecific crosses in the genus *Tulipa* L.: identification of pre-fertilization barriers.

2

Summary

Pollen tube growth in the pistil and pollen tube penetration in the ovules have both been studied in crosses between cultivars from *Tulipa gesneriana* L. and 13 tulip species from all eight sections of the genus *Tulipa* to identify pre-fertilization barriers. Depending on the cross, pollen tubes grew as far as the stigma or the style or continued growing down into the ovary. The pollen tubes did not penetrate in any or only a few percent of the ovules of some crosses, despite the presence of many more pollen tubes in the ovary. In other crosses, from which no or only a few hybrids have been obtained after seed maturation on the plant, pollen tube penetration was found in up to 79% of the ovules. Apparently, various kinds of barriers, preventing fertilization or normal embryogenesis, occur in interspecific tulip crosses.

Introduction

The genus *Tulipa* comprises about 55 species, of which 49 are described by Van Raamsdonk and De Vries (1992, 1995), who have revised this genus recently. The tulip species are classified in two subgenera, *Tulipa* and *Eriostemones*, which are subdivided into five and three sections, respectively (see Chapter 1, Table 1). The commercial assortment of tulips consists mainly of cultivars from *Tulipa gesneriana*, from the section *Tulipa*. Another group of cultivars, the Darwin hybrids, have mainly been obtained from interspecific crosses between members of the cultivar group *T. gesneriana* and genotypes of *T. fosteriana* Hoog ex W. Irving from the section *Eichleres*.

The present-day tulip assortment could be considerably improved by exploiting traits from other *Tulipa* species. In particular, introduction of resistances against tulip breaking virus (TBV), *Botrytis tulipae*, *Fusarium oxysporum* (bulb-rot), but also characteristics such as a short forcing period, good flower longevity and new flower colours and flower shapes are important targets for tulip breeding programs.

Interspecific crosses are usually made between genotypes of *T. gesneriana* and other *Tulipa* species. *T. gesneriana* proved to be compatible with other species of the same section. Crosses between *T. gesneriana* and representatives of the section *Eichleres* produced hybrids in several cases. Crosses between *T. gesneriana* and species of the other sections of the subgenus *Tulipa* produced no verified hybrids, except for *T. systola* Stapf (former name *T. stapfii* Turrill (Van Raamsdonk and De Vries 1995)) from the section *Tulipanum*. Crosses between the two subgenera have never been successful (Van Eijk et al. 1991, Van Raamsdonk et al. 1995).

Interspecific crossing barriers (incongruity) may result from a lack of genetic information in one partner about the other (Hogenboom 1973). Pre-fertilization barriers originate from the inability of pollen grains to germinate on the stigma or from malfunctioning of the pollen tube and/or sperm cells. Several methods have been developed for bypassing these barriers: the mentor pollen technique (Stettler 1968), a combination of bud pollination with the treatment of stigmas with an artificial medium (Gradziel and Robinson 1991), the cut-style method and the grafted-style method (Asano and Myodo 1977, Van Tuyl et al. 1988) and placental pollination (DeVerna et al. 1987, Zenkteler et al. 1987).

Post-fertilization barriers can cause premature degeneration of the embryo and/or endosperm. Hybrid breakdown and F1-sterility of hybrid plants, excluding them from further breeding, are also crossing barriers. Post-fertilization barriers are bypassed by using ovary culture and/or ovule culture and/or embryo culture in many crops, such as *Brassica* (Bajaj et al. 1986), *Nicotiana* (Nikova and Zagorska 1990), *Lycopersicon* (Chen and Adachii 1992), *Triticum* (Comeau et al. 1992) and *Lilium* (Asano 1978, Van Tuyl et

al. 1991). Custers et al. (1995) obtained hybrids of the cross *T. gesneriana* x *T. kaufmanniana* Regel after ovule culture. This cross also succeeds after pollination and pod maturation on the plant. In *Lilium*, F1-sterility can be restored through artificial chromosome doubling by treatment with colchicine or oryzaline (Van Tuyl et al. 1993).

The successful development of a method for bypassing crossing barriers in a specific cross requires knowledge of the crossing barriers. Kho and Baër (1971) analyzed pollen tube growth in tulip crosses between cultivars of *T. gesneriana* (mother) and species of the section *Eichleres*: *T. fosteriana*, *T. greigii* Regel and a hybrid of *T. kaufmanniana* x *T. greigii*. Only a few seeds were obtained from these crosses. They concluded that the low number of seeds produced was due to abnormal growth of the pollen tubes in the embryo sac regions of most ovules, with the result that fertilization did not occur in those ovules.

The aim of this study is to identify pre-fertilization barriers in interspecific crosses between *T. gesneriana* and a number of representative species of the other eight sections of the genus *Tulipa* (presented in Table 1), by studying pollen tube growth in the pistil and their penetration in the ovules.

Materials and methods

Plant material

Seventeen different genotypes (cultivars or species) were used in total, representing all sections of both subgenera of the genus *Tulipa*. The genotypes used are presented in Table 1. The cultivars of *T. gesneriana*, *T. fosteriana*, *T. praestans* and *T. clusiana* were obtained from commercial growers and other species and genotypes were derived from the *Tulipa* collection of CPRO-DLO. One genotype was used (*T. de novo species A*, an unpublished species) which resembles *T. praestans* in morphology except for flower colour, which is yellow instead of red. The species used are diploid (2n=2x=24), except *T. praecox* (2n=3x=36), which is triploid and *T. clusiana*, *T. sylvestris* and *T. turkestanica*, which are tetraploid (2n=4x=48).

Crossing procedure

Bulbs were planted in September-October in flats and subsequently cold stored at 5-9 °C for 15-18 weeks. The plants were placed in a greenhouse at a temperature of 15-17 °C in January-March. They flowered in 2 to 4 weeks.

The flowers were emasculated about 2 days before anthesis. One or 2 days after anthesis, when the stigma was receptive, the flowers were pollinated with fresh pollen

Table 1. The species with their collection numbers or cultivars of the genus *Tulipa L.*, according to the classification of Van Raamsdonk and De Vries (1992, 1995). Previously used names are given in brackets.

Subgenus	Section	Species	Cultivars/ CPRO-DLO numbers
Tulipa (*Leiostemones*)	*Tulipa*	*T. gesneriana* L.	Christmas Marvel Leen van der Mark Cassini Prominence
		T. didieri Jord. (*T. marjoletti* Jord.)	66040-4
	Eichleres (Hall) Van Raamsdonk	*T. fosteriana* Hoog *T. praestans* Hoog *T. de novo species A* *T. kaufmanniana* Regel (subsection *Spiranthera*)	Madame Lefeber Zwanenburg 71331-10 65252-1
	Tulipanum de Reboul (*Oculus-solis* Hall)	*T. agenensis* DC. (*T. oculus-solis* St. Amans) *T. praecox* Tenore	75145 83209
	Kolpakowskianae (Hall) Van Raamsdonk	*T. altaica* Pall. (*T. kolpakowskiana* Regel)	68596
	Clusianae Baker	*T. clusiana* DC. *T. montana* Lindley	Cynthia 65152 73116
Eriostemones (Boissier) Van Raamsdonk	*Australes*	*T. sylvestris* L.	77258
	Saxatiles	*T. pulchella* Fenzl.	68365
	Biflores	*T. turkestanica* Regel	70656

whenever possible. In some cases pollen was used that had been stored in the dark for at most 1 month in a desiccator with silica gel. The pollen was rehydrated before use at 100% relative humidity at 15 °C for 2 hours. Pollinations were carried out at 15 °C, according to Kho and Baër (1971). The ability of pollen to germinate was tested in liquid Brewbaker and Kwack (1963) medium, supplemented with 10% (w/v) sucrose.

Crossing scheme

Compatible crosses between *T. gesneriana* 'Christmas Marvel' and *T. gesneriana* 'Leen van der Mark' and incongruent crosses between 'Christmas Marvel' and *T. praestans* 'Zwanenburg' were made to determine the period needed from pollination to fertilization. Pollen tube growth and penetration of the pollen tubes in the ovules was analyzed 1, 3, 5, 7, 9, 11, 13 and 15 days after pollination (DAP). Five or six flowers were analyzed per cross for each time interval. Pollinations were carried out in February and March 1991.

The executed crosses between *T. gesneriana* and the cultivars and species used are presented in Table 2. These crosses were carried out in February - March 1992, in March - May 1993 and in February 1994. The plant material used for microscopical observations was collected at 12 DAP.

On average 14 pollinated flowers from each interspecific cross were analyzed in 1992 and 1993, except for the crosses 'Christmas Marvel' x *T. de novo species A*, *T. kaufmanniana* x 'Christmas Marvel' and *T. pulchella* x 'Christmas Marvel', of which 10, 27 and 5 pollinated flowers were studied, respectively. For compatible crosses, 25 pollinated flowers were analyzed. Five to 12 pollinated flowers were studied in 1994 of the following crosses: 'Christmas Marvel' x 'Leen van der Mark', 'Christmas Marvel' x *T. de novo species A*, 'Christmas Marvel' x *T. turkestanica*, *T. kaufmanniana* x 'Christmas Marvel', *T. praecox* x 'Christmas Marvel' and *T. pulchella* x 'Christmas Marvel'.

Microscopic techniques

A pistil of tulip is composed of three fused carpels. Two carpels per flower were used for studying the pollen tube growth in the pistil and the third one for analyzing pollen tube penetration in the ovules.

Pollen tube growth was analyzed using aniline blue staining and UV-fluorescence (Kho and Baër 1968). A squash preparation of both carpels was made before analysis. The length of the longest pollen tubes as a percentage of the total pistil length and the number of pollen tubes half-way down the pollen tube bundle were determined per preparation. The highest percentage of the two preparations of one flower was used for calculating the average percentage of pistil length traversed by pollen tubes per time interval and per cross. Because the number of pollen tubes could not be counted exactly, a division in classes was used (per carpel): 1: 1-10 pollen tubes, 2: 11-25, 3: 26-50, 4: 51-100, 5: 100-250 and 6: more than 250 pollen tubes. The average number of pollen tubes per cross is presented by the class(es) with the most representatives in it.

The pollen tube penetration in the ovules was studied after destaining ovules, while still attached to the placenta, in a mixture of water, glycerol and lactic acid (1:2:1). The

material was then stained in a solution of 1% aniline blue in the same mixture of water, glycerol and lactic acid and subsequently destained (Janson et al. 1993). The number of ovules penetrated by a pollen tube was determined using a bright field microscope.

Results

Time needed taken from pollination to ovule penetration

The upper part of the pistil of tulip consists of the stigma and a short style, which together make up about 20% of the total pistil length. The growing pollen tubes then reach the first ovules, those at the bottom of the ovarian cavity being reached at about 80% of the total pistil length. The rest of the pistil consists of the part of the ovary which does not contain ovules. Due to the use of squashing, sometimes ovules were observed at the end of the pistil.

Data on pollen tube growth (as a percentage of pistil length) for the compatible crosses between *T. gesneriana* 'Christmas Marvel' and 'Leen van der Mark' and the incongruent crosses between *T. gesneriana* 'Christmas Marvel' and *T. praestans* are shown in Fig. 1. For all crosses, pollen tubes reached the first ovules at 1 to 3 DAP. Pollen tubes grew in all flowers of the compatible crosses to the lowest ovules in 7 to 11 DAP. The pollen tubes never reached the lowest ovules in the incongruent crosses. In the cross 'Christmas Marvel' x *T. praestans*, the pollen tubes attained their maximum length of about 55% of the pistil length, at 7-9 DAP. The pollen tubes never exceeded an average percentage of pistil length of more than 26% in the reciprocal cross. The variation between the flowers of each observation date is comparable with the variation (range) shown in Table 2.

The percentages of ovules with pollen tube penetration in the crosses given above at different dates after pollination are shown in Fig. 2. The first ovules were found to be penetrated at 1 DAP. The penetration percentage increased in the compatible crosses between 3 and 9 DAP to 70%-73%, then slowed down and fluctuated between 68% and 83%. In the incongruent cross 'Christmas Marvel' x *T. praestans*, the percentage of ovules with pollen tube penetration increased up to 11 DAP, then fluctuated between 29%-40%. No pollen tube penetration was found in the reciprocal incongruent cross (*Tpr* x CM).

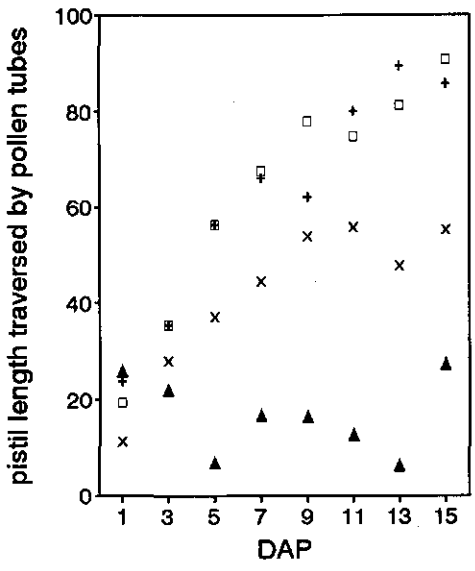


Fig. 1. The percentage of total pistil length traversed by pollen tubes at 1, 3, 5, 7, 9, 11, 13 and 15 days after pollination (DAP), in crosses between *T. gesneriana* 'Christmas Marvel' (CM) with *T. gesneriana* 'Leen van der Mark' (LvdM) and *T. praestans* 'Zwanenburg' (Tpr).

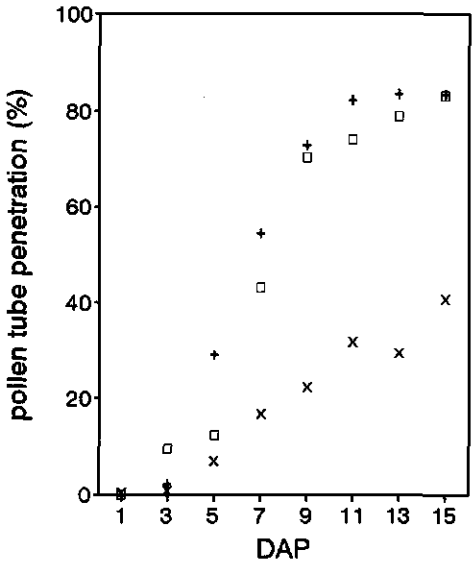


Fig. 2. The percentage of ovules penetrated by pollen tubes at 1, 3, 5, 7, 9, 11, 13 and 15 days after pollination (DAP), in crosses between *T. gesneriana* 'Christmas Marvel' (CM) with *T. gesneriana* 'Leen van der Mark' (LvdM) and *T. praestans* 'Zwanenburg' (Tpr)

Progamic characteristics of various interspecific crosses

The averages of pollen tube length, number of pollen tubes and number of penetrated ovules are presented in Table 2. The range of percentages, indicating the variation between flowers, is also shown. The percentages, presented in this table, were related to the actual lengths of the pistils and the actual numbers of ovules of the analyzed flowers. In fact, these characteristics vary strongly between species. The pistils of *T. gesneriana*, *T. kaufmanniana* and *T. agenensis* have average lengths between 4.0 - 4.5 cm. Pistils of *T. didieri*, *T. fosteriana* and *T. praecox* are about 3 cm long and the pistils of the other

Table 2. The averages are presented of the numbers of pollen tubes in the pistil (N, divided in classes), the percentages of total pistil length traversed by pollen tubes (%_PT) and the percentages ovules with pollen tube penetration (%_PE) after crosses between *Tulipa gesneriana* 'Christmas Marvel' (CM) or 'Leen van der Mark' (LvdM) with species of the genus *Tulipa*, executed in 1992, 1993 and 1994 (nd= not done). Per cross, of both percentages, the minimum and maximum values (Range) are also presented. The results of the pollen tube growth were averaged for subsequent years. The number of pollen tubes (N) was determined in one of the three carpels of the pistil. Classes: 1: 1-10, 2:11-25, 3:26-50, 4:51-100, 5:100-250, 6:more than 250 pollen tubes.

Cross	Pollen tube growth			Penetration in ovules			
				1992		1993/1994*	
	N	%_PT	Range	%_PE	Range	%_PE	Range
T. didieri x CM	4	74	27-100	nd		64	1-95
T. fosteriana x CM	2-3	50	34-67	nd		6	0-17
T. praestans x CM	4	8	5-36	0.0		nd	
T. de novo species A x CM	1-2	25	3-88	nd		5	0-46
T. kaufmanniana x CM	1	28	2-68	0.0		nd/0.0*	
T. agenensis x CM	2	28	3-64	0.2	0-3	nd	
T. praecox x CM	1	12	2-28	0.0		nd/0.1*	0-0.6
T. altaica x CM	2-3	23	5-55	nd		4	0-13
T. clusiana x CM	3-4	68	35-100	nd		11	0-87
T. sylvestris x CM	3-4	79	47-100	nd		0.6	0-5
T. turkestanica x CM	4-5	8	3-18	0.0		nd	
T. pulchella x CM	3-4	70	44-94	nd		4/9*	0-31
Cassini x *T. kaufmanniana*	2-3	84	59-100	52	0-81	nd	
Prominence x *T. kaufmanniana*	2-6	86	63-98	79	1-92	nd	

* crosses were repeated in 1994, the results of 1993 are presented before the slash, the results of 1994 after the slash

Table 2. continuation

Cross	Pollen tube growth			Penetration in ovules			
				1992		1993/1994*	
	N	%_PT	Range	%_PE	Range	%_PE	Range
CM x LvdM	5-6	85	69-100	72	50-95	65/72*	25-81
CM x *T. didieri*	3	78	59-92	nd		39	7-60
CM x *T. fosteriana*	4	82	64-100	nd		60	24-81
CM x *T. praestans*	3-4	68	55-98	58	34-69	21	15-29
CM x *T. de novo species A***	3/6	30/57	14-61	nd		0.5/23*	0-26
CM x *T. kaufmanniana*	3-4	83	42-100	75	43-91	37	5-55
CM x *T. agenensis*	3-4	70	57-81	76	60-85	35	22-53
CM x *T. praecox*	1-2	41	14-64	21	1-29	2	0-5
CM x *T. altaica*	2	33	16-51	nd		2	0-7
CM x *T. clusiana*	3-4	24	15-36	nd		0.0	
CM x *T. sylvestris*	4	27	19-33	nd		0.0	
CM x *T. turkestanica*	1-2	25	2-60	0.4	0-3	nd/1*	0-3
CM x *T. pulchella*	1-2	25	7-60	nd		1	0-7
LvdM x CM	3-4	73	14-95	71	18-92	73	61-93
LvdM x *T. didieri*	3	78	58-87	nd		74	63-87
LvdM x *T. fosteriana*	2-3	67	51-85	nd		40	5-68
LvdM x *T. praestans*	1-2	47	16-94	19	0-49	nd	
LvdM x *T. de novo species A*	2-3	50	40-64	nd		30	12-50
LvdM x *T. kaufmanniana*	2-3	66	14-97	48	25-79	nd	
LvdM x *T. agenensis*	2-3	44	11-81	38	0-74	nd	
LvdM x *T. praecox*	1-2	34	8-71	1	0-4	nd	
LvdM x *T. altaica*	2-3	31	2-43	nd		0.4	0-2
LvdM x *T. montana*	3	3	3	nd		0.0	
LvdM x *T. sylvestris*	3	3	2-3	nd		0.0	
LvdM x *T. turkestanica*	1	21	12-42	0.04	0-0.6	nd	
LvdM x *T. pulchella*	4-5	2	2	nd		0.0	

** the results of the pollen tube growth of both years are presented separately due to large variation between both years

analyzed species have lengths between 1.5 and 2 cm. The number of ovules per carpel varies between species as well. *T. gesneriana*, *T. didieri*, *T. fosteriana*, *T. agenensis* and *T. praecox* have between 100 and 150 ovules per carpel. *T. turkestanica* possesses about 50 ovules per carpel and the other species have between 70 and 90 ovules per carpel.

The crosses were executed in 1992, 1993 and 1994. The results of the pollen tube growth in the pistils were averaged for subsequent years, since differences between these percentages were at most 7%. No pollen tube growth was observed in at most 3 flowers of most crosses. In total 2% of the pistils from the compatible crosses, 1% from interspecific crosses with 'Christmas Marvel' as mother and 5% from interspecific crosses with 'Leen van der Mark' as mother did not show pollen tube growth. No pollen tube growth was found in 8% of the pistils from the reciprocal crosses with 'Christmas Marvel' as pollen donor. These flowers were disregarded. In the cross *T. kaufmanniana* x 'Christmas Marvel', however, pollen tube growth was found in only 9 of the 27 flowers. In crosses of *T. turkestanica* as pollen donor with 'Christmas Marvel', 15 of the 19 flowers showed pollen tube growth, and with 'Leen van der Mark' only 4 of the 13 flowers. Pollen tube growth was observed in 14 of the 21 pistils of *T. praecox* pollinated with 'Christmas Marvel'.

Pollen used for the different pollinations germinated abundantly in vitro, except for the triploid species *T. praecox*. This species appeared to have a pollen viability of only 4%.

A central bundle of pollen tubes can be seen after pollination in the ovarian cavity, from which pollen tubes bend sideways and grow towards the ovules (Fig. 3A). After compatible pollination, the pollen tubes traversed 73% ('Leen van der Mark' x 'Christmas Marvel') and 85% ('Christmas Marvel' x 'Leen van der Mark') of the pistil length and penetrated in 65% to 73% of the ovules.

There was large variation in the number of pollen tubes formed, the percentage of pollen tube growth in the pistils and the percentage of penetrated ovules between the various interspecific crosses. For most interspecific crosses, the number of pollen tubes was lower compared to the compatible crosses. Pollen tubes reached lengths comparable with the compatible crosses in crosses with pollen from *T. didieri*, *T. fosteriana* and *T. kaufmanniana* and the penetration percentages were in several crosses similar as well. Relatively high percentages of pollen tube length (44%-70%) and pollen tube penetration (19%-76%) were found in crosses with *T. praestans* and *T. agenensis* as pollen donors. In the cross 'Christmas Marvel' x *T. de novo species A*, the pollen tube growth and penetration was poor in 1993 (30% and 0.5%, respectively), while in 1994 and in the cross 'Leen van der Mark' x *T. de novo species A*, pollen tubes had penetrated in 23%-30% of the ovules. In crosses with *T. praecox*, *T. altaica* and *T. turkestanica*, pollen

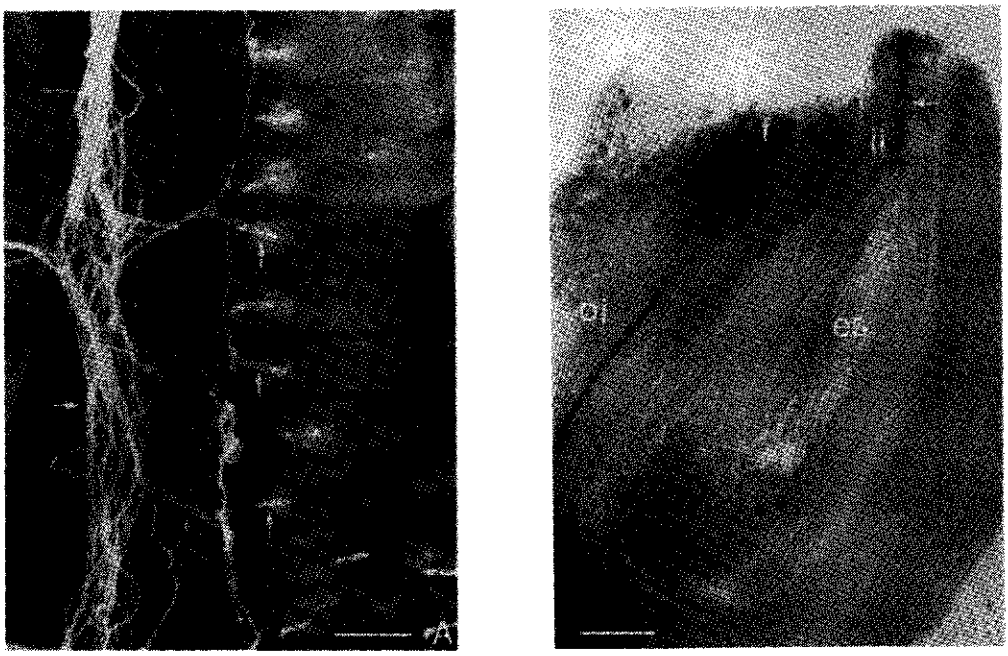


Fig. 3. Pollen tube growth in the pistil of *T. gesneriana* 'Christmas Marvel'. **A** Central bundle of pollen tubes from *T. gesneriana* 'Leen van de Mark' in the ovary bending towards the ovules (*arrows*). *Bar*: 0.04 cm. **B** Pollen tube of *T. turkestanica* penetrating an ovule (*arrow*). *Bar*: 0.01 cm. *es* embryo sac, *f* funiculus, *ii* inner integument, *oi* outer integument.

tubes reached the ovules in a low number of flowers and pollen tubes had penetrated in at most 2% of the ovules (Fig. 3B), except in the cross 'Christmas Marvel' x *T. praecox*, executed in 1992 (21% penetration). Pollen tubes reached the ovules in a number of flowers when 'Christmas Marvel' was pollinated with *T. clusiana*, *T. sylvestris* or *T. pulchella*. Only the cross 'Christmas Marvel' x *T. pulchella* showed pollen tube penetration in the ovules. Pollen tubes had not entered the ovary in any flower in crosses between 'Leen van der Mark' as mother with *T. montana*, *T. sylvestris* and *T. pulchella*.

Usually a lower number of pollen tubes was found in crosses with pistils of 'Leen van der Mark' than in crosses with pistils of 'Christmas Marvel'. In the interspecific crosses, the pollen tube penetration percentages were lower in the crosses with 'Leen van der Mark', as compared to 'Christmas Marvel', except for crosses with *T. didieri* and *T. de novo species A*.

Differences in pollen tube growth in the pistils have not been found in crosses

between four cultivars of *T. gesneriana* (mother) and *T. kaufmanniana*. The penetration percentages, however, were lower in crosses with 'Leen van der Mark' and 'Cassini' than with 'Christmas Marvel' and 'Prominence'.

The pollen tube penetration percentages were higher in crosses between 'Christmas Marvel' and *T. fosteriana*, *T. praestans*, *T. kaufmanniana*, *T. agenensis*, *T. praecox* or *T. turkestanica* as pollen donors in comparison with the reciprocal crosses. In crosses with *T. didieri*, *T. altaica* and *T. pulchella*, the absolute number of ovules penetrated by a pollen tube was similar between the reciprocal crosses with 'Christmas Marvel'. More pollen tubes had penetrated the ovules in the cross *T. de novo species A* x 'Christmas Marvel' of 1993 as compared to the reciprocal cross. Pollen tube penetration was found when *T. clusiana* and *T. sylvestris* were pollinated with 'Christmas Marvel', however, not in the reciprocal crosses.

Discussion

Pollen tube growth

The number of flowers with maximum pollen tube length and maximum pollen tube penetration did, at a temperature of 15 °C, increase until 9-11 DAP in compatible *T. gesneriana* crosses and crosses between *T. gesneriana* and *T. praestans*. Pistils used for the identification of crossing barriers were, therefore, fixed at 12 DAP. However, Sayama et al. (1982) reported that in *T. gesneriana* fertilization is already completed 5 DAP under field conditions. Differences in temperature can explain the earlier time of fertilization as observed by Sayama et al. (1982) compared to our results, because the pollen tube growth rate is influenced by the temperature (Ascher and Peloquin 1966, Chang and Struckmeyer 1976, Van Herpen and Linskens 1981).

The largest increase in ovule penetration of the compatible crosses was from 3 to 9 WAP, which finally resulted in an average maximum penetration percentage of 83% for compatible *T. gesneriana* crosses. This percentage was about 90% in compatible *Lilium longiflorum* Thunb. crosses (Janson 1992), 80% in crosses within *Brassica napus* L. and 77% in crosses within *Sinapis alba* L. (Lelivelt 1993). In *L. longiflorum*, probably not all ovules are penetrated, because ultimately only 80-90% of the ovules reach maturity (Van Went et al. 1985).

The results of the analysis of the pollen tube growth in interspecific tulip crosses will be discussed in relation to pre-fertilization barriers, seed set, taxonomy and the perspectives for tulip breeding.

Pre-fertilization barriers

Pre-fertilization barriers were observed in thirteen interspecific tulip crosses during various developmental stages of the progamic phase, e.g. pollen germination, pollen tube growth and pollen tube penetration. Absence of pollen germination in all flowers of a specific cross was never found. However, in many crosses pollen did not germinate in some of the flowers, even in compatible crosses. Low viability of the pistillate parents seems to be the cause of this absence of pollen germination, rather than a crossing barrier. The higher percentage of flowers without pollen germination in crosses with 'Christmas Marvel' as pollen donor can be due to problems in the recognition of the receptivity of the stigma at pollination, especially in crosses with *T. kaufmanniana*. However, the absence of pollen germination in the cross 'Leen van der Mark' x *T. turkestanica* can not be explained by low viability of the pistillate parent or low pollen quality. Probably, in this case a crossing barrier is present on the stigma.

Inhibition of pollen tube growth is indicated by a lower number of pollen tubes and/or a shorter length of the pollen tubes as compared to compatible crosses. The number of pollen tubes was usually lower in the interspecific crosses than in the compatible crosses. Apparently, pollen germination and/or pollen tube growth is hampered at an early stage. In interspecific crosses, the pollen tubes often remained shorter than in compatible crosses, indicating that crossing barriers limit pollen tube growth.

The percentage of ovules to be penetrated by pollen tubes is dependent on the number of pollen tubes and the lengths of pollen tubes. For most crosses, comparable pollen tube lengths were obtained in each year studied. However, pollen tube penetration percentages of the interspecific crosses differed greatly between the years. Apparently, year-effects influence pollen tube penetration in interspecific crosses. In many crosses, the number of pollen tubes observed in the pistil was comparable to the number of ovules which could be penetrated by a pollen tube, in at least one year. This indicates that in these crosses pre-fertilization barriers do not prevent pollen tube penetration. In other crosses, however, for example *T. sylvestris* x 'Christmas Marvel', the number of pollen tubes in the ovary is much higher as compared to the number of penetrated ovules, indicating the prevention of pollen tube penetration by pre-fertilization barriers.

Considerable differences in pollen tube growth and pollen tube penetration were found between individual flowers of most tulip crosses, like in *Lilium longiflorum* (Janson et al. 1993). In other tulip experiments, performed at CPRO-DLO, considerable variation in seed set is always found. The penetration percentage might influence the further development of the ovary. Janson (1992) found, after cut-style pollination in the intraspecific cross *Lilium longiflorum* 'Gelria' x *L. longiflorum* 'White American', absence of seed set at a penetration percentage of about 5%, while some seed set was

obtained when the penetration percentage was 10%.

Beside year-effects, cultivar-effects as well were found to influence pollen tube penetration. In general, more pollen tubes penetrated the ovules of 'Christmas Marvel' than of 'Leen van der Mark'. Cultivar-effects, effects of accessions and year-effects influenced seed set in interspecific tulip crosses (Van Eijk et al. 1991, Custers et al. 1995). Cultivar/accession-effects are due to genetic differences. Year-effects can originate from differences between lots of cultivars used each year, resulting from differences occurring during the growing periods of the bulbs and the forcing periods of the flowers.

Seed set

Our results are to a great extent in accordance with the results of Van Eijk et al. (1991) and Van Raamsdonk et al. (1995), who obtained hybrids from several crosses within the subgenus *Tulipa*. The best results were obtained with *T. gesneriana* as female parent, which is in agreement with the higher penetration percentages we did observe as compared to the reciprocal crosses. Many hybrids can be produced from crosses between *T. gesneriana* and other species from the section *Tulipa*. Van Raamsdonk et al. (1995) found differences in seed set between reciprocal crosses of *T. gesneriana* with *T. didieri*. In these crosses, most hybrids were produced with *T. didieri* as pollen donor, despite comparable penetration percentages of the reciprocal crosses (Table 2).

Small numbers of seeds can be obtained from several crosses between *T. gesneriana* and species from the section *Eichleres*, such as *T. kaufmanniana* and *T. fosteriana*. The production of seeds from crosses between *T. gesneriana* and *T. praestans* has never been reported. The absence of pollen tube penetration in the cross *T. kaufmanniana* x *T. gesneriana* (Table 2) is in disagreement with the seed set obtained from this cross by Van Raamsdonk et al. (1995) (0.2 F1 bulbs per pod). This discrepancy between the absence of pollen tube penetration and seed set can be caused by cultivar and accession effects and by year-effects.

Crosses between *T. gesneriana* and species of the section *Tulipanum* have been successful with *T. systola* (former name *T. stapfii* (Van Raamsdonk and De Vries 1995)) and *T. julia* C. Koch, but not with *T. agenensis* and *T. praecox* (Van Eijk et al. 1991, Van Raamsdonk et al. 1995). More seedlings were obtained when pistils of *T. systola* (65 seedlings per pod) were used in comparison with the reciprocal cross (2 per pod) (Van Raamsdonk et al. 1995). This is in contrast with the lower penetration percentages we generally observed when *T. gesneriana* was used as pollen donor. The hybrids with *T. julia* were never verified.

Van Eijk et al. (1991) did not obtain hybrids of crosses between *T. gesneriana* and species of the sections *Kolpakowskianae* and *Clusianae*. Van Raamsdonk et al. (1995)

obtained plants after pollination of *T. linifolia* forma *chrysantha* (Boissier) Van Raamsdonk (former name *T. batalinii* Regel (Van Raamsdonk and De Vries 1995)) and *T. clusiana* DC. 'Cynthia' (both section *Clusianae*) with *T. gesneriana*. The plants from crosses with *T. linifolia* forma *chrysantha* appeared to be no hybrids. The plants of the crosses with *T. clusiana* were never verified on their hybrid origin (Van Raamsdonk, personal communication). We found pollen tube penetration percentages up to 87% in the cross *T. clusiana* 'Cynthia' x *T. gesneriana*.

Crosses between *T. gesneriana* and species of the subgenus *Eriostemones* have never been successful (Van Eijk et al. 1991). We observed absence of penetration or a very low penetration percentage, except in several flowers of the cross *T. pulchella* x *T. gesneriana*.

Despite high penetration percentages in crosses between *T. gesneriana* and several other tulip species, no (*T. praestans*, *T. agenensis*) or only a few (*T. fosteriana*, *T. kaufmanniana*) seeds were produced after pod maturation on the plant. Apparently, barriers occur which prevent either fertilization and/or normal embryogenesis. Kho and Baër (1971) concluded that the poor seed yield from crosses between *T. gesneriana* and species of the section *Eichleres* (pollen donor) was due to abnormal pollen tube growth in the embryo sac of numerous ovules, preventing fertilization. We have also found this abnormal growth, but only in some ovules (data not shown).

Taxonomy

Results of pollen tube growth and pollen tube penetration can be related to the taxonomy of the genus *Tulipa* (Van Raamsdonk and De Vries 1992, 1995). Crosses between *T. gesneriana* and *T. didieri* (both section *Tulipa*) showed pollen tube growth percentages comparable to those of the intraspecific *T. gesneriana* crosses. Crosses between *T. gesneriana* and species of the sections *Eichleres* and *Tulipanum* showed high percentages of pollen tube penetration, except for the triploid species *T. praecox*. Differences between crosses of *T. gesneriana* and species of the same section can be related to the division of these sections in series (Van Raamsdonk and De Vries 1995). *T. fosteriana*, *T. praestans* and *T. kaufmanniana* are classified in three of the eight different series of the section *Eichleres*. This section shows large differences in geographic distribution and morphology. *T. agenensis* and *T. praecox* are classified both in another series of the two series of the section *Tulipanum* (Van Raamsdonk and De Vries 1995). The species of the section *Tulipanum* resemble species of the section *Tulipa* (Hall 1940) and, for several morphological characters, species of the section *Eichleres* (Van

Raamsdonk and De Vries 1995).

The sections *Kolpakowskianae* and *Clusianae* are less related to the section *Tulipa* than the above mentioned sections. The section *Clusianae* has an isolated position within the subgenus *Tulipa* (Van Raamsdonk and De Vries 1995). In most crosses between species of these sections and *T. gesneriana*, only some pollen tube penetration was observed. *T. clusiana* was the only species of the subgenus *Tulipa* used, which showed more pollen tube penetration when used as pistillate parent. This was also found in crosses with *T. sylvestris* and *T. pulchella* of the subgenus *Eriostemones*. Pollen tube growth and pollen tube penetration, however, remained poor in these crosses and in crosses with *T. turkestanica*.

Perspectives for breeding

Pre-fertilization barriers were identified in this study of several interspecific tulip crosses. In crosses with no or hardly any pollen tube penetration, floral manipulation techniques might be successful for bypassing these barriers. In several crops, pre-fertilization barriers are bypassed through the application of techniques such as the cut-style method and placental pollination.

No or hardly any seeds were obtained of several interspecific crosses (Van Eijk et al. 1991, Custers et al. 1995, Van Raamsdonk et al. 1995), despite high percentages of pollen tube penetration. A study of the fertilization process and embryo and endosperm development of those crosses will be important to get insight in the number of embryos formed and, when embryos are formed, on the time of embryo abortion.

The progamic phase and embryo and endosperm development in a compatible *T. gesneriana* L. cross and in the incongruent cross *T. gesneriana* x *T. agenensis* DC.

3

Summary

The development of the embryo and endosperm has been investigated in a compatible cross within *Tulipa gesneriana* and in the incongruent cross *T. gesneriana* x *T. agenensis* at intervals of ten days, from 12 to 82 days after pollination. In these tulip crosses, the zygote gives rise to an apparently undifferentiated cell-mass, the proembryonal cell-mass, on which then a suspensor develops. The first proembryonal cell-masses were found at 22 DAP and most suspensors were observed from 32 DAP on. Subsequently a globular embryo is formed on top of the suspensor. This embryo finally elongates, giving rise to a spindle shaped embryo. Most globular and spindle shaped embryos were observed from 42 and 62 days after pollination, respectively. The cellular endosperm fills the whole embryo sac in mature seeds, except a region immediately around the embryo. Even in the compatible cross, an embryo and endosperm was not always found in ovules with pollen tube penetration. The pollen tube did not open in a number of ovules. The pollen tubes seemed to have opened in many ovules, but an embryo or endosperm was not found or only endosperm was observed. Ovules with a proembryonal cell-mass or with a globular embryo were still found at the time pods can be harvested. Fewer pollen tubes entered the ovules in the cross *T. gesneriana* x *T. agenensis* than in the compatible cross. Differences between the compatible cross and the incongruent cross were mainly found in the number of ovules with deformations in embryo and/or endosperm development. Between 87%-100% of the ovules with embryo and endosperm development showed normal development in the compatible cross, while in the incongruent cross, from 22 DAP, between 17%-56% of the ovules showed normal development. Embryos of the incongruent cross might be saved after the application of embryo rescue techniques.

Introduction

No or low numbers of seeds are obtained in tulip after crossing different species, like in many other crops. Barriers preventing the production of hybrid seeds can occur during the progamic phase (pre-fertilization barriers), fertilization and embryogenesis (post-fertilization barriers). Several pre-fertilization barriers are identified in crosses between *Tulipa gesneriana* and thirteen other species from the two subgenera of the genus *Tulipa* L. (Chapter 2). Pollen tubes penetrate the ovules in many incongruent crosses. Pollen tube penetration percentages up to 76% were found, even in crosses which have never produced seeds.

Fertilization can still be blocked after pollen tube penetration in the ovules, for example through in ovular pollen tube arrest (Williams et al. 1986, Gradziel et al. 1993) or through the failure of gametes to fuse (Laurie and Bennett 1990, Samimy 1991). After successful fertilization, post-fertilization barriers can still prevent hybridization, e.g. the growth of the embryos can be arrested or the embryos can abort in a premature stage often due to the absent or retarded development of the endosperm (Vijendra Das 1970, Dowrick and Brandram 1970, Bennett et al. 1975, Gritton and Wierzbicka 1975, White and Williams 1976, Barbano and Topoleski 1984, Thörn 1993). Hybrid breakdown and F1-sterility can also hamper interspecific hybridization.

Sayama et al. (1982) concluded that the low number of seeds obtained from the cross *T. gesneriana* x *T. fosteriana* Hoog ex W. Irving, as compared to crosses within either species, may be associated with the poor development of the endosperm and a disharmony between the embryo and endosperm. If post-fertilization barriers limit or prevent hybridization, embryo rescue techniques could be useful for the production of hybrids. Tulip embryos can be germinated through ovule culture (Custers et al. 1995). When embryos are formed, identification of post-fertilization barriers could help to determine the period in which embryo rescue techniques must be applied. However, the analysis of embryogenesis in incongruent crosses of tulip has only been reported for the cross *T. gesneriana* x *T. kaufmanniana* Regel (Custers et al. 1995), from which seeds can be produced on the plant (Van Raamsdonk et al. 1995). Only 10%-25% of the embryos of the swollen ovules from this cross continued to grow, but the sizes reached by these embryos were shorter (0.5-2.8 mm) than after self-pollination of *T. gesneriana* (2.7-3.5 mm).

Ernst (1901) described embryo and endosperm development for a compatible cross within *T. gesneriana*. Haccius and Hausner (1972) have analyzed embryo development within *T. gesneriana*, *T. altaica* Pall ex Sprengel (former name *T. kolpakowskiana* Regel (Van Raamsdonk and De Vries 1995)) and *T. tarda* Stapf. Embryo development in *T.*

clusiana DC. has been described by Wafai and Koul (1982). After fertilization, the zygote divides and grows into a cell complex, which, in the case of *T. gesneriana*, is irregularly segmented ("proembryonal cell-mass"). The suspensor is formed at the chalazal side of this cell complex. A globular embryo develops on top of the suspensor and will finally elongate in length.

Ernst (1901), Haccius and Hausner (1972) and Wafai and Koul (1982) described only regular embryo development in tulip. The only report concerning abberations during embryogenesis of compatible tulip crosses is from Sayama et al. (1982). They observed seeds with endosperm but without embryo in compatible crosses within *T. gesneriana* and within *T. fosteriana*. Aberrant embryo and/or endosperm development is observed in other crops in a number of cases, for example in *Sophora japonica* L. (O'Donnell and Bawa 1993), *Lilium pumilum* DC. (Dowrick and Brandram 1970) and *Ipomoea batatas* Lam. and *I. trifida* (H.B.K.) G. Don. (Mont et al. 1993).

The aim of our work is to identify crossing barriers occurring after pollen tube penetration into the ovules of the incongruent cross *T. gesneriana* x *T. agenensis* (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)). Seeds of this cross have never been produced despite pollen tube penetration percentages varying between 22%-85% (Chapter 2). Embryo and endosperm development was studied in the cross *T. gesneriana* x *T. agenensis* and, for reference, in a compatible cross of *T. gesneriana*.

Materials and methods

Plant material

Cultivars of *T. gesneriana* L., Christmas Marvel (2n=24) and Leen van der Mark (2n=24), were obtained from commercial growers, while *T. agenensis* DC. (CPRO-DLO number 75145) (2n=24) was derived from the *Tulipa* L. collection of CPRO-DLO.

Bulbs were planted in September-October in flats and subsequently stored at 5-9 °C for 15-18 weeks. The plants were placed in a greenhouse in January-March 1993 at a temperature of 15-17 °C. They flowered after two to three weeks.

The flowers were emasculated about two days before anthesis. One or two days after anthesis, when the stigma is receptive, the flowers were pollinated. The pollen was stored in the dark in a desiccator with silica gel for at most one month. Pollen was rehydrated at 100% relative humidity for two hours at 15 °C. Pollinations were carried out at 15 °C, which has been shown to be optimal for interspecific tulip crosses (Kho and Baër 1971). The ability of pollen to germinate was tested in liquid Brewbaker and Kwack (1963)

medium, supplemented with 10% (w/v) sucrose.

Microscopical techniques

Ovaries were collected at intervals of ten days, from 12 to 82 days after pollination (DAP). At each time interval, 3 to 5 ovaries of *T. gesneriana* 'Christmas Marvel' pollinated with *T. gesneriana* 'Leen van der Mark' were used and eight ovaries of 'Christmas Marvel' pollinated with *T. agenensis*. Each ovary was longitudinally dissected into six parts, each consisting of one row of ovules attached to the placenta.

The material was fixed in FAA (formalin : acetic acid : 50% ethanol, 5:5:9, v:v:v) and placed under vacuum for 1 hour to facilitate the penetration of the fixative. The material was then maintained for one week in FAA at room temperature. Thereafter, the material was dehydrated in an ethanol series of 30%, 50% to 70% and subsequently stored at room temperature. The ovules were cleared in Herr solution (Herr 1973). The outer and inner integuments of the ovules collected from 42 DAP were removed. The embryo and endosperm development was analyzed in one row of ovules of each ovary using differential interference contrast (DIC) microscopy.

Results

Development in the compatible cross

An average of 38% of all ovules studied of the cross 'Christmas Marvel' x 'Leen van der Mark' had normal embryo and endosperm development. Aberrant development or absence of development occurred in the other ovules. First, normal ovule organization, pollen tube penetration and embryogenesis will be presented. Thereafter, aberrant development will be described.

Morphology of the ovule and pollen tube penetration

The mature embryo sac of *T. gesneriana* contains eight nuclei: one egg nucleus, two synergid nuclei, two polar nuclei and three antipodal nuclei. The embryo sac is much broader at the micropylar side than at the chalazal pole. A large vacuole is visible in the middle of the embryo sac (Fig. 1). The embryo sac is surrounded by the nucellus, which consists of one cell layer. The embryo sac and nucellus are enveloped by an inner integument and an outer integument.

The pollen tube penetrates the micropyle, which is formed by the inner integument, and grows to the nucellus at the micropylar basis of the embryo sac. In several ovules, a pollen tube with a spherical tip was positioned between two nucellus cells. These pollen

tubes still contained the vegetative nucleus. Released pollen tube cytoplasm was found at the micropylar pole of the embryo sac of these ovules. At the chalazal end, the antipodals were present. In several other ovules, antipodals were degenerating. The pollen tube tip was bulb-shaped in these ovules and no vegetative nucleus could be detected inside the pollen tube. In ovules which contained at least two endosperm nuclei, the remains of the wall of the pollen tube tip could be recognized as a line between two nucellus cells.

Development after fertilization

About 43% of the ovules contained a zygote at 12 DAP (Table 1A). Two third of these ovules with zygotes had two endosperm nuclei (Fig. 2). The remaining one third of ovules had one, three, four or six endosperm nuclei. Until 32 DAP, a portion of the fertilized ovules were still at the zygote stage. Most of the ovules with zygotes at 22 and 32 DAP had up to 16 endosperm nuclei. Only a limited number of ovules contained a higher number of endosperm nuclei. The size of the endosperm nuclei diminished after the initial divisions. The nuclei were sometimes of different sizes. When two nuclei were present, the vacuole was situated in between them. The vacuole was smaller or had disappeared when the number of endosperm nuclei had increased. The chalazal pole had broadened after fertilization.

The zygote started to divide after the endosperm nuclei had divided several times. An apparently undifferentiated mass of cells is formed from the zygote. This structure will be called henceforth the proembryonal cell-mass (Fig. 3). The micropylar region of the embryo sac was fully filled by this proembryonal cell-mass. Ovules could contain a proembryonal cell-mass from 22 DAP on.

The endosperm nuclei were located at the periphery of the embryo sac, near the nucellus, at the stages in which a proembryonal cell-mass was present. More endosperm nuclei were situated in the broadened chalazal region than in the other parts of the embryo sac. Strings of plasma were present just below the proembryonal cell-mass (Fig. 3). Endosperm nuclei were positioned along these plasma strings, but only in the central region of the embryo sac. The divisions of the endosperm nuclei did not occur synchronously. The successive phases of cell division, e.g. from prophase until telophase, were observed from the chalazal pole towards the micropylar pole. In each region of the embryo sac, the same division stage of the endosperm nuclei was found.

The cell walls of the inner integument and outer integument of the fertilized ovules developed yellow pigmentation from 22 DAP. The cell walls of the outer integument thickened from 32 DAP.

A suspensor was found at the chalazal side of the proembryonal cell-mass from 22 DAP onward to mainly 42 DAP. The globular embryo developed on top of the

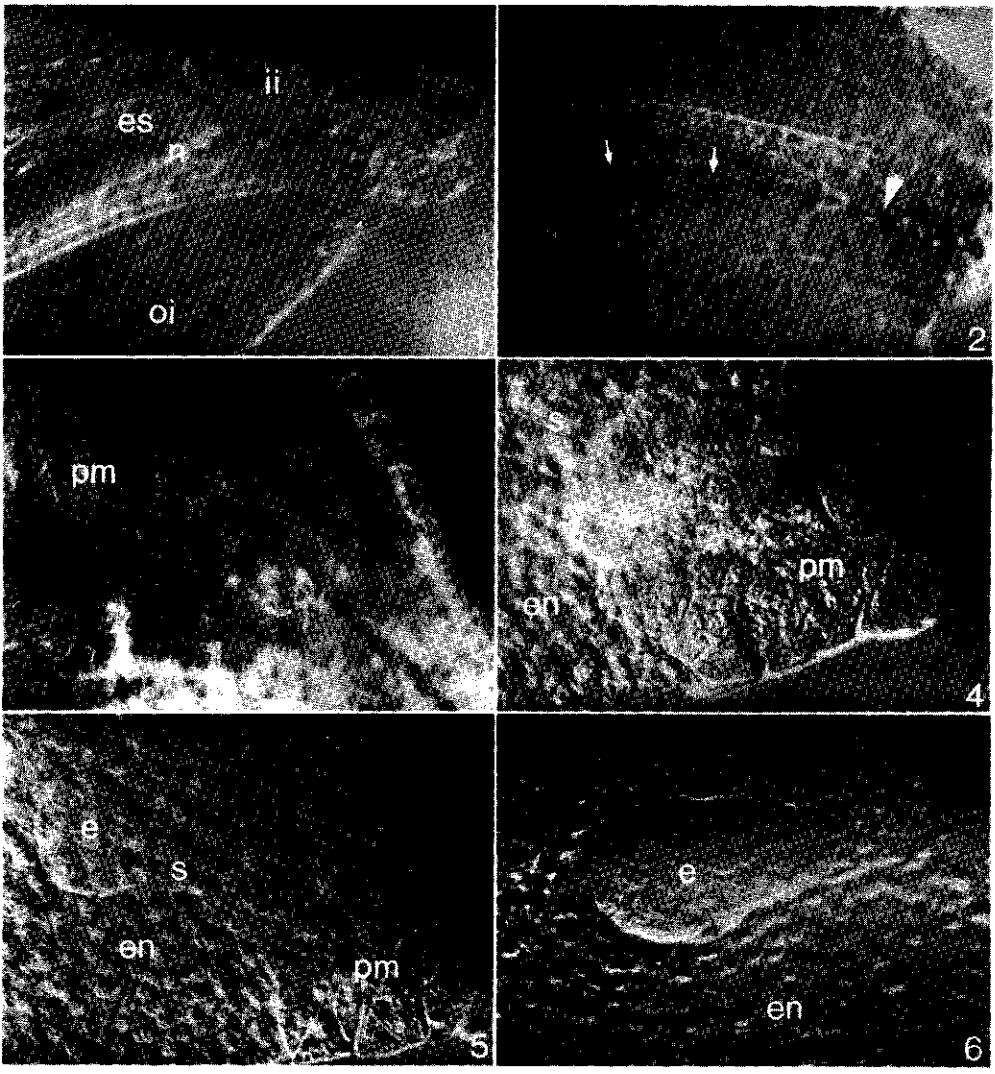


Fig. 1. Mature ovule of *T. gesneriana* 'Christmas Marvel'. The embryo sac (es) is surrounded by the nucellus (n), the inner integument (ii) and the outer integument (oi). DIC x150.

Fig. 2-6. Subsequent stages of embryogenesis of the cross *T. gesneriana* 'Christmas Marvel' x *T. gesneriana* 'Leen van der Mark'. DIC x150. **Fig. 2.** Ovule just after fertilization. The pollen tube is still discernable in the micropyle (*arrowhead*). In the embryo sac already 3 endosperm nuclei are formed of which 2 are visible in this optical section (*arrows*). **Fig. 3.** Fertilized embryo sac with proembryonal cell-mass (pm). **Fig. 4.** Proembryonal cell-mass with thickened cell walls (pm). At one place, a suspensor (s) is formed. The endosperm (en) is already cellular. **Fig. 5.** Proembryonal cell-mass (pm), with suspensor (s) and globular embryo (e), surrounded by endosperm (en). **Fig. 6.** An elongating embryo (e) surrounded by endosperm (en).

suspensor. The suspensor pushed the embryo through the plasma string, into the central part of the embryo sac. The cell walls of the proembryonal cell-mass became thickened at that stage (Fig. 4). The first globular embryos were observed at 22 DAP. However, most globular embryos were found from 42 DAP. The average diameter of globular embryos of the cross 'Christmas Marvel' x 'Leen van der Mark' was 0.10 mm at 42 DAP (se=0.002) and 0.15 mm at 52 DAP (se=0.01). The suspensor and the embryo were surrounded by endosperm (Fig. 5). From 42 DAP, the endosperm shifted from nuclear to cellular. Cellular endosperm was formed from the periphery of the embryo sac towards the centre. At that stage, the cells of the proembryonal cell-mass were surrounded by thick cell walls and nuclei were not visible. The suspensor degenerated at the advanced globular embryo stage.

The globular embryo elongated and developed into a spindle shaped embryo (Fig. 6). Most spindle shaped embryos were observed from 62 DAP. The average length of these embryos of the compatible cross was 1.0 mm (se=0.05) at 62 DAP, 1.5 mm (se=0.08) at 72 DAP and 1.9 mm (se=0.08) at 82 DAP. At the spindle shaped embryo stage, the embryo sac was almost completely filled with endosperm. The endosperm around the embryo was digested and, ultimately, the embryo was positioned in a cavity filled with fluid.

Irregular embryo and/or endosperm development

Between 171 and 350 ovules (mean=278) were studied per time interval of the cross 'Christmas Marvel' x 'Leen van der Mark'. Spindle shaped embryos were observed in only 30% and 21% of the ovules fixed at 72 and at 82 DAP, respectively (Table 1A). For the remaining ovules, the process of embryogenesis had not started, was retarded or had stopped at various prior stages of development. The percentage of ovules in certain defined developmental stages at the different fixation dates are presented in Table 1A.

The ovule was not penetrated by a pollen tube in 38% of the ovules (average of all studied ovules). Frequently, the nuclei of these unfertilized embryo sacs were degenerated at 12 DAP. The ovule was penetrated by a pollen tube, but fertilization had not taken place, in at most 5.2% of the ovules. The pollen tube stopped in these ovules in the micropyle or did enter the embryo sac, but did not discharge its content (Fig. 7, 8).
Pollen tube growth may also have stopped between the nucellus and the embryo sac. Pollen tubes in other ovules appeared to be normal, but no zygote or endosperm nuclei were detected. The percentage of such ovules increased between 12 and 42 DAP and remained around 25% from 42 to 82 DAP. In a number of ovules, only endosperm development was found. Endosperm development alone was found to increase to 11% of ovules at 32 DAP and decreased thereafter.

Table 1(A-B). The percentage of ovules divided in classes of developmental stages at different days after pollination (DAP) for the crosses *T. gesneriana* 'Christmas Marvel' x *T. gesneriana* 'Leen van der Mark' (A) and *T. gesneriana* 'Christmas Marvel' x *T. agenensis* (B).

no penetration	:	no pollen tube had entered the ovule
no fertilization	:	a pollen tube had entered the ovule, but no fertilization had occurred
no development	:	a pollen tube had entered the ovule and had opened, but no nuclei were visible in the embryo sac
zygote - spindle shaped embryo	:	different stages of embryo development were observed
only endosperm	:	endosperm was present, but no embryo

A. *T. gesneriana* 'Christmas Marvel' x *T. gesneriana* 'Leen van der Mark'

DAP	12	22	32	42	52	62	72	82
number of ovaries	4	5	4	5	3	5	3	4
no penetration	54.3	36.3	25.2	23.9	53.3	39.0	32.6	39.4
no fertilization	0.3	3.7	1.8	1.9	3.8	5.2	2.3	4.1
no development	2.4	9.1	12.7	27.3	25.8	23.4	25.6	25.8
only endosperm	0.3	0.6	11.2	5.8	1.1	0.3	0.0	0.4
zygote	42.7	29.1	1.4	0.0	0.0	0.0	0.0	0.0
proembryonal cell-mass	0.0	14.0	25.3	10.9	3.2	1.4	1.2	1.1
suspensor	0.0	6.9	21.7	13.5	0.5	0.0	0.0	0.0
globular embryo	0.0	0.3	0.7	16.7	11.8	8.7	8.1	7.9
spindle shaped embryo	0.0	0.0	0.0	0.0	0.5	22.0	30.2	21.3

The percentages of ovules with normal and aberrant embryo development from 12 to 82 days after pollination are presented in Figure 13. The percentage of ovules with developing embryos varied between 16% and 50%. It can be deduced from Figure 13 that between 87% (32 and 42 DAP) - 100% (72 DAP) of these ovules with embryo development showed normal embryo and endosperm development. Pooling all observation dates, 5.4% of the ovules with embryos showed aberrations in embryo and/or endosperm development. Most of these ovules showed abnormal endosperm (4.3%), of which the

majority also had a deformed embryo (3.0%). The development from zygote to spindle shaped embryo was retarded in several ovules (Table 1A). Seeds are mature and can be harvested around 80 DAP. A proembryonal cell-mass, instead of the expected spindle shaped embryos, was found in 1.2% of all ovules fixed at 62-82 DAP. Globular embryos were observed in 8.3% of all ovules of these classes.

B. *T. gesneriana* 'Christmas Marvel' x *T. agenensis*

DAP	12	22	32	42	52	62	72	82
number of ovaries	8	8	8	7	6	3	2	4
no penetration	68.7	71.4	61.8	45.7	53.5	51.4	46.1	31.9
no fertilization	7.4	5.1	5.2	4.1	5.7	1.6	7.7	4.9
no development	9.5	7.6	15.3	32.4	28.4	40.9	30.7	49.8
only endosperm	0.0	1.1	6.4	7.6	4.6	1.5	3.5	2.2
zygote	14.4	6.6	2.0	0.2	0.0	0.0	0.0	0.0
proembryonal cell-mass	0.0	6.6	7.1	3.9	1.8	0.5	2.6	0.0
suspensor	0.0	1.6	2.2	5.1	1.9	1.0	0.0	0.0
globular embryo	0.0	0.0	0.0	1.0	4.1	2.1	6.0	8.5
spindle shaped embryo	0.0	0.0	0.0	0.0	0.0	1.0	3.4	2.7

Development in the incongruent cross

In the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*, 2.1% of all ovules at 62-82 DAP contained a spindle shaped embryo. All the other ovules contained no embryo, or the embryo had degenerated, or the development of the embryo was retarded.

The percentages of ovules in certain defined developmental stages at the various fixation dates for the incongruent cross are presented in Table 1B. Eight ovaries were pollinated per time interval. However, several ovaries died and could not be studied. Only 2 to 4 ovaries could, therefore, be analyzed from 62 DAP. Between 117 and 608 ovules (mean=384) were studied per time interval. Several ovaries of the incongruent cross showed a retarded development.

Pollen tube penetration was not found in on average 68% of all ovules at 12-32 DAP. Of the individual flowers, pollen tube penetration percentages varied between 0%-77% at these first three fixation dates. The few ovaries left at the last three fixation dates showed

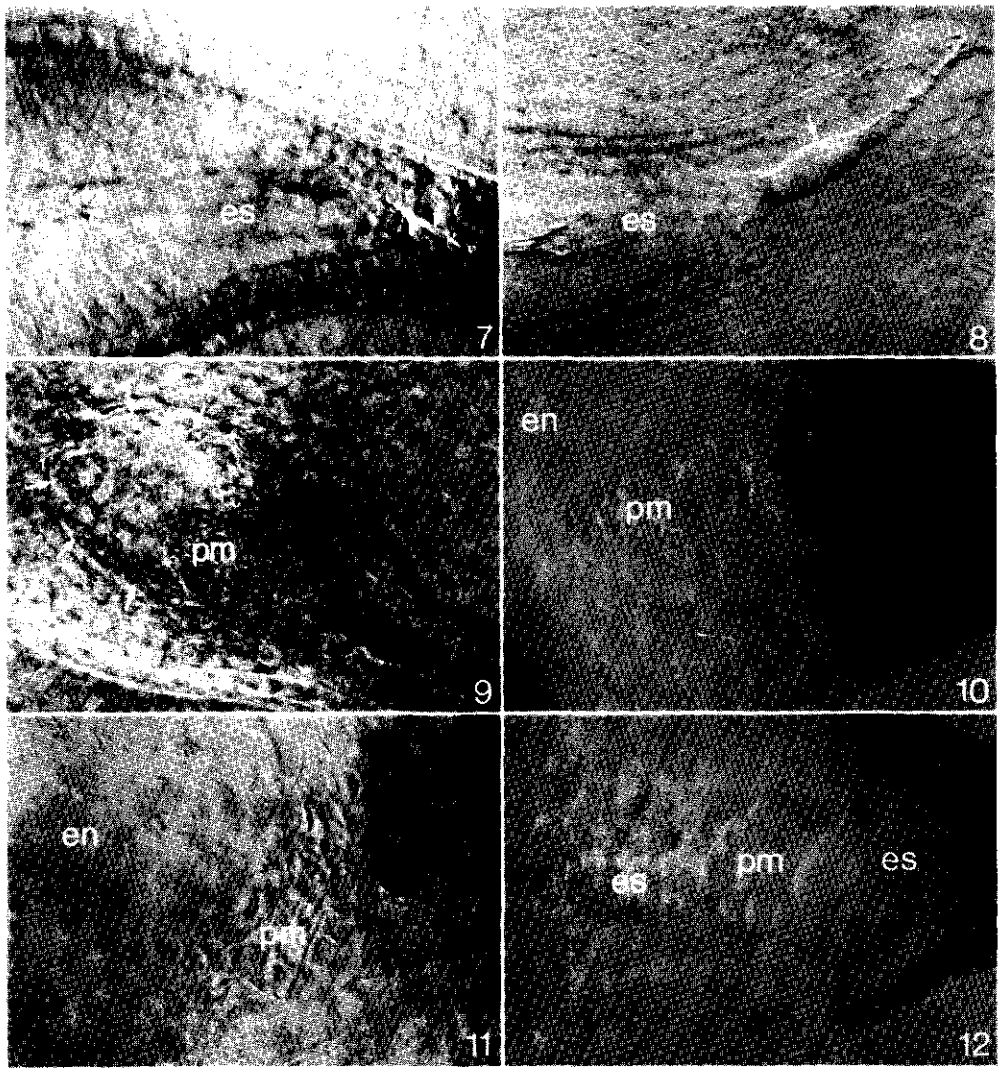


Fig. 7-8. Pollen tube entrance in the micropyle. The pollen tube (*arrow*) did not open and failed to discharge its content into the embryo sac (es). DIC x150.

Fig. 9-12. Irregularities during embryogenesis in the cross *T. gesneriana* x *T. agenensis*. DIC x150. **Fig. 9.** Ovule after fertilization. Embryo sac with proembryonal cell-mass (pm) and endosperm nuclei (*arrows*). The number of endosperm nuclei is too low in relation to the developmental stage of the embryo. **Fig. 10-11.** A proembryonal cell-mass with thickened cell walls (pm) surrounded by endosperm (en). The formation of an embryo has failed. **Fig. 12.** A proembryonal cell-mass (pm) situated in the centre of the embryo sac (es).

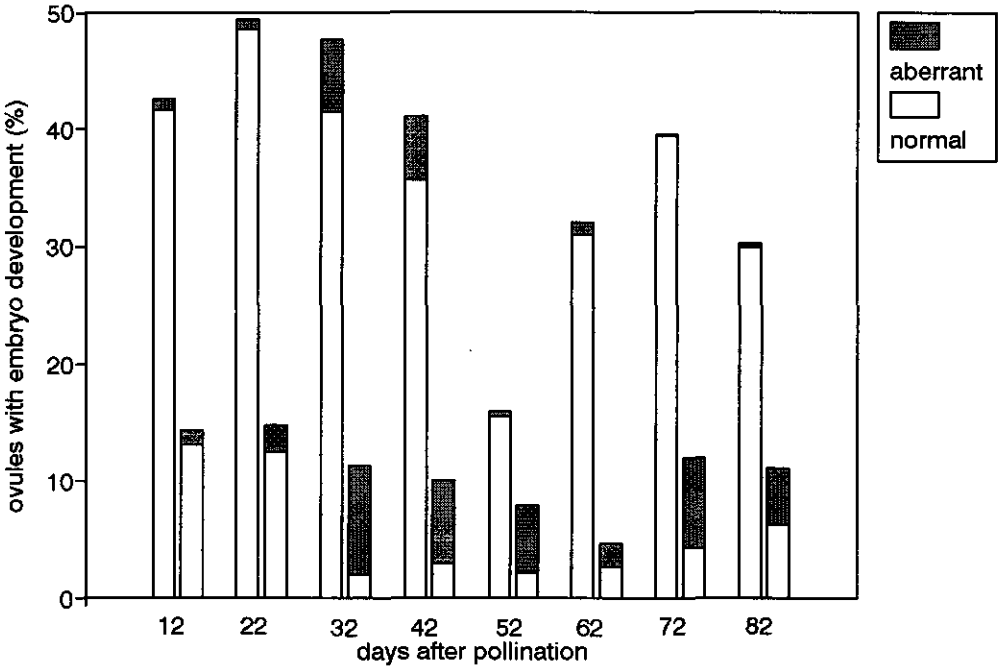


Fig. 13. The percentage of ovules with normal (white block) and aberrant (black block) embryo development from 12 to 82 days after pollination for the crosses *T. gesneriana* 'Christmas Marvel' x *T. gesneriana* 'Leen van der Mark' (left column) and *T. gesneriana* 'Christmas Marvel' x *T. agenensis* (right column).

penetration percentages between 35%-79%. It is likely that some of the ovaries with a low percentage of pollen tube penetration died at an early developmental stage. Percentages of penetrated ovules and percentages of ovules with embryo development at later fixation dates must, therefore, be interpreted with caution.

The percentage of ovules in which the pollen tube did not open (no fertilization) varied between 1.6% and 7.7%. Several aberrant pollen tube pathways were observed in the incongruent cross, which were not found in the compatible cross. Pollen tubes had penetrated the outer integument in 60 of the total of 3075 ovules studied. Ovules with penetration in the outer integument only were scored as ovules with no pollen tube penetration. More than one pollen tube had penetrated the micropyle in 5 ovules. The pollen tubes of these ovules were often coiled in the micropyle. In 15 ovules, the pollen tube did not stop growing and did not open after entering the embryo sac and showed a

curly pathway.

The percentage of ovules with apparent normal pollen tube penetration, but without visible nuclei in the embryo sac (no development), increased up to 42 DAP and fluctuated thereafter between 28%-50%, as shown in Table 1B. The percentage of ovules with only endosperm varied between 1.1% and 7.6%.

The total percentages of ovules with developmental stages from zygote to spindle shaped embryo per time interval fluctuated between 4.6%-14.8% (Fig. 13). From 62 DAP, globular embryos were observed in most ovules with embryos, instead of the expected spindle shaped embryos. The globular embryos had an average diameter of 0.13 mm (se=0.01), 0.14 mm (se=0.02), 0.24 mm (se=0.04) and 0.21 mm (se=0.02), at 52, 62, 72 and 82 DAP, respectively. At 82 DAP, 6 spindle shaped embryos were observed, with an average length of 1.3 mm (se=0.2).

It can be deduced from Figure 13 that between 84%-91% of the ovules with embryos appeared to develop normally until 12 and 22 DAP. Only 17%-56% of the ovules with an embryo showed normal embryo and endosperm development from 32 DAP. Across all observation dates, 42% of the ovules with an embryo showed deformations in embryo and/or endosperm development, while for 35%, the endosperm was abnormal. The embryo was also deformed in about half of these ovules. Normal endosperm development with a deformed embryo occurred in 7% of the ovules with aberrant development.

Various aberrant structures were found in ovules with abnormal endosperm or with deformed embryos. The nuclei of apparently deformed endosperm were, in some cases, not surrounded by a distinct envelope. Several ovules had a low number of endosperm nuclei in relation to the developmental stage of the embryo (Fig. 9). Different deformations of the embryo were observed. The proembryonal cell-mass could have thick cell walls while no suspensor and/or embryo was found (Fig. 10), or could have degenerated before a globular embryo was formed (Fig. 11), or could be situated in the middle of the embryo sac (Fig. 12), or the embryos could have an irregular outline instead of a globular or spindle shaped.

Discussion

General processes

The development from zygote to mature embryo in tulip follows another pathway than found in most other monocot angiosperms. The zygote of most species divides transversely resulting in an apical cell, which gives rise directly to the embryo, and a basal cell, which divides to form the suspensor. In tulip, the basal cell develops into a proembryonal cell complex and the apical cell gives rise to a part of this cell complex,

the suspensor and the embryo (Ernst 1901, Haccius and Hausner 1972, Wafai and Koul 1982). During (pro-)embryo development, cell multiplication proceeds from the base to the apex. The developmental sequence of cell division is precisely ordered in *T. tarda*, while it is more variable in *T. altaica* (former name *T. kolpakowskiana* (Van Raamsdonk and De Vries 1995)). Irregularities in division order are frequently found in *T. gesneriana* (Haccius and Hausner 1972). This embryogenesis with delayed embryo-differentiation has been observed in several other crops (Haccius and Bhandari 1975).

The endosperm of *T. gesneriana* is diploid (Pecenicyn 1972, Sayama 1982). In tulip species with a Fritillaria type of embryo sac, as is found in *T. gesneriana* (Romanov 1959), the polar nuclei do not fuse prior to fertilization (Pecenicyn 1972). This implies the fusion of a sperm cell nucleus with the haploid polar nucleus and not with the triploid polar nucleus. The result is a fertilized embryo sac which contains beside the zygote, two genetically and morphologically different nuclei, a diploid endosperm nucleus and a triploid polar nucleus. We did have observed nuclei of different sizes in the embryo sac.

In most crops, the first divisions of the endosperm nuclei appear to proceed synchronously, resulting in two, four, eight id endosperm nuclei. However, we observed in tulip also three and six endosperm nuclei. Pecenicyn (1972) and Sayama et al. (1982) found in tulip three or two to nine endosperm nuclei, respectively. These numbers of nuclei could be explained by non-synchronous divisions of the first endosperm nuclei, like observed in ovules with numerous endosperm nuclei. Such a waved sequence of divisions of endosperm nuclei, resulting in non-synchronous divisions, was also reported by Ernst (1901). Another explanation might be that in some cases the second polar nucleus was taken for an endosperm nucleus. This is confirmed by the differences in sizes of nuclei we have observed in the embryo sac. Three nuclei in the embryo sac could than be explained by the presence of one triploid polar nucleus and two diploid endosperm nuclei. Six nuclei could arise after non-synchronous divisions of the endosperm nuclei. Alternatively, the presence of six nuclei can be explained by two division cycli of the endosperm nuclei, resulting in 4 endosperm nuclei, and one division of the polar nucleus. However, since the endosperm is diploid, the divisions of the triploid polar nucleus must stop, if the polar nucleus would divide, because otherwise diploid endosperm cells and triploid cells would be expected in the embryo sac.

Aberrant embryo and/or endosperm development was found in both the compatible and the incongruent cross. The embryo sacs of ovules classified in the categories 'no development' and 'only endosperm' did not contain nuclei despite apparent normal pollen tube penetration or contained only endosperm nuclei. The low percentages of ovules in these categories at 12 DAP and the low percentages of ovules with aberrant embryogenesis at 12 DAP indicate the occurence of fertilization. However, after fertilization of the egg nucleus, of the polar nucleus or of both nuclei, the development of

the endosperm and/or embryo has been interrupted, leading to degeneration. This is supported by the observed changes of the inner integument and outer integument, which were found to go along with normal embryogenesis. Mature ovaries also contained ovules with retarded embryo development which indicates malfunctioning in embryogenesis.

Barriers in the incongruent cross T. gesneriana x T. agenensis

The lower penetration percentages at 12-32 DAP in the incongruent cross compared to the compatible one indicate the occurence of crossing barriers preventing pollen tube penetration. We found, as described in Chapter 2, however, pollen tube penetration percentages which were comparable for both crosses in one year (72%-76%), but which differed greatly in another year (65% and 35%, respectively for the compatible and the incongruent cross).

The percentage of ovules with pollen tube penetration and no fertilization, was somewhat higher in the incongruent cross as compared to the compatible cross. However, only in the incongruent cross some pollen tubes had penetrated the outer integument, sometimes more than one pollen tube had entered the micropyle and some pollen tubes had continued growth inside the embryo sac. These phenomena were also found in other crops. In hexaploid crosses of *Triticum aestivum* L. x *Zea mays* L., two pollen tubes entered one embryo sac in about 14% of the penetrated ovules (Laurie and Bennett 1990). Pollen tube growth inside the embryo sac cavity was found in this cross and in the crosses *Lycopersicon esculentum* Mill. x *L. peruvianum* Mill. and *Solanum melongena* L. x *S. sisymbriifolium* Lam. (Gradziel et al. 1993).

Fewer embryos were formed from the incongruent cross as compared to the compatible cross and embryogenesis was retarded. The first globular and spindle shaped embryos were found at later fixation dates and the relatively lower number of spindle shaped embryos at 82 DAP had a shorter average length than in the compatible cross. This retarded development, in combination with the higher percentages of ovules with aberrations in development from 32 DAP, mainly in endosperm formation, reveal that post-fertilization barriers occur at this level.

When the percentages of ovules without embryogenesis (no development) or with only endosperm formation are related to the percentage of ovules with pollen tube penetration, it appears that these percentages are higher in the incongruent cross as compared to the compatible cross. This will have resulted from the relative higher percentage of ovules with degeneration of the endosperm and/or the embryo.

Causes for embryo abortion

The percentage of ovules with aberrant development, mainly of the endosperm, of the incongruent cross *T. gesneriana* x *T. agenensis* increased from 22 to 32 DAP. The number of endosperm nuclei augments rapidly during this stage. This increase in endosperm must go together with a higher nutrient supply. In *Vicia faba* L., the endosperm absorbed degradation products of the maternal tissue and used these products both for its own growth and for the transport of nutrients to the embryos (Johansson and Walles 1993).

Problems with the transport of nutrients into the embryo sac could account for the increased level of malformations observed in the cross *T. gesneriana* x *T. agenensis* from 32 DAP on. In the cross *Medicago sativa* L. x *M. scutellata* (L.) Mill, a relative inactivity of dictyosomes and endoplasmic reticulum was observed at the late heart stage. According to Sangduen et al. (1983), this suggests failure of nutrient metabolism and transport in all nutritive tissues including the nucellus, integumentary tapetum, endosperm and suspensor of the embryo. In the cross *T. gesneriana* x *T. agenensis*, it might be that at early developmental stages (12 and 22 DAP) sufficient reserves were available in the embryo sac for growth of the pro-embryo and the endosperm. At that stage, when mostly zygotes were present, the division rate of the endosperm nuclei is low.

The endosperm is considered to be a tissue that supports and interacts with embryogenesis. The nature of the interaction of the embryo and endosperm is, however, not clear (reviewed by Lopes and Larkins 1993). Descriptive studies supported the view that suspensors play an active part in transporting nutrients to the young embryo proper. Factors needed for the growth of the embryo may also be synthesized in the suspensor (reviewed by Yeung and Meinke 1993). The embryo proper also seems to regulate processes in the ovule. In various angiosperms, it appears that suspensors have a developmental potential that is revealed only when an inhibitory effect of the embryo proper is removed (see review Yeung and Meinke 1993). An aberrant endosperm development could, possibly in combination with malfunctioning of the suspensor and the embryo, result in insufficient nutrient supply for the embryo. Deformations in embryo development combined with normal endosperm development do not seem to differ relatively between the compatible and the incongruent cross, confirming that the endosperm is not always the cause of embryo abortion. Differences in cell structures of the suspensor between intraspecific and interspecific crosses were found in *Lupinus* (Busmann-Loock et al. 1992) and in *Medicago* (Sangduen et al. 1983).

Problems with the nutrient supply of the embryo could cause embryo abortion, but also a retarded embryo development. The retarded embryo development seems to be initiated before 32 DAP, when most ovules contained a pro-embryo. These pro-embryos seem not to be nourished by the endosperm. However, Matzk (1991) suggested that the

developing endosperm exerts regulatory effects on the development of the pro-embryo. The retarded embryo development could, therefore, be initiated by the endosperm.

Perspectives

It is likely that endosperm degeneration is the major cause of starvation of the embryo before seed maturity in the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*. Hybrids were produced in various interspecific crosses of many crops after the application of embryo rescue techniques. The efficiency of these techniques is determined by the number of embryos formed and the time the embryos abort. The number of ovules with embryos results in turn from the pollen tube penetration percentage, which differed greatly between the analyzed crosses of *T. gesneriana* with other *Tulipa* species (Chapter 2) and the percentage of fertilization.

The presented results give an overview of embryogenesis in both a compatible cross and the incongruent cross *T. gesneriana* x *T. agenensis*. Further research can give additional information on interspecific crossing barriers in tulip. The development of embryo rescue techniques gives opportunities for the production of hybrids (Chapters 5, 6).

Application of three pollination techniques and of hormone treatments for bypassing interspecific crossing barriers in *Tulipa* L.

4

Summary

Interspecific crossing in tulip is restricted by both pre-fertilization barriers and post-fertilization barriers. These barriers must be bypassed in order to introduce traits from wild species into the cultivar assortment. Through the application of embryo rescue techniques, unique hybrids have been obtained from several interspecific tulip crosses.
Four techniques were tested for their capacity to overcome interspecific crossing barriers in tulip: the cut-style method, the grafted-ovary method, placental pollination and hormone treatments of ovaries. After the application of the cut-style method, the percentages ovules with pollen tube penetration did not increase in crosses between *T. gesneriana* L. and five other *Tulipa* species. Apparently, crossing barriers were not bypassed 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, after placental pollination, most of the ovules with pollen tube penetration showed subsequent embryo germination. 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* DC. Seed production on the plant from this cross has not been previously reported. Treating ovaries with 1% BAP seemed to have a negative effect on seed set in compatible crosses, while 1% NAA had no significant effect.

Introduction

Commercial tulips are mainly cultivars of *T. gesneriana* and of Darwin hybrids, the latter of which are obtained by interspecific hybridization between *T. gesneriana* and *T. fosteriana* Hoog ex W. Irving. The genus *Tulipa* comprises about 55 species, of which 49 are described by Van Raamsdonk and De Vries (1992, 1995). These species represent an enormous gene pool from which traits can be used for the improvement of present-day cultivars. For example, genes for disease resistance, for a short forcing period, for new flower colours and for new flower shapes could enrich the assortment. Conventional interspecific hybridization of tulips generates few if any seeds per pod (Van Eijk et al. 1991, Van Raamsdonk et al. 1995).

Pre-fertilization barriers and post-fertilization barriers are both found to occur in interspecific tulip crosses. Pre-fertilization barriers have been identified for crosses between *T. gesneriana* and thirteen other tulip species (Chapter 2). Depending on the cross, the pollen tube growth may already be stopped in the stigmatic tissue or not until they grew into a part of the ovarian cavity. These barriers result in pollen tubes penetrating between 0%-79% of the ovules (Chapter 2). Seeds have never been harvested on the plant of the cross *T. gesneriana* x *T. agenensis* (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)) (Van Eijk et al. 1991). In this cross, some embryos were formed, but they died prematurely or showed a retarded development. The endosperm showed an aberrant development or was degenerated (Chapter 3).

The post-fertilization barriers expressed during several interspecific tulip crosses can be bypassed by using embryo rescue techniques (Chapter 5). Pre-fertilization barriers in several crops can be bypassed by using methods such as the mentor pollen technique (Stettler 1968, Van Tuyl et al. 1988), gamma-ray irradiation of pollen or of the ovary (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 (Swaminathan and Rabhakrishna Murty 1957, Van Tuyl et al. 1991, Wietsma et al. 1994), the grafted-style method (Van Tuyl et al. 1991) and placental pollination (Kanta and Maheshwari 1963, Marubashi and Nakajima 1985, De Verna et al. 1987, Zenkteler 1990a). Hormone treatments have been suggested as a method to bypass 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 bypassing pre-fertilization barriers and to determine the effect of hormone treatments on the number of seeds or emerged plantlets which can be produced from interspecific *Tulipa* crosses.

Materials and methods

Plant material

T. gesneriana L. 'Christmas Marvel' was used in all crosses as maternal genotype. Compatible pollinations were carried out with 'Leen van der Mark' and 'Prominence'. *T. praestans* Hoog 'Zwanenburg', *T. kaufmanniana* Regel (CPRO-DLO number 65252-1), *T. agenensis* DC. (75145) (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)), *T. praecox* Tenore (83209), *T. altaica* Pall. ex Sprengel (68596) (former name *T. kolpakowskiana* Regel (Van Raamsdonk and De Vries 1995)) and *T. turkestanica* Regel (70650) were used for interspecific crosses. Pistils of *T. gesneriana* 'Cassini' were used as donor 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, the culture conditions and pollination methods are described in Chapter 2.

Methods

Hormone treatments and the cut-style method were applied to flowers pollinated on the plant. 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 5-7 days before anthesis. The petals and anthers were excised and the remaining parts of the flower (henceforth called 'flower') were placed in test-tubes. Additional information concerning this in vitro pollination method is described in Chapter 7. The methods for ovary-slice culture, ovule culture and verification of hybrids are presented in Chapter 5, while the microscopic techniques are presented in Chapter 2.

Experiments

Cut-style method (CSM)

For this method (CSM), the (short) style was cut just above the ovary one day after stigma receptivity and pollen was applied immediately on to the cut surface. Untreated pistils were pollinated as a control. The pollen tube growth in the pistil and pollen tube penetration into the ovules was studied for all flowers.

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 with in vitro pollination medium. Seven weeks after

pollination, the ovules from swollen ovaries were excised and placed on ovule culture medium. The pollen tube growth was analyzed in two flowers per cross.

Placental pollination

The ovaries were cut longitudinally into six sectors one day after the stigma was receptive. Each sector contained a placenta with a row of ovules and the ovary wall. The explants were transferred to medium for ovary-slice culture. Pollen (exp. I, 1992) or pre-germinated pollen (exp. II, 1993), the latter 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). Ten rows of ovules were analyzed per cross for pollen tube penetration into the ovules. The explants remained 3 weeks (exp. I) or 2 days (exp. II) in the light, before being placed in the dark. Ovule culture was carried out between 8 and 9 weeks after pollination. Only the swollen ovules were placed in vitro in exp. II. Five flowers per cross were used for normal in vitro stigmatic pollination in exp I.

Hormone treatments

The hormones 6-benzylaminopurine (BAP) and α-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. The hormone mixtures were replaced 2 weeks later by fresh mixtures in experiment II. Ovary-slice culture was applied to swollen ovaries 8 (exp. I) or 6 (exp. II) weeks after incongruent pollinations. 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 (Payne 1993).

Results

Table 1 shows the results of pollen tube growth in the pistil and pollen tube penetration in the ovules after stigmatic pollination and after using the cut-style method. The pollen tube growth in the pistil after the cut-style method was less or comparable to the pollen tube growth after stigmatic pollination, depending on the cross. The penetration percentage of pollen tubes into the ovules was generally not different between the two pollination methods. However, fewer pollen tubes penetrated the ovules in the cross

Table 1. The average percentage of total pistil length traversed by pollen tubes (% PT) and the average percentage ovules with pollen tube penetration (% PE) after normal pollination on the stigma (NP) and after cut-style method (CSM) for crosses between *Tulipa gesneriana* 'Christmas Marvel' (CM) with 'Leen van der Mark' (LvdM) or species of the genus *Tulipa* L. Per cross, also the number of analyzed pistils (N) and the minimum and maximum percentages (Range) found for the individual flowers of both percentages are presented.

Cross	Method	N	% PT	Range	% PE	Range
Leen vd Mark	NP	9	85	78-100	72	50-95
	CSM	10	48	2.0-100	60	39-81
T. praestans	NP	9	68	55-82	58	34-69
	CSM	8	34	2.1-78	59	44-65
T. kaufmanniana	NP	10	85	75-90	75	43-91
	CSM	8	70	5-100	82	70-90
T. agenensis	NP	8	72	58-81	76	60-85
	CSM	8	81	67-98	72	25-95
T. praecox	NP	9	42	14-64	21	0.8-30
	CSM	8	11	2.4-15	1.5	0.0-4.5
T. turkestanica	NP	8	9.6	2.1-24	0.4	0.0-2.6
	CSM	8	4.5	2.4-7.7	0.0	

'Christmas Marvel' x *T. praecox* after the use of the cut-style method. Nevertheless, there was an overlap of the ranges.

The pollen tubes had penetrated between 44%-100% of the total length of the donor pistils of 'Cassini' (see Fig. 1), with an average of 88%, after application of the grafted-ovary method. About one week after pollination, the donor pistils dried up, shrivelled and finally released from the mother ovaries. None of the ovules of the two analyzed mother ovaries had been penetrated by a pollen tube in crosses with 'Prominence', whereas pollen tubes had penetrated in 1.8% of the ovules in crosses with *T. kaufmanniana*. Germination of ovules in ovule culture (started 7 weeks after pollination) from the single swollen ovary from crosses with 'Prominence' and the three swollen ovaries from crosses with *T. kaufmanniana* are shown in Table 2. Of both crosses, 0.6 germinated ovules per pollinated flower were obtained.

Table 2. 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' and *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 (ovules) and of germinated ovules (germ), the percentage of germinated ovules (% ovules) and the number of germinated ovules per pollinated flower (N flower).

Cross	Flowers (A)	Ovaries	Ovules (B)	Germ (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

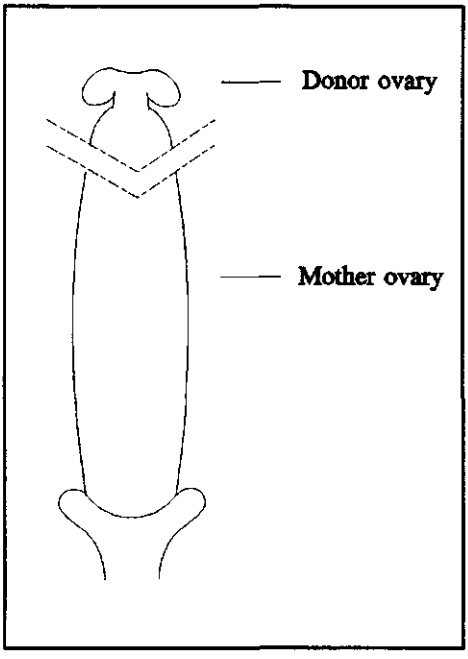


Fig. 1. The grafted-ovary method

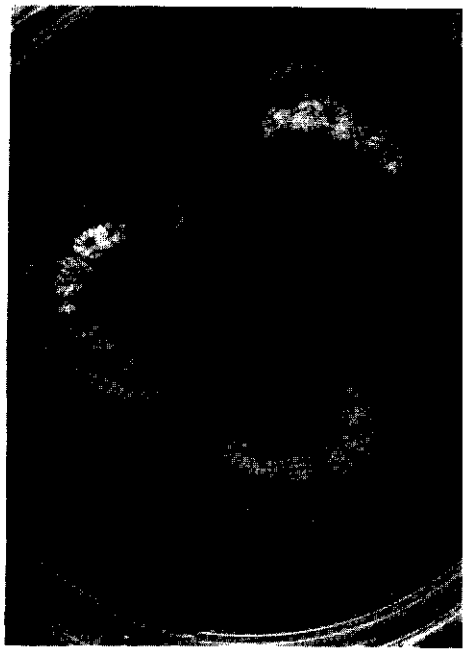


Fig. 2. Swollen ovules after placental pollination (photo 31 DAP)

The results obtained after placental pollination are shown in Table 3. After placental pollination, ovules were swollen (Fig. 2). Of all crosses, at most 1.2% of the ovules showed germination. For the compatible crosses with 'Prominence' and 'Leen van der Mark' as pollen sources, both the penetration percentage (1.8%-2.3%) and the number of seeds per pollinated flower (0.20-3.0) was lower after placental pollination, compared to stigmatic pollination on the plant and in vitro. Fewer ovules which showed germination per pollinated flower were also obtained after placental pollination as compared to the number of seeds harvested after stigmatic pollination on the plant in the cross with *T. kaufmanniana*. Finally, only one ovule showed germination from the cross with *T. altaica*. No pollen tube penetration was observed in the cross with *T. turkestanica*.

Table 3. 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; nd= not done)

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

Table 4. The lengths of the pods of *T. gesneriana* 'Christmas Marvel' at 40 days after pollination from crosses with different pollen donors (cross) after the treatment of the ovaries with no hormones or with NAA or BAP. Results were analyzed statistically by means of the t-test (LSD=10). (--: ovaries died before 40 DAP)

Cross	no hormones	1% NAA	1% BAP
unpollinated	--	61	44
Leen vd Mark	70	72	63
T. praestans	51	66	59
T. altaica	--	--	--

The average lengths of the ovaries of 'Christmas Marvel' treated with 0.1% BAP in lanolin varied between 70-76 mm at 40 DAP for crosses with 'Prominence', *T. kaufmanniana* and *T. agenensis* (exp. I, 1992). The average lengths reached at 40 DAP in crosses between 'Christmas Marvel' and 'Leen van der Mark', *T. praestans* and *T. altaica* are shown in Table 4 (exp. II, 1993). The unpollinated and untreated ovaries died before 40 DAP. The unpollinated ovaries treated with 1% BAP or 1% NAA were at 40 DAP smaller than those from the compatible cross, but they were still green and swollen. Although the hormone treatments did not promote ovary growth in the cross with 'Leen van der Mark', the application of mainly NAA enhanced ovary growth in the cross with *T. praestans*. In crosses with *T. altaica*, all ovaries were brown and shrivelled before 40 DAP.

The number of seeds or the number of germinated ovules from untreated and hormone treated (at 12 DAP) ovaries are presented in Table 5. There was no significant effect of treatment with 0.1% BAP in the crosses with 'Prominence' and *T. kaufmanniana* (exp. I). After pollination with *T. kaufmanniana*, 0.4 seeds per flower (se=0.2) germinated from the 2.4 seeds produced per flower (se=0.7) from untreated ovaries. Of ovaries treated with 0.1% BAP, 1.5 seeds per flower (se=0.5) germinated from the 2.5 seeds obtained per flower (se=0.4). In this cross, 49% of the ovules was penetrated by a pollen tube. Each of the 3 ovaries treated with 0.1% BAP produced 1 seed after maturation on the plant of the cross with *T. agenensis* (exp. I). A total of 2233 ovules of this cross were generated from 5 flowers and placed in vitro, of which 6% germinated. A pollen tube had penetrated in 13% of the ovules. For the crosses with 'Leen van der Mark' (exp. II),

the percentage of the ovules penetrated by a pollen tube varied between 54% and 70% per flower. 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 total 3447 ovules placed in vitro germinated. The percentage ovules with pollen tube penetration per flower varied between 6% and 8% for this cross.

Table 5. The number of seeds per pod 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.). (nd = not done).

Exp.	pollen donor	pl/ iv	no hormones	BAP 0.1%	BAP 1%	NAA 1%
I	Prominence	pl	196	192	nd	nd
	T. kaufmanniana	pl	2.4	2.5	nd	nd
	T. agenensis	pl	0.0	1.0	nd	nd
		iv	nd	27	nd	nd
II	Leen vd Mark	pl	179	nd	109	129
	T. praestans	iv	0.0	nd	0.0	0.0

Discussion

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

The cut-style method did not promote pollen tube growth. The desired effect of bypassing barriers in the stigma or style, which are found in crosses with for example *T. turkestanica*, has not been achieved. Apparently, pre-fertilization barriers can not be

circumvented by this method in tulip, in contrast to what is found in *Lilium* and *Fritillaria* (Van Tuyl et al. 1988, Wietsma et al. 1994). Pollen tube growth of these latter crops is mostly inhibited in the style following incongruent crosses (Ascher and Peloquin 1968, Wietsma et al. 1994).

Pollen tubes frequently failed to grow to the bottom of the donor ovary after the use of the grafted-ovary method in tulip. 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 (Chapter 2). A critical point was the passage of pollen tubes from the donor ovary into the mother ovary, which was also observed for the grafted-style method in *Lilium* (Van Tuyl et al. 1991). In *Lilium*, the grafted-style method was used, because in this species it was found that the cut-style method resulted in reduced pollen tube penetration percentages of the ovules. In *Tulipa*, however, this disadvantage of the cut-style method was not found. Thus, the application of the grafted-ovary method has in tulip no advantages over the cut-style method.

Pollen tubes had penetrated a maximum of 2.3% of the ovules after placental pollination. These percentages were not higher than those obtained after stigmatic pollination (Chapter 2). Thus, pre-fertilization barriers seem not to be bypassed. A high percentage of ovules with pollen tube penetration after placental pollination showed embryo germination. Apparently, a high percentage of tulip embryos can be rescued by using the method applied after pollination of the placenta. One ovule showed germination in the cross with *T. altaica*. However, this embryo died and was, therefore, not verified on hybrid character. Crosses between *T. gesneriana* and *T. altaica* have never succeeded, not even after the application of embryo rescue techniques (Chapter 5).

The pollen tube penetration percentages obtained after placental pollination might be increased after optimization of the pollination procedure. Zenkteler (1990a) observed pollen germination only on those ovules which were not wet and Janson (1993) reported higher penetration percentages when the ovules were placed on filter paper. In experiment II, the ovules were covered with fluid by the use of pregerminated pollen in BK-medium. Several other factors can be investigated to optimize the procedure of placental pollination, for example, the age of the ovaries (Balatkova and Tupy 1972), the culture medium (Kameya and Hinata 1970) and the type of placental pollination (Zenkteler 1990a, Slusarkiewicz-Jarzina and Zenkteler 1983, Marubashi and Nakajima 1985). Further research needs also to be carried out on the transfer of the seedlings into the soil, since many germinated embryos showed abnormalities, similar to that reported by Custers et al. (1992).

Seed set after pod maturation on the plant was obtained in the cross *T. gesneriana* x *T. agenensis* after treatment of the ovaries at 12 DAP with 0.1% BAP. The production of

seeds on the plant from this cross has never been previously reported. Seed set in compatible crosses was not affected by this hormone treatment. For the cross *T. gesneriana* x *T. agenensis*, we found that 13% of ovules were penetrated by a pollen tube and that 6% of the ovules showed germination after in vitro culture (8 weeks after pollination). These are higher levels than reported in other experiments, where only 3% of the ovules germinated when cultured from 7 weeks after pollination, while 21% showed pollen tube penetration (Chapter 5). This suggests that application of 0.1% BAP is potentially an useful method for achieving interspecific tulip hybrids.

The seed set did not differ significantly in the compatible cross between the untreated ovaries and the ovaries treated with 1% BAP or 1% NAA. However, three of the seven ovaries treated with 1% BAP died prematurely. Compared to the use of 0.1% BAP, these poorer results may be caused by the higher concentration of BAP or through the accumulative effect of the double application of the hormones (12 and 26 DAP). However, the treatment of *Lilium* ovaries with 1% naphthalene acetamide reduced seed set in several crosses, while in others seeds were only obtained after application of this hormone (Emsweller and Stuart 1948). No hybrid seeds were generated in both treated and untreated ovaries of the cross with *T. praestans*. Of this same cross, 21% of the ovules were penetrated by a pollen tube in another experiment and finally 0.2 ovules per flower showed germination (embryo-rescue started 3 and 5 weeks after pollination) (Chapter 3). The poor results in the present crosses with *T. praestans*, reported here, could be due to the low penetration percentage (6%-8%) and/or the later date of embryo rescue (6 weeks after pollination). Further studies are needed before conclusions can be drawn on the effect of the treatments with 1% NAA and 1% BAP.

Four methods have been examined for their ability to bypass pre-fertilization barriers or post-fertilization barriers. The cut-style method and the grafted-ovary method appeared not to bypass 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, might give an better idea on the practical use of hormone treatments for tulip breeding.

The effect of ovule age on ovary-slice culture and ovule culture in intraspecific and interspecific crosses with *Tulipa gesneriana* L.

5

Summary

The efficiency of two embryo rescue techniques, direct ovule culture and ovary-slice culture followed by ovule culture, is studied in tulip. The cultures were started at 2-9 weeks after pollination on the plant. Intraspecific *Tulipa gesneriana* crosses and interspecific crosses of this species with *T. praestans* Hoog, *T. de novo species A*, *T. kaufmanniana* Regel, *T. agenensis* DC. and *T. altaica* Pall. ex Sprengel were made. Germinating embryos have been obtained from cultures started 2 weeks after pollination. The percentage of germinating embryos increased, in most cases, significantly with a more advanced developmental stage of the embryos at the start of the culture. The analysis of embryogenesis in compatible intraspecific *T. gesneriana* crosses showed that the lower germination percentage in the cultures started at 3 weeks after pollination, in comparison to cultures started at 5 weeks after pollination, was caused by a higher rate of embryo abortion and by a retarded embryo development. The germination percentages for ovary-slice culture followed by ovule culture started at various dates was for some culture dates comparable to direct ovule culture. For other culture dates, it was significantly higher. The length of the period of ovary-slice culture prior to ovule culture mostly did not affect the germination percentage. By using ovary-slice culture and/or ovule culture, unique hybrids have been obtained from the crosses *T. gesneriana* x *T. agenensis* and *T. gesneriana* x *T. praestans*.

Introduction

The tulip assortment used for cut flower production, mainly cultivars of *T. gesneriana*, could be improved considerably by introducing traits, such as disease resistance and a shorter forcing period, from other tulip species. The genus *Tulipa* L. comprises approximately 55 species, of which 49 are described by Van Raamsdonk and De Vries (1992, 1995). However, *T. gesneriana* has been crossed successfully with only 12 other tulip species by using conventional breeding methods, for example with *T. kaufmanniana* (Van Eijk et al. 1991, Van Raamsdonk et al. 1995). In most interspecific crosses, crossing barriers prevent the formation of hybrids.

Pollen tubes were found to penetrate the ovules in crosses between *T. gesneriana* and other tulip species (Kho and Baër 1971, Custers et al. 1995, Chapter 2). Pollen tube penetration percentages up to 76% were observed, even in crosses which have never been successful, such as *T. gesneriana* x *T. praestans* and *T. gesneriana* x *T. agenensis* (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)) (Chapter 2). In the latter cross, embryos were formed, but these died prematurely or showed a retarded development (Chapter 3). The endosperm showed a disturbed development or degenerated in most cases (Chapter 3). If embryos are formed after pollen tube penetration, like in the cross *T. gesneriana* x *T. agenensis*, post-fertilization barriers seem to prevent hybridization due to embryo degeneration.

Embryo rescue techniques might be applied to enable the embryos to survive in those cases in which embryos abort prematurely. For this purpose, ovary culture, ovary-slice culture, ovule culture and embryo culture can be applied. The use of methods for embryo rescue has been reviewed by Raghavan and Srivastava 1982, Rangan 1982, 1984, Collins and Grosser 1984, Williams 1987, Williams et al. 1987 and Sharma et al. 1996. The number of embryos which can be saved from a specific cross is influenced by the developmental stage at which the embryos are placed in vitro. In most cases, the embryos must have a minimum age before they can be rescued (Mukherjee et al. 1991, Kishi et al. 1994). The success of survival is often higher with increasing embryo age (Wakizuka and Nakajima 1975, Scemama and Raquin 1990, Niederwieser et al. 1990, Kobayashi et al. 1993). Custers and Bergervoet (1990) observed, however, an optimum age for embryo recovery after self-pollination of *Cucumis* spp. The developmental stage at which embryos can be recovered also depends on the type of embryo rescue technique applied (Przywara et al. 1989, Nomura and Oosawa 1990, Espinasse et al. 1991, Van Tuyl et al. 1991, Lazaridou et al. 1993).

The application of embryo rescue techniques in tulip breeding has been reported by Van Tuyl et al. (1990) and Custers et al. (1992, 1995). With ovule culture, more

embryos could be rescued from an earlier developmental stage (4 weeks after pollination) as compared to embryo culture. Also more embryos could be rescued at each culture date with ovule culture than with embryo culture (Custers et al. 1995). Preliminary research showed that embryos cultured 1 week after pollination could even be rescued by using the ovary-slice culture technique (Van Tuyl et al. 1990).

We present in this paper the results of experiments with the ovary-slice culture technique and the ovule culture technique in tulip. We compared the efficiency of direct ovule culture and ovary-slice culture followed by ovule culture, for cultures started at different dates after pollination. For the experiments we used intraspecific crosses within *T. gesneriana* and interspecific crosses between *T. gesneriana* and *T. praestans*, *T. de novo species A*, *T. kaufmanniana*, *T. agenensis* and *T. altaica* (former name *T. kolpakowskiana* Regel (Van Raamsdonk and De Vries 1995)). *T. gesneriana* was used as the maternal genotype in interspecific crosses, because the percentages ovules with pollen tube penetration proved to be higher as compared to the reciprocal crosses (Chapter 2). We also studied embryogenesis in vitro for ovary-slice culture followed by ovule culture for intraspecific *T. gesneriana* crosses.

Materials and Methods

Plant material and pollination method

The cultivars of *T. gesneriana* L., Christmas Marvel, Leen van der Mark and Prominence and *T. praestans* Hoog 'Zwanenburg' were obtained from commercial stocks. *T. de novo species A* (CPRO-DLO number 71331-10), *T. kaufmanniana* Regel (65252-1), *T. agenensis* DC. (75145) (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)) and *T. altaica* Pall. ex Sprengel (68596) (former name *T. kolpakowskiana* Regel (Van Raamsdonk and De Vries 1995)) were derived from the CPRO-DLO *Tulipa* L. collection. *T. de novo species A* is an unpublished species which resembles *T. praestans* in morphology except for flower colour, which is yellow instead of red.

Bulbs were planted in September-October in flats and then stored at 5-9 °C for 15-18 weeks. The plants were placed in a greenhouse in January-March at a temperature of 15-17 °C and flowered after two to three weeks. The flowers were emasculated about two days before anthesis. One or two days after anthesis, the stigma is receptive and the flowers were pollinated with fresh pollen. In some cases pollen was used, that had been stored in the dark for at most one month in a desiccator with silica gel. Pollen was rehydrated before pollination at a 100% relative humidity for two hours at 15 °C.

Experiments

Two experiments were performed with compatible intraspecific crosses, using pods (henceforth called ovaries when used for in vitro culture) of crosses between 'Christmas Marvel' and 'Leen van der Mark'. Pollinations were carried out in March 1991 (experiment I (exp. I)), March 1992 (experiment II (exp. II)) and February 1993 (experiment III (exp. III)). Ovaries were collected 2 to 9 weeks after pollination (WAP). Ovules were dissected directly from the ovaries at 3, 5, 7 and 9 WAP and cultured. Ovary-slice cultures were started at 2, 3, 4 and 5 WAP. The ovules were excised from these ovary-slices at 5, 7, 9 and 11 WAP for subsequent ovule culture. Between 9-13 ovaries of 'Christmas Marvel' and 6 ovaries of 'Leen van der Mark' (exp. I only) were used for each treatment. During each experiment, 12 to 28 pods per cross matured on the plant to determine the number of seeds obtained after pollination on the plant. Results were statistically analyzed by means of the t-test (Payne et al. 1993).

Five interspecific crosses were studied, using *T. gesneriana* as the maternal genotype and *T. praestans* 'Zwanenburg', *T. de novo species A*, *T. kaufmanniana*, *T. agenensis* and *T. altaica* as the pollen donor. Pollinations were carried out in 1991 (crosses with *T. praestans* only) and in 1993 (all crosses). Ovary-slice culture was started at 3 and at 5 WAP, followed by ovule culture 6 or 4 weeks later, respectively (9 WAP). In crosses with *T. agenensis*, also direct ovule culture was applied at 3, 5 and 7 WAP. The total numbers of ovaries used for embryo rescue in the different crosses are summarized in Table 2. Twelve pods from crosses with *T. agenensis* matured on the plant.

Plant treatments

Ovaries were collected 2-9 WAP and sterilized by soaking the ovaries during 1 minute in 70% ethanol, followed by a 20 minute rinse with a commercial bleach containing 2% chlorine and subsequently, three rinses with sterile water. For ovary-slice culture, ovaries were cut transversely in eight sections and placed with the basal cut end on medium. Four ovary-slices were placed per Petri dish of 9 cm diameter. Ovules used for ovule culture were dissected directly of the ovaries or of the ovary-slices. All the ovules of 1 (exp. I), 2 (exp. II) or the 3 carpels (other experiments) were cultured per ovary(-slice). In each 9 cm Petri dish, 50 ovules at most were placed separately.

All cultures were placed in a climate room at 15 °C until 16-17 WAP. Subsequently they were placed at 5 °C to induce germination. Twelve weeks later (28-29 WAP), the Petri dishes were transferred to 15 °C. All cultures were incubated in the dark. These conditions are the most optimal culture conditions for seedling and bulblet formation in tulip as found by Custers et al. (1992). From July to January, once or twice a month, ovules which showed germination were removed from the Petri dishes.

Media

All media contained the organic and inorganic components at half strength and the vitamins and myo-inositol at full strength of the medium of Murashige and Skoog (1962), supplemented with 2.0 mg/l glycine (indicated as ½MS). The medium for ovary-slice culture was composed of ½MS supplemented with 9% (w/v) sucrose, 1 mg/l α-naphthalenacetic acid (NAA), the fungicide Nystatin (50 mg/l) (Duchefa), the antibiotics Vancomycin and Cefotaxime sodium (both 100 mg/l) (Duchefa) and 0.7% bacteriological agar (Oxoid) at pH=6.0. The same medium was used for ovule culture, except for sucrose (3% (w/v)), antibiotics (both 50 mg/l) and for pH (5.6). The pH was adjusted before the addition of agar and before autoclaving the medium during 20 min at 120 °C. Nystatin was dissolved in dimethyl sulphoxide (DMSO). NAA, Nystatin and antibiotics were filter sterilized and added after autoclaving.

Microscopical observations

The development of the embryo and endosperm in vitro was studied in the compatible intraspecific cross 'Christmas Marvel' x 'Leen van der Mark' at 4, 6, 8, 12, 16, 24, 32 and 42 WAP. Flowers were pollinated in March 1993. Pollen tube penetration in the ovules was studied in 2 pistils, as described in Chapter 2. Five pods matured on the plant. Twelve ovaries per treatment were used for ovary-slice culture, applied at 3 and at 5 WAP, both followed by ovule culture 6 or 4 weeks later, respectively (9 WAP). Two to 6 Petri dishes containing 4 ovary-slices or 50 ovules at most were studied per time interval per treatment. Only the ovules with thickened embryo sac and integuments (henceforth called swollen ovules), as these structures indicate fertilization has taken place (Chapter 3), were studied. This resulted in the analysis of 26-77 ovules per time interval for each treatment. The method used for microscopical observations is described in Chapter 3.

The pollen tube growth in the pistil and the penetration of the pollen tubes in the ovules were studied in 7 pistils of both the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis* and the cross *T. gesneriana* 'Christmas Marvel' x *T. praestans* 'Zwanenburg', as described in Chapter 2.

Verification of hybrids

The hybrid character of several bulblets obtained from interspecific crosses was verified by the polymorphisms in the isozyme esterase, as described for tulip by Booy et al. (1993).

Table 1. The germination percentage after direct ovule culture and after ovary-slice culture followed by ovule culture started at different weeks after pollination (WAP) in crosses between *T. gesneriana* 'Christmas Marvel' (CM) and T. gesneriana 'Leen van der Mark' (LvdM), executed in 3 years (exp. I-III) and in the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*. For each cross and experiment, the percentage of ovules developed into seeds on the plant (Plant) is also shown. The LSD, as determined with the t-test, is presented for the different experiments. (nd= not determined)

Start of culture (WAP)	weeks ovules cultured in ovary-slices	LvdM x CM	CM x LvdM			CM x *T. agenensis*
		exp I	exp I	exp II	exp III	
Ovule culture:						
3		4.7	2.3	nd	1.6	0.2
5		7.7	13.1	nd	11.1	1.3
7		18.8	26.8	nd	29.1	3.1
9		44.0	41.3	nd	nd	nd
Ovary-slice culture and ovule culture:						
2	3	2.8	2.0	nd	nd	nd
2	5	5.1	8.8	nd	nd	nd
2	7	4.2	9.0	20.8	nd	nd
3	6	nd	nd	20.1	5.0	0.1
4	5	nd	nd	38.5	14.8	nd
5	2	13.2	14.3	26.0	nd	nd
5	4	14.1	25.0	29.0	20.3	1.2
5	6	nd	nd	23.7	nd	nd
Plant:		35.4	38.5	64.8	41.1	0.0
LSD		7.7	7.7	7.5	6.0	0.9

Results

Ovule culture versus the combination of ovary-slice culture with ovule culture

The mean germination percentages of the ovules after using different embryo rescue techniques, together with the percentages of ovules developed into seeds on the plant, are presented in Table 1. Pods matured on the plant in about 12 weeks. Embryos of both the compatible intraspecific crosses and the incongruent cross *T. gesneriana* x *T. agenensis* germinated in all in vitro treatments. For the intraspecific crosses between 4%-100% of the ovules which developed on the plant into seeds could be recovered in vitro, depending on the developmental stage of the ovules at the start of the in vitro culture.

The cross 'Christmas Marvel' x 'Leen van der Mark' has been made in three years (exp. I-III). In most cases of exp. I and exp. III, more embryos germinated when the embryo rescue techniques were started at later dates after pollination. However, in exp. II, ovary-slice culture started at 4 WAP resulted in more germination than the cultures started at 2, 3 and 5 WAP. When ovule culture was applied with ovules from ovary-slices, the time ovules had remained in the ovary-slice either had no effect on the germination percentage or the germination percentage increased with a longer period of ovary-slice culture. The combination of ovary-slice culture with ovule culture resulted in all crosses in a germination percentage that was for some culture dates comparable to direct ovule culture or, for other culture dates, significantly higher (exp. I and exp. III). The percentage of ovules which developed on the plant in mature seeds did not differ between exp. I and exp. III. However, a higher percentage of seeds was obtained in exp. II.

The cross 'Christmas Marvel' x 'Leen van der Mark' and the reciprocal cross have both been made in exp. I. Two out of the ten treatments, showed a significantly higher germination percentage when 'Christmas Marvel' was used as the mother. The germination percentages did not differ between the two crosses for the other treatments.

Germination was found from June to January. However, depending on the experiment, 81% to 86% of the embryos germinated from August to November. The experiments were finished in January.

Interspecific crosses

The germination percentage increased significantly from 0.2% for 3 WAP to 3.1% for 7 WAP in the cross 'Christmas Marvel' x *T. agenensis* (Table 1). Between the two embryo rescue methods, the germination percentages did not differ. On average 5.4 ovules germinated per flower for all treatments taken together (Table 2). No seeds could be harvested after pod maturation on the plant. Analysis of pollen tube growth showed that

the pollen tubes had traversed 72% of the total pistil length and had penetrated in 24% of the ovules. Nine plantlets were tested on their hybrid origin with isozyme analysis and proved to be intermediate between both parents.

The number and the percentages of germinated embryos, taken together over all treatments, of the interspecific crosses are presented in Table 2. On average 0.2 and 0.3 embryos germinated per flower of the crosses *T. gesneriana* 'Leen van der Mark' x *T. kaufmanniana* and *T. gesneriana* 'Christmas Marvel' x *T. praestans* 'Zwanenburg', respectively. The number of germinated embryos of both crosses was too small to detect differences between the two applied embryo rescue techniques. In the cross 'Christmas Marvel' x *T. praestans*, the pollen tubes traversed 67% of the total pistil length and penetrated in 21% of the ovules. One plantlet was tested on hybrid origin of the ten embryos obtained. This plantlet showed, in comparison with both parents, an intermediate character (Fig. 1).

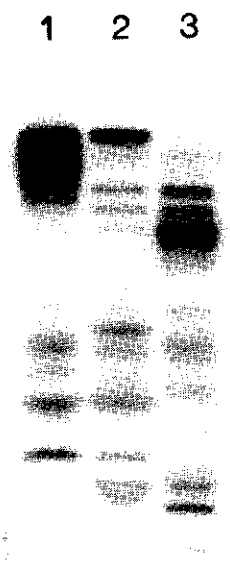


Fig. 1. Esterase banding pattern showing the hybrid character of a bulb obtained from the cross *T. gesneriana* x *T. praestans*. (1= *T. gesneriana* 'Christmas Marvel', 2= hybrid, 3= *T. praestans* 'Zwanenburg').

No embryo germination was obtained from the cross *T. gesneriana* 'Leen van der Mark' x *T. praestans* 'Zwanenburg', from the cross *T. gesneriana* 'Prominence' x *T. de novo species A* and from the cross *T. gesneriana* 'Prominence' x *T. altaica*. In the cross with *T. de novo species A*, of the 8 ovaries which would be used for ovary-slice culture started at 5 WAP, 5 had died between 3 and 5 weeks after pollination. Twelve of the 28 ovaries of the cross with *T. altaica* had died within 3 weeks after pollination.

Table 2. Germination of embryos of crosses between the cultivars Christmas Marvel (CM), Leen van der Mark (LvdM) and Prominence (Pro) of *T. gesneriana* with several tulip species. For each cross, the total number of pollinated flowers (a), the total number of ovules used for ovule culture (b), the total number of germinated embryos (c), the percentage of ovules showing germination (c/b) and the number of germinated embryos per pollinated flower (c/a) are shown.

Cross	number of flowers (a)	number of ovules (b)	germination (c)	germination percentage (c/b)	germ. per flower (c/a)
CM x *T. agenensis*	65	30486	353	1.2	5.4
LvdM x *T. kaufmanniana*	34	13387	10	0.07	0.3
CM x *T. praestans*	52	22973	10	0.04	0.2
LvdM x *T. praestans*	20	8042	0	--	--
Pro x *T. de novo species A*	28	337	0	--	--
Pro x *T. altaica*	15	996	0	--	--

Embryo development in a compatible cross

The results of the analysis of 520 ovules, cultured at 3 or at 5 WAP in ovary-slices both followed by ovule culture at 9 WAP, are presented in Table 3(A-B). Only the swollen ovules were studied, which means that 13% of all ovules of ovary-slice cultures started at 3 WAP were analyzed and 23% of cultures initiated at 5 WAP. Analysis of pollen tube penetration in two intact flowers revealed that in 55% of all ovules a pollen tube had entered.

All ovules studied showed normal pollen tube growth. However, no (pro-)embryo and endosperm were found (37%-76%) when ovary-slice culture was started at 3 WAP. This percentage was lower for cultures started at 5 WAP (5%-34%). The percentage of ovules with endosperm only ranged between 0%-26% for both treatments.

Mainly pro-embryos were found in ovules with embryogenesis at the first dates of analysis (4-8 WAP) in cultures started at 3 WAP, whereas ovules contained pro-embryos and globular embryos in cultures started at 5 WAP. The diameter of the globular embryos found at the various observation dates was comparable for both treatments (data

not shown). From 12 WAP on, which is normally the time of seed harvest on the plant, the percentage of ovules with spindle shaped embryos was in general higher for cultures started at 5 WAP than for cultures started at 3 WAP. Embryo germination was found from 32 WAP onward. At 42 WAP, however, also non-germinated spindle shaped embryos were found. Even globular embryos were still present at 32 and at 42 WAP. Only 2.3% of the ovules showed germination at the end of all cultures started at 3 WAP. For the cultures started at 5 WAP, 23% of the ovules showed germination. About 37% of the ovules had developed into seeds (179 seeds per flower) of the pods ripened on the plant.

Table 3(A-B). The percentage of swollen ovules divided in classes of developmental stages at different weeks after pollination (WAP) for the intraspecific cross 'Christmas Marvel' x 'Leen van der Mark' after the application of ovary-slice culture at 3 and at 5 WAP, both followed by ovule culture 6 or 4 weeks later (9 WAP), respectively.

no development	:	a pollen tube had entered the ovule and had opened, but no nuclei were visible in the embryo sac
pro-embryo - germinated embryo	:	different stages of embryo development
only endosperm	:	endosperm was present, but no embryo

A. Ovary-slice culture at 3 WAP, followed by ovule culture 6 weeks later (9 WAP).

	Ovary-slice culture		Ovule culture				
WAP	4	8	12	16	24	32	42
no development	45	37	49	50	76	73	40
pro-embryo	50	30	10	4	6	0	0
globular embryo	5	11	27	12	12	3	10
spindle shaped embryo	0	0	5	8	6	9	0
germinated embryo	0	0	0	0	0	6	50
only endosperm	0	22	9	26	0	9	0

B. Ovary-slice culture at 5 WAP, followed by ovule culture 4 weeks later (9 WAP).

	Ovary-slice culture		Ovule culture				
WAP	6	8	12	16	24	32	42
no development	5	29	25	34	9	22	8
pro-embryo	43	5	7	0	0	0	0
globular embryo	38	40	16	12	3	9	8
spindle shaped embryo	0	5	42	39	71	10	13
germinated embryo	0	0	0	0	0	54	71
only endosperm	14	21	10	15	17	5	0

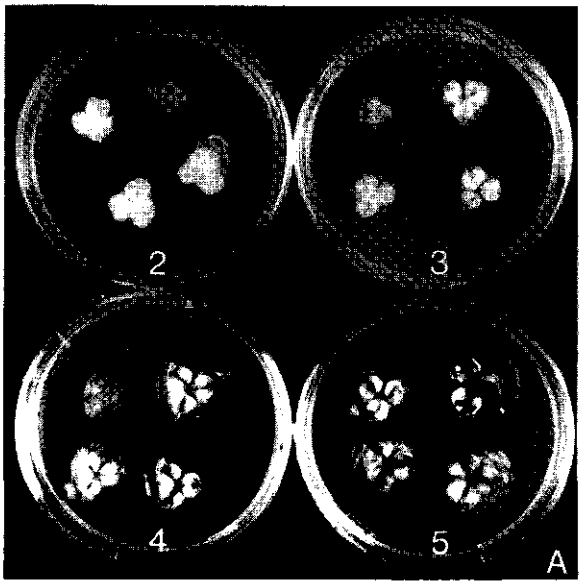
Several abnormalities in (pro-)embryo and/or endosperm development were found in ovules with embryogenesis. The embryo and/or endosperm showed abnormalities in 40% of all (pro-)embryo containing ovules cultured at 3 WAP, whereas this percentage was 17% in cultures started at 5 WAP. The endosperm and the integuments seemed in both treatments to be more rigid than from seeds developed on the plant. The size of ovary-slices and of the ovules did not seem to increase during the culture period (Fig. 2(A-B)).

Discussion

Culture date

Embryos were recovered from cultures started 2-9 weeks after pollination (WAP) (Table 1). Since most pollen tubes penetrate the ovules between 3-9 days after pollination, fertilized embryo sacs are between 5-11 days old at 2 WAP (Chapter 2). The number of germinated embryos of the compatible crosses increased in exp. I and exp. III as the cultures were started at later dates. This is in accordance with the results obtained for ovule culture with compatible *T. gesneriana* crosses (Custers et al. 1995).

An optimum stage for starting ovary-slice culture was, however, found in experiment II. The highest germination percentage was observed when ovary-slice culture was started at 4 WAP. The germination percentages for cultures started 2 to 4 WAP were in this experiment much higher in comparison to those found in the other experiments. These higher germination percentages are in agreement with the higher percentages of ovules



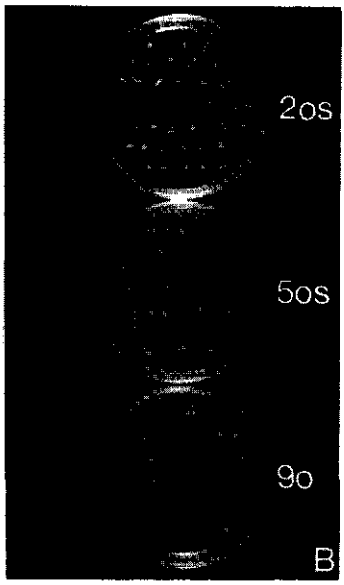


Fig. 2(A-B). Ovary-slice culture and ovule culture of the cross *T. gesneriana* 'Leen van der Mark' x *T. gesneriana* 'Christmas Marvel'. **A.** Ovary-slice cultures at 7 WAP, started 2, 3, 4, 5 WAP. **B.** Ovule cultures at 10 WAP with ovules from ovary-slice cultures started 2 (2os) or 5 (5os) WAP followed by ovule culture 7 or 4 weeks later (9 WAP), respectively, or from direct ovule culture at 9 WAP (9o).

which have developed into viable seeds in pods matured on the plant. An important factor which influences the germination percentage is the developmental stage of the embryos at the start of the culture. A more rapid development of the embryos in pods on the plant, as compared to the other experiments, could result in higher percentages of globular embryos at early culturing dates. The final result would be that more embryos germinated at early culture dates. However, if embryo development in exp. II had been more rapid than in the other experiments, also higher germination percentages would be expected for cultures started at 5 WAP. Possibly, the nourishment of the embryos is not sufficient for older embryos in ovary-slices. This could in this experiment also be caused by the higher number of ovules with embryos per ovary-slice as compared to exp. I and exp. II. Differences in seed set and embryo development can also be explained by year-effects resulting from different plant material and culture conditions. An optimum stage for tulip embryo recovery has only been found in this experiment and not in all the other experiments described in this chapter and chapter 6. These results must therefore be interpreted with caution.

The germination percentage for cultures started at 3 to 5 WAP used for the analysis of embryogenesis were 2.3% and 23%, respectively, which is higher than the percentage of globular embryos after one week in culture (cultures started at 3 WAP: 5% of the swollen ovules contained a globular embryo, 13% of all ovules was swollen (Table 3A), thus of all ovules of a flower 0.7% (5% of 13%) seems to contain a globular embryo; 5 WAP: 38%, 23% and 9%, respectively (Table 3B)). This indicates the ability of pro-embryos to develop in vitro into spindle shaped embryos. However, the low percentage of swollen ovules at 3 WAP (13%) in comparison with the percentage of ovules developed into seeds on the plant (37%), show that many (pro-)embryos die during the first weeks of culture.

The lower rate of embryo abortion observed in cultures started at 5 WAP as compared to cultures started at 3 WAP (Table 3) is in accordance with the higher germination percentages at later culturing dates. The higher germination percentages can partly be caused by less retarded embryo development. Embryo development was only retarded for cultures started at 3 WAP and not for cultures started at 5 WAP in comparison with embryo development on the plant (Chapter 3). In for example *Gossypium* (Joshi and Johri 1972), *Papaver* (Maheshwari 1957) and *Capsella* (Lagriffol and Monnier 1985), embryo development was also reported to be slower in vitro. However, in *Zea mays* L., embryo development was not retarded compared to the development on the plant (Schel and Kieft 1986, Campenot et al. 1992).

Culture method

The results of ovary-slice culture were comparable to or better than the results of direct ovule culture. Culturing the ovules for a longer period in the ovary-slices did not improve the germination percentage except for one case (Table 1). Apparently, the ovary wall and/or the placenta have a more or less positive influence on embryogenesis in vitro, like in *Capsella bursa-pastoris* Medic. (Lagriffol and Monnier 1985) and *Petunia hybrida* Vilm. (Wakizuka and Nakajima 1975).

The start of cultures at early dates reduces the efficiency of both culture methods. This is due to embryo abortion and retarded embryo development. Apparently, culture conditions are less optimal when the embryos are younger at the start of the culture. Media composition influenced embryo and endosperm development in several crops (Rangan 1982, Campenot et al. 1992, Comeau et al. 1992, Lippmann and Lippmann 1993). Younger embryos often need more sophisticated media (Williams et al. 1987). Development of a more complex medium might improve the efficiency to recover tulip embryos at early culture dates.

Some spindle shaped embryos did not germinate at all. This might be due to a

mechanical barrier caused by the structure of both the endosperm and the integuments, which were more rigid in vitro than on the plant. Slicing the ovules, as done in e.g. *Cucumis* (Custers and Bergervoet 1990) and *Alstroemeria* (Buitendijk et al. 1995), or applying embryo culture after ovule culture, like in *Medicago* (McCoy and Smith 1986), might increase germination.

Hybrids

For the first time, hybrids have been rescued from interspecific incongruent crosses of *T. gesneriana* with *T. agenensis* and *T. praestans*, with the aid of ovary-slice culture and ovule culture. Less embryos of the cross *T. gesneriana* 'Christmas Marvel' x *T. praestans* germinated in cultures started at the same dates than in the cross with *T. agenensis*, despite comparable pollen tube penetration percentages. Apparently, less fertilization occurs or post-fertilization barriers more frequently cause earlier embryo abortion in the cross with *T. praestans*. Of the cross 'Leen van der Mark' x *T. praestans*, no embryos germinated at all. This could be the result of a lower pollen tube penetration percentage generally observed in crosses with 'Leen van der Mark' as compared to those of 'Christmas Marvel' (Chapter 2) and/or by stronger (post-)fertilization barriers. The influence of the maternal genotype on seed set in interspecific tulip crosses is also observed after pollination and pod maturation on the plant (Van Eijk et al. 1991). No ovules of the cross *T. gesneriana* 'Prominence' x *T. de novo species A* showed germination. Many ovaries of this cross had died before 5 WAP indicating that embryos, if formed, die at early developmental stages. The percentage of embryos which can be rescued is rather low at early developmental stages (Table 1).

Hybrids from the cross *T. gesneriana* x *T. kaufmanniana* can also be produced on the plant (Van Raamsdonk et al. 1995, Custers et al. 1995). The higher germination percentage (21%) after ovule culture as reported by Custers et al. (1995), compared to our experiments (0.3%), might be due to differences between the maternal genotypes used and the time embryo rescue was started (5 to 13 WAP). No hybrids were obtained from the cross *T. gesneriana* 'Prominence' x *T. altaica*. This species is less related to *T. gesneriana* than the other species used (Van Raamsdonk and De Vries 1995). Probably, the pollen tube penetration percentages were rather low, like in other crosses between this species and cultivars of *T. gesneriana*. Pollen tubes had penetrated in at most 2% of the ovules in an experiment executed in 1993 (Chapter 2). Of this cross, 12 of the 28 ovaries had died before 3 WAP, when ovary-slice culture was started. Apparently, embryos were not formed or had died at early developmental stages. Starting ovary-slice culture before 3 WAP, could probably be necessary to enlarge the chance of embryo rescue, if embryos are formed.

Perspectives

Interspecific hybridization is an important target in tulip breeding, because it can enrich the tulip assortment used for cut flower production with desirable traits from other tulip species (Van Eijk et al. 1985). The ovary-slice culture technique and the ovule culture technique proved to be powerful methods for bypassing post-fertilization barriers, which hamper the production of hybrids from many interspecific tulip crosses. The described methods can, however, still be optimized by studying several factors important to the production of viable hybrid plants.

The percentage of germinated embryos using embryo rescue techniques in vitro is reported in this article. About 95%-98% of the seeds produced on the plant germinated, of which most seedlings will produce bulblets. However, the transfer of seedlings obtained in vitro into the soil is rather difficult, caused by abnormalities after embryo germination as described by Custers et al. (1992). The next barrier which can prevent further breeding in interspecific crosses is hybrid breakdown and sterility of F1-hybrids. In the latter case, chromosome doubling, by means of colchicine or oryzalin treatment, as done in *Lilium*, may restore fertility (Van Tuyl et al. 1993).

The germination percentage was for the intraspecific crosses lower, up to 9 WAP, after the application of the two embryo rescue techniques than the seed set on the plant. The applied methods appear, however, to be useful for rescuing interspecific hybrids. An optimum is expected for the recovery of hybrids in interspecific incongruent tulip crosses, caused by an increase in the efficiency of the embryo rescue techniques in time and a decrease in the number of viable hybrid embryos in time. However, the germination percentage in the cross *T. gesneriana* x *T. agenensis* improved when cultures were started at later dates. In this cross, embryos are still found at about 12 WAP, when seeds can be harvested on the plant. The optimum date for embryo rescue in this cross might, therefore, be at 7 WAP (last date studied) or at later dates. In other interspecific crosses, embryos might abort at earlier developmental stages, when the embryo rescue techniques are less optimal for embryo recovery. Further improvement of culture methods and culture media can still enhance the efficiency of embryo rescue in tulip, resulting in an improvement of the numbers of embryos rescued at early culture dates.

The effect of medium composition on ovary-slice culture and ovule culture in intraspecific *Tulipa gesneriana* L. crosses

6

Summary

The effect of several media components on the percentage of ovules showing germination in intraspecific *T. gesneriana* crosses was studied after the application of two embryo rescue techniques, ovary-slice culture followed by ovule culture or direct ovule culture. The addition of 9% sucrose to the medium for ovary-slice culture, started at 3 or at 5 weeks after pollination (WAP), significantly improved the germination percentage as compared to 5% sucrose. The germination percentage did not differ between both sucrose concentrations (3% and 5%) used in ovule culture started 4 weeks later (at 9 WAP). Similar germination percentages were obtained with media containing the full or half of the concentrations micronutrients and macronutrients of the MS-medium during ovary-slice culture and ovule culture. For direct ovule culture, started at 4, 6, and 8 WAP, the germination percentages did not differ between ovules cultured on media with 3%, 6% or 9% sucrose. No effect on the germination percentage was observed of the addition of the cytokinin BAP (0.01 or 0.1 mg/l). The use of liquid-shaken culture gave germination percentages which were comparable to those on agar solidified media. Analysis of the carbohydrate concentration of the media revealed that, in both media for ovary-slice culture and for ovule culture, ultimately all sucrose is converted into glucose and fructose. The total concentration of carbohydrates decreased with 22%-50% in media for ovary-slice culture, whereas the total concentration of carbohydrates remained rather constant in media for ovule culture.

Introduction

Embryo rescue techniques, such as embryo culture, ovule culture and ovary culture, are often used in interspecific hybridization programs. In tulip, ovary-slice culture and ovule culture were applied successfully for the recovery of unique hybrids (Chapter 5). The efficiency of seed(ling) production of interspecific crosses, from which on the plant only small amounts of seeds are produced (Van Eijk et al. 1991, Van Raamsdonk et al. 1995), might also be raised with embryo rescue techniques. More seedlings of the cross *T. gesneriana* x *T. kaufmanniana* Regel were obtained after ovule culture than after pod maturation on the plant (Custers et al. 1995).

The efficiency of ovary-slice culture and ovule culture in tulip increases when embryo rescue is started at more advanced embryo ages (Custers et al. 1995, Chapter 5). However, in many incongruent interspecific crosses, embryo rescue methods must be started at early culture dates, because embryos often die prematurely. When the efficiency of embryo rescue procedures for tulip could be improved for early culturing dates, more embryos might be recovered from crosses from which already unique hybrids were obtained by using these methods (Chapter 3). This is especially important for crosses from which momentarily only few hybrids are obtained, like *T. gesneriana* x *T. praestans* Hoog. When embryos can be rescued at early developmental stages, hybrids might be rescued from crosses which will not succeed with the currently available methods.

The media used for embryo rescue are often more complex for young embryonal stages. Media described for embryo rescue of a range of crops differ in composition. Different mixtures of macronutrients and micronutrients have been used, for example those of White, Murashige and Skoog, Gamborg (B5) and Linsmaier and Skoog (for review and references see Williams et al. 1987). Sucrose is often applied as carbon source, in concentrations up to 13%. The sucrose concentration used often declines with increasing embryo age. Sucrose functions as energy source, but also for the establishment of the osmolarity. Influence of vitamin mixture, hormones, agar and pH have been reported. Other components like amino acids, individually adjusted or in the form of casein hydrolysate, activated charcoal and picloram have been used in media for embryo rescue. Further, more complex nutrient mixtures are added to media, such as coconut milk, cucumber juice (Przywara et al. 1989), juice of immature white clover seeds (Yamada and Fukuoka 1986), extracts of cotton ovules (Joshi and Johri 1972) and yeast extracts (Inomata 1977).

Results are presented in this article on the influence of sucrose, macronutrients and micronutrients, 6-benzylaminopurine (BAP) and agar on the germination percentage of embryos of intraspecific *T. gesneriana* crosses, cultured from different dates after

pollination. The change of the pH and the concentration of carbohydrates during culture have been studied.

Material and methods

General

The procedures employed for ovary-slice culture and ovule culture are described in Chapter 5. Only essential points and supplementing information are described here.

Crosses were made between *T. gesneriana* L. 'Christmas Marvel' and *T. gesneriana* 'Leen van der Mark'. The standard medium for ovary-slice culture was composed of half of the concentration macronutrients and micronutrients and the normal concentration vitamins and myo-inositol of the medium of Murashige and Skoog (1962) and 2.0 mg/l glycine (indicated as ½MS), supplemented with 9% (w/v) sucrose, 1 mg/l α-naphthalenacetic acid (NAA), 50 mg/l Nystatin (Duchefa), 100 mg/l Vancomycin (Duchefa), 100 mg/l Cefotaxime sodium (Duchefa) and 0.7% bacteriological agar (Oxoid) at pH=6.0. The same medium was used for ovule culture, except for sucrose (3%), antibiotics (both 50 mg/l) and for pH (5.6). Only the media components which were changed as compared to the standard media for ovary-slice culture and ovule culture are described in the different experiments. The statistical analysis of the experiment in which both ovary-slice culture and ovule culture were applied (Table 1 and 2) has been executed on probit scale (McCullagh and Nelder 1989). The other results have been analyzed statistically by means of the t-test (Payne et al. 1993).

Combination of ovary-slice culture and ovule culture

Four media were tested for ovary-slice culture, e.g. ½MS supplemented with 5% or 9% sucrose and media with the normal concentration macronutrients and micronutrients (MS) supplemented with 5% or 9% sucrose. The 4 different media used for ovule culture consisted of ½MS or MS, both with 3% or 5% sucrose.

Thirty flowers of 'Christmas Marvel' and 30 of 'Leen van der Mark' were pollinated reciprocally in February 1992. Ten pods per cross matured on the plant. The ovaries were cut transversely in 8 slices and brought into culture at 5 weeks after pollination (WAP). The 4 slices at the top of the ovary (stigmatic side) were placed on medium which differed in composition from that used for the four slices at the basis of the ovary. Four weeks later, at 9 WAP, ovules were extracted from the ovary-slices and placed individually on medium. Ovules of two subsequent slices of each flower were placed on each of the 4 media used in ovule culture. Approximately the same number of (ovules

from) slices from each position in the ovary were placed on each medium.

Ovule culture

The influence of the sucrose concentration (3%, 6% and 9%) and of bacteriological agar (liquid (0.0%) and 0.7%) and of the cytokinin 6-benzylaminopurine (BAP: 0, 0.01 and 0.1 mg/l) on the percentage of ovules showing germination was investigated in three different experiments. Liquid media were placed on a shaker at 15 rpm. Ovules cultured in liquid media were placed on agar-solidified medium at 16 WAP. At this time, all cultures were transferred from 15 °C to 5 °C to induce germination.

Eightyfour flowers in total of 'Leen van der Mark' were pollinated in March 1992 and 1993, of which 30 pods matured on the plant. In each experiment, at 4, 6 and 8 WAP, the ovules of 6 ovaries were extracted and placed on medium. Each of the three carpels of an ovary was used for a different treatment within an experiment.

Measurement of carbohydrates and pH

The concentration of carbohydrates and the pH have been measured in media used for ovary-slice culture and for ovule culture.

Thirtythree flowers of 'Christmas Marvel' were pollinated in March 1993. Five pods were left on the plant until seed harvest. Ovary-slice culture was started at 3 WAP and at 5 WAP. Fourteen ovaries were used per date. Ovules were extracted from the ovary-slices at 9 WAP and placed individually on medium.

The pH of the medium was determined and samples for carbohydrate analysis were taken weekly from 4 to 10 WAP and at 12, 16, 24, 32 and 42 WAP. The pH of the medium was determined in the same Petri dishes as used for the analysis of carbohydrates. Measurements were made and samples were taken at each date of on average 3 Petri dishes from cultures started at 3 WAP or at 5 WAP and of 1 Petri dish without explants. The pH was determined three times in each Petri dish with pH indicator paper, which was placed in medium which had been in contact with the explant. For sugar analysis, samples were taken from medium located just below the explant. Each sample was weighed and contained between 40-70 mg medium. Samples were freeze-dried and stored at -80 °C until analysis. Water was added to the samples until a final volume of 1 ml and each sample was heated for 15 min at 75 °C. After dilution, the samples were injected directly in a Dionex HPLC system equipped with a CarboPac PA1 column and a pulsed-amperometric detection system. Carbohydrates were identified by their co-migration with authentic standards.

Results

Combination of ovary-slice culture and ovule culture

The mean germination percentages of ovules cultured first from 5 to 9 WAP in ovary-slices and then individually on medium are presented in Table 1 and Table 2. The germination percentages for the different ovary-slices are given in Table 1. For both the cross 'Christmas Marvel' x 'Leen van der Mark' and the reciprocal cross, considerable lower percentages of ovules germinated from the slices originating from the top (1) and the bottom (8) of the ovary. These slices represented 6% of all cultured ovules of the cross 'Christmas Marvel' x 'Leen van der Mark' and 13% of all cultured ovules of the reciprocal cross. Interactions between experimental factors were found, caused by the deviating germination percentage and the low number of ovules from ovary-slices 1 and 8 for both cultivars. Results obtained from these ovary-slices were, therefore, disregarded in the further statistical analysis. The percentage ovules showing germination from slice 7 was significantly (F pr. <0.001) lower than those from ovary-slices 2-6.

Table 1. The effect of the place of the ovary-slices in the ovary on the percentage of ovules showing germination for the cross 'Christmas Marvel' x 'Leen van der Mark' (CM) and the reciprocal cross (LvdM). Ovary-slices 1 to 8 are successively cut from the top towards the bottom of the ovary.

cultivar	1	2	3	4	5	6	7	8
CM	5.6	39.9	41.4	36.3	36.4	29.9	24.5	5.7
LvdM	8.5	22.5	19.5	24.0	31.0	23.6	13.7	1.7

The mean germination percentages, per combination of media used, for the ovules of ovary-slices 2 to 7 of the cross 'Christmas Marvel' x 'Leen van der Mark' are presented in Table 2. In the reciprocal cross (data not shown), significantly (F pr. <0.001) less ovules showed embryo germination (overall mean 21%). However, the conclusions with regard to the effects of media composition did not differ between the two crosses. Significantly more (F pr. <0.001) embryos germinated by using media for ovary-slice culture containing 9% sucrose in comparison with media with 5% sucrose. No difference was found in germination percentage between the sucrose concentrations tested (3% and 5%) for the subsequent ovule culture. The germination percentage was not influenced by

the concentration of macronutrients and micronutrients for both ovary-slice culture and ovule culture. On average 33% of all cultured ovules with 'Christmas Marvel' as mother showed germination. On the plant, 56% of the ovules of this cross had developed into seeds and 33% in case of the cross with 'Leen van der Mark' as mother.

Table 2. The percentage of ovules showing germination for different combinations of media composition after the application of ovary-slice culture at 5 WAP, followed by ovule culture 4 weeks later (9 WAP) for the cross 'Christmas Marvel' x 'Leen van der Mark'. Media contained either the full concentration macronutrients and micronutrients (MS) or half of these concentrations (½MS) and either 5% or 9% sucrose for ovary-slice culture and 3% or 5% sucrose for ovule culture. The results of ovules from the ovary-slices 2-7 were analyzed.

ovary-slice culture	ovule culture			
media	½MS+3	MS+3	½MS+5	MS+5
½MS+5	34.7	21.0	26.8	27.4
MS+5	31.3	26.9	24.2	31.3
½MS+9	37.4	40.0	39.6	31.0
MS+9	47.9	49.5	38.0	46.7

Ovule culture

The germination percentages after ovule culture started at 4, 6 and 8 WAP of ovules cultured on media containing 3%, 6% or 9% sucrose are presented in Table 3. The percentages of germination increased in time, from on average 19% to 52%. However, the germination percentages did not differ significantly between the three media, at none of the three starting dates of ovule culture. On average 34% of the ovules on the plant had developed into seeds.

Table 3. The effect of sucrose concentration (3%, 6%, 9%) of the MS-medium on the percentage of ovules showing germination of cultures started at 4, 6 and 8 WAP for the cross 'Leen van der Mark' x 'Christmas Marvel'.

WAP	3% sucrose	6% sucrose	9% sucrose	mean
4	16.6	23.4	17.6	19.2
6	45.7	47.0	40.0	44.2
8	50.8	55.8	49.9	52.2
mean	37.7	42.1	35.8	

LSD_{WAP} : 6.8; $LSD_{sucrose}$: 6.8; $LSD_{WAPxsucrose}$: 11.7

Table 4. The effect of 6-benzylaminopurine (BAP) (0, 0.01 or 0.1 mg/l) added to MS-medium on the percentage of ovules showing germination of cultures started at 4, 6 and 8 WAP for the cross 'Leen van der Mark' x 'Christmas Marvel'.

WAP	0 mg/l BAP	0.01 mg/l BAP	0.1 mg/l BAP	mean
4	3.0	1.7	0.6	1.8
6	9.4	9.2	8.1	14.5
8	30.5	31.7	30.9	31.0
mean	14.3	19.8	13.2	

LSD_{WAP} : 10.0; LSD_{BAP} : 10.0; $LSD_{WAPxBAP}$: 17.3

No influence was observed of the addition of BAP (0.01 and 0.1 mg/l) to media for ovule culture, at none of the three starting dates (Table 4). Interactions between the application time of ovule culture and the medium used was not found. The germination percentages increased in time to 31% at 8 WAP on average. After maturation on the plant, 20% of the ovules had developed into seeds.

The germination percentages were not influenced by placing the ovules on liquid shaken medium (until 16 WAP) as compared to solidified medium (Table 5). The germination percentages increased in time. Ultimately on average 70% of the ovules germinated. On the plant, on average 34% of the ovules had developed into seeds.

Table 5. The percentage of ovules showing germination on MS-media containing 0.0% (liquid) or 0.7% bacteriological agar (agar) of cultures started at 4, 6 and 8 WAP for the cross 'Leen van der Mark' x 'Christmas Marvel'.

WAP	liquid	agar	mean
4	25.3	29.9	27.6
6	59.0	55.9	57.5
8	72.2	66.9	69.6
mean	52.2	50.9	

LSD_{WAP} : 6.8; LSD_{agar} : 5.6; $LSD_{WAPxagar}$: 9.7

Carbohydrates and pH

The results of the analysis of carbohydrate concentrations in medium for ovary-slice culture (3 and 5 WAP) are presented in Figure 1 and those for ovule culture (9 WAP) in Figure 2. Other carbohydrates than glucose, fructose and sucrose were not found in the media. The carbohydrate concentrations in the Petri dishes without explants remained relatively constant (se=0.06) in time. The results of the carbohydrate concentrations in the Petri dishes without explants were therefore averaged and taken as reference for the results of the analysis of media on which ovary-slices or ovules were placed. After autoclaving, 6% (ovary-slice culture) to 15% (ovule culture) of the sucrose was already converted into glucose and fructose. The total concentration of carbohydrates in the medium for ovary-slice culture decreased in the first week(s) of culture and stabilized thereafter. Averaged for analysis from 6 to 9 WAP, the total carbohydrate concentrations had diminished with 26% for cultures started 3 WAP and 30% for cultures started 5 WAP, as compared to the Petri dishes without explants. The concentration of sucrose decreased considerably during the culture period, whereas the concentration of glucose and fructose first increased and stabilized later on, each being 22% in relation to the total carbohydrate concentration of the control (average of 6-9 WAP). The total carbohydrate concentration in the medium for ovule culture (Fig. 2) was, for both cultures, about 90% (averaged for 16-42 WAP) of the carbohydrate concentration of the Petri dishes without explants. The concentration of glucose and fructose increased to about 16 WAP and stabilized thereafter to on average (both cultures) 40% and 52%, respectively. Less than 1% of the concentration carbohydrates consisted of sucrose at 24 WAP (cultures started at 5 WAP) or 32 WAP (cultures started at 3 WAP).

The pH of the medium for ovary-slice culture and for ovule culture was 6.0 and 5.6, respectively (before autoclaving). One week after autoclaving, the pH of the medium without explants was 4.4-5.0 and fluctuated between these levels during the remaining culture period. The pH of both the media for ovary-slice culture (both 3 and 5 WAP) and the media for ovule culture, fluctuated in time between 3.9-4.5.

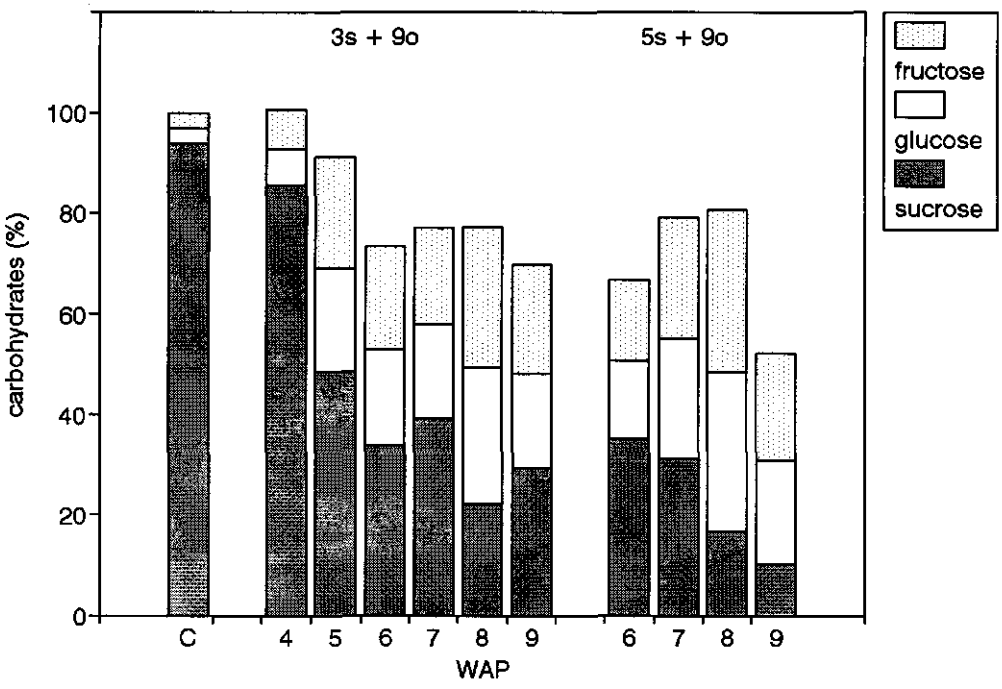


Fig. 1. The sucrose, glucose and fructose concentrations in time (in weeks after pollination (WAP)) in medium used for ovary-slice culture (9% sucrose), related to the concentrations of these carbohydrates in the same autoclaved medium, however, without explants. Ovary-slice cultures were started at 3 WAP (3s) and at 5 WAP (5s), followed by ovule culture 6 or 4 weeks later (9o), respectively.

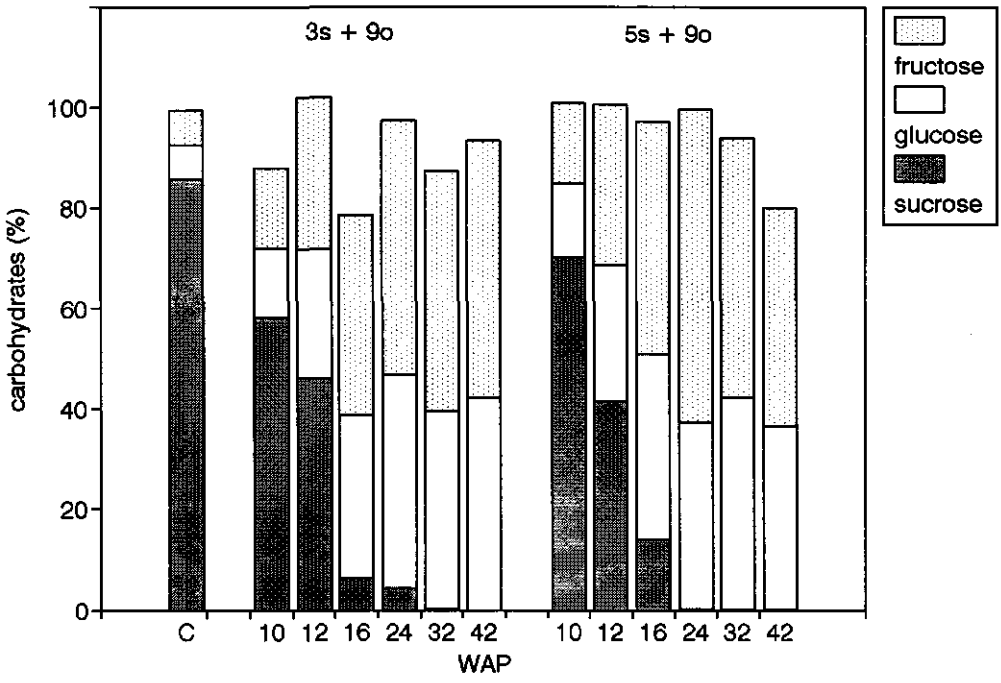


Fig. 2. The sucrose, glucose and fructose concentrations in time (in weeks after pollination (WAP)) in medium used for ovule culture (5% sucrose), related to the concentrations of these carbohydrates in the same autoclaved medium, however, without explants (C). Ovary-slice cultures were started at 3 WAP (3s) and at 5 WAP (5s), followed by ovule culture 6 or 4 weeks later (9o), respectively.

Discussion

General

The germination percentage after ovary-slice culture followed by ovule culture depended on the sucrose concentration of the medium for ovary-slice culture, 9% being better than 5%, but not on the sucrose concentration used for ovule culture subsequently. The concentrations micronutrients and macronutrients of MS tested gave both comparable germination percentages. In direct ovule culture, the germination percentages did not differ between the concentrations sucrose, BAP and agar used.

Carbohydrates

Autoclaving of the medium resulted in the conversion of 6%-15% of the sucrose into glucose and fructose, which was also reported by Singha et al. (1987). The addition of 9% sucrose to media for ovary-slice culture resulted in more germination than the addition of 5% sucrose. If sucrose is free available in the medium and only necessary as energy source, media with 5% sucrose would already support embryo growth optimally, because the concentration of carbohydrates in the medium of ovary-slice culture containing 9% sucrose diminished only with 22%-50%. Therefore, sucrose seems to be needed as osmoticum in media for ovary-slice culture.

The germination percentage for ovary-slice culture (9% sucrose) followed by ovule culture (3% sucrose) was either comparable to direct ovule culture (3% sucrose) or significantly higher (Chapter 5). The high carbohydrate concentration in media for ovary-slice culture seems, at least partly, to be more important for processes in the ovary-slices themselves, or for interactions between the ovary-slices and the ovules, rather than for processes within the ovule. The rapid uptake of carbohydrates in the first week(s) of ovary-slice culture followed by a period of a relatively constant carbohydrate concentration also indicates the absorption of most carbohydrates by the ovary-slice itself rather than being consumed by the ovules.

Each Petri dish with 4 ovary-slices contained 2.25 g sucrose (9% sucrose, 25 ml) at the start of the culture. The concentration carbohydrates remained rather constant in Petri dishes without explants. The concentration carbohydrates decreased with 26%-30% in ovary-slice culture. This implies, assuming that the measured concentrations carbohydrates are representative for the total concentration carbohydrates in the Petri dishes, the absorption of 0.63 g carbohydrates by the 4 ovary-slices. At 3 WAP, 4 ovary-slices had a fresh weight of about 2.6 g (data not shown). The ovules in these 4 ovary-slices had a fresh weight of in total about 0.8 g. The fresh weight of 4 ovary-slices was about 5.2 g at 5 WAP and of the ovules about 2.5 g. Most carbohydrates were absorbed by the ovary-slices within 1 to 2 weeks after starting the culture. When carbohydrates were consumed by the ovules, this implies the use of 0.63 g carbohydrates in 1 to 2 weeks by 0.8 g (3 WAP) or 2.5 g (5 WAP) ovules (fresh weight). In callus culture of *Solanum tuberosum* (Van der Plas and Wagner 1986, Gude et al. 1988) the respiration rate was 72-77 nmol O_2 min^{-1} g FW^{-1} (gram fresh weight) at 25 °C. One mol of glucose (180 g mol^{-1}) is needed for the respiration of 6 mol O_2. This implies the need of 3.2 mg carbohydrates day^{-1} g FW^{-1}, when 75 nmol O_2 min^{-1} g FW^{-1} was used for respiration. For 5.2 g fresh weight (weight ovary-slices at 5 WAP), this means that after 2 weeks of culture 0.23 g sucrose would be used for respiration. However, 0.63 g was absorbed by the ovary-slices, which was much more than the amount of sucrose needed in actively

growing callus culture of *S. tuberosum* (Van der Plas and Wagner 1986, Gude et al. 1988). The respiration rate was expected to be lower under the culture conditions of tulip as compared to the callus cultures of *S. tuberosum*, because tulip cultures were placed in the dark and at lower temperatures (15 °C or, from 16-28 WAP, at 5°C). The above data show, therefore, that it seems probable that at least a part of the carbohydrates are not used for respiration of the ovules, but absorbed and/or used for respiration by the ovary-slices itselves.

The germination percentage did not differ for the sucrose concentrations tested in ovule culture of tulip, which was also found in *Pelargonium x hortorum* Bailey (Scemama and Raquin 1990) and *Helianthus* (Espinasse et al. 1991). The concentrations of carbohydrates in the medium remained rather constant during the culture of isolated ovules, pre-cultured in ovary-slices. At the start of ovary-slice culture (3 and 5 WAP), most ovules contained only small amounts of nuclear endosperm. Ultimately 2.3% and 23% of the ovules cultured from 3 and 5 WAP, respectively, germinated (Chapter 5). For embryogenesis, it seems that the ovules must have absorbed carbohydrates from the medium. The uptake from the medium is, however, minimal but an (osmotic) effect of the sucrose concentrations used was not found. Also in somatic embryogenesis of *Picea mariana* Mill., embryos matured despite undetectable uptake of sugars (Tremblay and Tremblay 1995).

The percentage carbohydrate uptake in relation to the fresh weight of ovary-slices was 12%-24% (0.63 g carbohydrates, 2.6 g fresh weight (3 WAP) or 5.2 g fresh weight (5 WAP) ovary-slices). Four ovary-slices contained about 245 ovules which had a fresh weight of about 0.8 g at 3 WAP and of 2.6 g at 5 WAP. For ovule culture, at most 50 ovules were placed per Petri dish. This means that in each Petri dish at most 0.16 g (3 WAP) or 0.53 g (5 WAP) of ovules (fresh weight) was placed. Supposing that the uptake of carbohydrates in ovule culture was also about 12%-24%, this implies the absorption of about 0.02-0.04 g (3 WAP) or 0.06-0.12 g (5 WAP) carbohydrates. The total concentration of sucrose in media for ovule culture was 0.75 g (3% sucrose, 25 ml). Uptake of between 0.02-0.12 g carbohydrates by the ovules means the absorption of between 3%-16% of the available carbohydrates by the tissue. The decrease in total concentration carbohydrates of 10%, averaged for 16-42 WAP, is with this in agreement. However, this low decrease in carbohydrate concentration as compared to the total concentration of carbohydrates in each Petri dish, together with the variability between the concentrations carbohydrates of Petri dishes with explants, makes it difficult to study the carbohydrate uptake in ovule culture precisely.

Other components

Comparable germination percentages were found in tulip for culturing ovary-slices on media with ½MS and MS, which is in contrast with the results found for *Rosa hybrida* L. (Gudin 1994) and *Ipomoea batatas* L. (Kobayashi et al. 1993). More embryos germinated on MS-medium than on ½MS in *Rosa hybrida* (Gudin 1994), while in *Ipomoea batatas* embryo development was improved on ½MS as compared to MS (Kobayashi et al. 1993).

Positive results were obtained by using liquid shaken-culture in ovule culture of *Populus deltoides* Bartr. (Savka et al. 1985) and in half-ovule culture of *Alstroemeria* spp. (Buitendijk et al. 1995). However, in tulip, culturing the ovules of different ages until 16 WAP in liquid shaken-culture did not enhance germination in comparison with culture on solidified medium.

No effect on the germination percentage was detected in tulip after the addition of the cytokinin BAP (0.01 or 0.1 mg/l) to medium for ovule culture, containing the auxin NAA (1 mg/l), at none of the ovule ages tested. In other crops, cytokinins, including BAP, had positive or negative results, or no effect, depending on the concentrations and types of cytokinin used (Cohen et al. 1984, Savka et al. 1985, Campenot et al. 1992, Marchant et al. 1994).

The constant germination percentages upon varying the concentration of MS, agar and BAP, can be explained by several factors. Firstly, the tested components could really do not influence the germination percentage. Secondly, the concentration of other media components or the culture conditions might be suboptimal with as consequence that detection of an effect of the tested components is not possible. Thirdly the interaction between a medium component and the components tested might restrict the uptake. The influence of one medium component on the effect of another has been reported (Raghavan and Torrey 1963, Dougall 1980, Neal and Topoleski 1983, Schmitz and Lörz 1990).

The pH of medium without explants decreased from 6.0 and 5.6 to 4.4-5.0 from 1 week after autoclaving, which has also been reported by Skirvin et al. (1986). In ovary-slice culture and ovule culture of tulip the pH decreased even more, to 3.9-4.5. Skirvin et al. (1986) observed the pH fluctuating between 4.6-4.9 after 48 hours of culturing *Cucumis* callus on media with pH ranging between 3.3-8.0. The shift of a wide range of initial pH of the medium to the same final pH has been reported for many crops (Minocha 1987). Apparently, the initial pH does not determine the pH after the culture of a specific explant. However, the uptake of several media components is influenced by the pH in different in vitro cultures (Thorpe and Meier 1973, Martin and Rose 1976, Veliky et al. 1977, Steiner and Dougall 1995). The pH of the medium had significant effects on growth and differentiation of cells in several cases, whereas the growth rates were not

affected over a wide range of initial pH of the medium in other cases (Minocha 1987). The number of seeds obtained after in vitro pollination of *T. gesneriana* did not increase significantly when the buffer MES was added to agar solidified media, whereas more seeds were harvested in several cases when liquid media were used (Chapter 7). Research on the effect of the addition of buffers to media for embryo rescue can give more insight in the influence of the pH on the germination percentage of tulip embryos.

Perspectives

The efficiency of direct ovule culture has not been improved through the addition of 6% or 9% sucrose instead of 3% sucrose, the addition of BAP and through the use of liquid-shaken culture. A concentration of 9% sucrose in media for ovary-slice culture gave better results as compared to 5% sucrose. Other media components can still be tested, such as other (combinations of) hormones and casein hydrolysate, and other culture conditions to increase the germination percentage. For ovary-slice culture it remains of interest to analyze whether 9% sucrose is the optimum concentration, or if concentrations higher than 9% or between 5%-9% are optimal. The addition of an inert osmoticum to the medium should be tested to detect whether sucrose is really needed as osmoticum.

Culture conditions could also be studied. Mature tulip seeds need a cold treatment (5 °C) to induce germination. All in vitro cultures are placed at 5 °C at 16 WAP. However, ovules cultured from 3 WAP show a retarded development compared to ovules cultured from 5 WAP and ovules developing on the plant (Chapter 5). Possibly, ovules cultured from an early developmental stage get a cold treatment at a too early embryonal stage.

The observations are focused on the germination percentages in the described experiments. The formation of tulip bulblets in vitro and subsequently the transfer into the soil are known to be difficult and surely further research is needed (Custers et al. 1992, 1995). For in vitro bulblet formation of tulip, induced on floral stem explants, a sucrose concentration of between 3%-6% appeared to be most optimal. Both auxins and cytokinins are added to media in concentrations between 0.03 mg/l and 1 mg/l (Nishiuchi 1980, Taeb and Alderson 1990, Custers et al. 1992, Wilmink et al. 1995, Chanteloube et al. 1995). While in ovule culture, the concentration of both sucrose and BAP did not influence the percentage germination, concentrations could be used which enhance bulblet development.

Influence of medium composition on in vitro pollination of *Tulipa gesneriana* L.

7

Summary

A procedure for in vitro pollination in tulip has been developed, using compatible intraspecific *Tulipa gesneriana* crosses as model system. The effect of several media components on seed set and seed germination has been studied. As standard medium MS with 7% sucrose and 0.7% bacteriological agar was used. Differences in effects of media components were observed between reciprocal crosses of 'Leen van der Mark' and 'Christmas Marvel'. The number of seeds produced per ovary was higher on media with agar than on liquid media. The use of Daichin agar improved the seed set as compared to bacteriological agar. The application of 5%-7% sucrose proved to be optimal for seed production. Analysis of carbohydrate uptake revealed the absorption of significantly more carbohydrates on liquid media than on media with agar. Similar levels of seed set were observed for ovaries cultured on agar solidified media with 3.5% fructose and 3.5% glucose and media with 7% sucrose. The addition of either 1 or 10 mg/l NAA had a positive effect on the seed set in crosses with 'Christmas Marvel' as maternal genotype, whereas no or a negative effect, respectively, was found in the reciprocal cross. The seed set was not improved when 10 mg/l BAP, 1 mg/l GA_3 or 0.2 g/l spermine was added to the medium or when the concentration of macronutrients and micronutrients of MS was halved. Buffering of the medium by the addition of 5.3 g/l 2-morpholinoethanesulfonicacid-monyhydraat (MES) to liquid media affected the seed set positively. The pH declined in media without MES from 5.8 before autoclaving to 3.8-4.8 in media with agar or to 3.1-5.2 in liquid media. Casein hydrolysate had a positive effect on the seed set when added to media with agar, but the germination percentage was affected negatively sometimes when added to liquid media. A lower germination percentage, as compared to the other media tested, was also found for seeds produced on liquid media with 9% sucrose.

Introduction

The genus *Tulipa* L. comprises about 55 species, of which 49 are described by Van Raamsdonk and De Vries (1992, 1995). However, the majority of commercial important tulips are cultivars of *T. gesneriana* or of Darwin hybrids, the latter of which are obtained from crosses between *T. gesneriana* and *T. fosteriana* Hoog ex W. Irving. The tulip assortment could be improved considerably by introducing traits from other tulip species, such as resistance for diseases and short forcing period. By using conventional breeding methods, hybrids can only be obtained from a limited number of interspecific tulip crosses (Van Eijk et al. 1991, Van Raamsdonk et al. 1995). Most interspecific crosses are hampered by crossing barriers, caused by incongruity (Hogenboom 1973). Pre-fertilization and post-fertilization barriers have both been identified in interspecific tulip crosses (Chapters 2, 3).

Pre-fertilization barriers can be bypassed in several crops by the application of methods such as the cut-style method, the grafted-style method and placental pollination (Kameya and Hinata 1970, Marubashi and Nakajima 1985, Zenkteler 1990a, Van Tuyl et al. 1991). Placental pollination can be executed in different ways, ranging from injecting pollen into the ovary (intra-ovarian pollination) to the pollination of isolated ovules (Kanta and Maheshwari 1963, Stewart 1981, Zenkteler 1990a). Non-germinative seeds of *T. gesneriana* were obtained after self-pollination of ovules attached to the placenta (Zubkova and Sladky 1975).

Ovary-slice culture and ovule culture were applied for bypassing post-fertilization barriers in tulip. Unique hybrids of two interspecific crosses have been rescued by the application of these techniques (Chapter 5). However, the number of embryos which can be saved at an early developmental stage is still low. In *Vicia* spp., it has been shown that larger numbers of embryos can be rescued at younger stages when whole ovaries are cultured in vitro (Lazaridou et al. 1993). Seeds could be harvested after ovary culture in many other crops (Rangan 1984, Gengenbach 1984, Barratt 1986, Takahata and Takeda 1990, Sharma et al. 1995).

In vitro pollination is a method which proved to be successful for the production of seeds from crops, such as *Nicotiana* spp. (Rao 1965, Dulieu 1966), *Antirrhinum majus* L. (Usha 1965), *Trifolium* spp. (Leduc et al. 1990), *Gossypium* (Refaat et al. 1984), *Zea mays* L. (Higgins and Petolino 1988), *Brassica napus* L. (Lardon et al. 1993), *Lilium* spp. (Van Tuyl et al. 1991) and *Nerine bowdenii* DC. (Van Tuyl et al. 1992b). Instead of normal stigmatic pollination, also other pollination methods can be executed in vitro. In this way, pollination, fertilization and embryogenesis can be achieved under optimal environmental conditions. The in vitro pollination procedure can also be applied for the

post-fertilization culture of whole ovaries.

Medium composition is an important factor for the rate of success of in vitro pollination (Richards and Rupert 1980, Gengenbach 1984, Van Tuyl et al. 1991). For the development of an in vitro pollination procedure for tulip, we have examined the effects of media components, such as carbohydrates, hormones, casein hydrolysate and agar on the number of seeds obtained and the germination percentage of those seeds. The pH and the concentration of the carbohydrates has been studied during the culture period. Compatible intraspecific *T. gesneriana* crosses were used as model, because *T. gesneriana* is mostly used as maternal genotype in interspecific crosses (Van Eijk et al. 1991, Van Raamsdonk et al. 1995).

Materials and methods

Plant material

The cultivars of *T. gesneriana* L., Christmas Marvel, Leen van der Mark, Prominence and Cassini were obtained from commercial stocks. Bulbs were planted in September-October in flats and subsequently stored at 5-9 °C for 15-18 weeks. The plants were placed in a greenhouse in January-March of 1991-1993 at a temperature of 15-17 °C and flowered two to three weeks later. Flowers were emasculated two days before anthesis for controlled pollination on the plant.

In vitro pollination

Flower-buds, of which the tepals were colouring, were collected 5-7 days before anthesis and sterilized 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, closed with cotton plugs. The test-tubes were placed in a climate room (light intensity 12 Wm^{-2}, photoperiod 16 hours, temperature 17 °C).

Flower-buds were collected 3-5 days before anthesis to obtain aseptic pollen. They were rinsed during 1 minute in alcohol (70%) followed by two rinses in sterile water. Flower-buds were placed in an airflow cabinet in flasks containing the medium of Murashige and Skoog (1962) without sucrose until anther dehiscence. The anthers were subsequently stored in a desiccator with silica gel, during one week at most.

The flowers were pollinated at stigmatic receptivity. Pollen was rehydrated before pollination at 100% relative humidity for two hours at 15 °C. The ability of pollen to germinate was tested in liquid Brewbaker and Kwack (1963) medium, supplemented with 10% (w/v) sucrose. The test-tubes with pollinated flowers were placed in the climate

room at a temperature of 15 °C, which proved to be a good temperature for pod maturation after interspecific crosses of tulip (Kho and Baër 1971). The pollen tube growth in the pistil and the penetration of the pollen tubes in the ovules were studied in each experiment in 2 flowers per medium of each cross, as described in Chapter 2.

Thirteen flowers, grown on the plant, were pollinated with aseptic pollen and thirteen with fresh pollen collected from flowers grown in the greenhouse, to check the ability of the aseptic pollen to effect fertilization.

Experiments: media comparison

The medium of Murashige and Skoog (1962) (MS) was used as routine, supplemented with 2.0 mg/l glycine, 7% sucrose and a pH of 5.8. All media used in 1992 and 1993 contained 1 mg/l α-naphthalenacetic acid (NAA), which is in contrast with the media used in 1991 that contained no NAA. Solidified and liquid media were both tested. Standard 0.7% (w/v) bacteriological agar (Oxoid) was added to solidified media.

In four experiments (Table 1-4) the following components were changed as compared to the standard medium (either solidified or liquid) or were added to the standard medium: 3%, 5%, 9% sucrose, 3.5% fructose and 3.5% glucose, half of the concentration macronutrients and micronutrients of MS (½MS), 1 mg/l or 10 mg/l NAA ($5.3\ 10^{-3}$ mM or $5.3\ 10^{-2}$ mM respectively), 10 mg/l 6-benzylaminopurine (BAP) ($4.4\ 10^{-3}$ mM), 1 mg/l gibberellic acid (GA_3) ($2.9\ 10^{-3}$ mM), 2 g/l casein hydrolysate (CH) (Oxoid), 0.2 g/l spermine (1 mM), 5.3 g/l buffer 2-morpholinoethanesulfonicacid-monohydraat (MES) (25 mM) (Merck) and 0.7% Daichin agar.

Flowers were first cultured for 0, 3, 6, 9 or 12 weeks on liquid standard medium and subsequently during 12, 9, 6, 3 or 0 weeks, respectively, on standard agar solidified medium in two experiments (Table 5). The sucrose concentration in the liquid medium was lowered from 7% to 4% at 6 weeks after pollination in another treatment of this experiment (Table 5).

The pH was adjusted before the addition of agar and before autoclaving the medium during 20 min at 120 °C. Hormones, CH, spermine and MES were filter sterilized and added after autoclaving. Each test-tube of 3 cm diameter contained 25 ml medium. The media were refreshed every two weeks.

On average 15 flowers were pollinated per medium per cross of each experiment. On average 26 flowers were pollinated in the greenhouse (15 °C) per cross of each experiment and the pods matured on the plant to determine the seed set. The seeds harvested after pollination on the plant and after in vitro pollination were sown in 9 cm diameter Petri dishes on moisture filter paper to study the germination percentage. These Petri dishes were placed in the dark in a climate room of 5 °C to induce germination.

Seed set was analyzed statistically with a generalized linear model using the Poisson distribution with logarithmic scale and the percentages germination with the t-test (Payne et al. 1993).

pH and carbohydrates

The pH of the medium was estimated and samples for carbohydrate analysis were taken at the day flowers were pollinated (6 days after in vitro culture was started) and thereafter weekly until 8 weeks after pollination (WAP) (experiment I (exp. I)) or 12 WAP (experiment II (exp. II)). The standard medium solidified with bacteriological agar was used in exp. I. Liquid media were used in exp. II. The media used in both experiments were not refreshed. The pH of the medium was measured in the medium of the same test-tubes as from which samples were taken used for the determination of carbohydrates. At each date, measurements were made and samples were taken of on average 3 test-tubes and of 1 test-tube without explant. The pH was determined three times in each test-tube with pH indicator paper (exp. I) or one time with a pH meter (exp. II). Samples of on average 68 mg (se=5) (exp. I) or of 50 ml (exp. II) were taken for sugar analysis. Medium located just below the explant was collected in exp. I. Samples were freeze-dried and subsequently stored at -80 °C until analysis. Water was added to the samples until a final volume of 1 ml and each sample was heated for 15 min at 75 °C. After dilution, the samples were injected directly in a Dionex HPLC system equipped with a CarboPac PA1 column and a pulsed-amperometric detection system. Carbohydrates were identified by co-migration with authentic standards.

The buffer MES was added to media in two experiments (Tables 2 and 4). The pH of the media with MES and the standard media (liquid or agar) was measured in on average 15 test-tubes per treatment as described above.

Results

General

The pollen germination tests showed abundant germination of all pollen samples. No differences in pollen tube growth in the pistil and pollen tube penetration in the ovules were found between various treatments of an experiment or between different experiments. Averaged for all experiments with crosses between 'Leen van der Mark' and 'Christmas Marvel', 80% (se=2) of the total pistil length was traversed by pollen tubes and 68% (se=3) of the ovules was penetrated by a pollen tube. The number of seeds obtained after pollination and pod maturation on the plant did not differ

Table 1. Effect of medium composition on the maturation of ovaries, the number of seeds per ovary and the mean germination percentage after in vitro pollination using reciprocal crosses between 'Leen van der Mark' and 'Christmas Marvel'. MS-medium with 7% sucrose and 0.7% bacteriological agar was used as standard medium. The sucrose concentration was varied and the concentration macronutrients and micronutrients halved (½MS) as compared to the standard medium and NAA or BAP were added to the standard medium. As a control flowers were pollinated and pods matured on the plant in the greenhouse (plant). Means followed by different letters differ significantly at the 1% level. (nd= not determined)

Media	Leen van der Mark x Christmas Marvel			Christmas Marvel x Leen van der Mark		
	matured ovaries (%)	seeds per ovary	germination (%)	matured ovaries (%)	seeds per ovary	germination (%)
standard medium (agar)	100	16.4 c	73 bc	100	2.4 ab	83 a
3% sucrose	58	1.1 b	43 ab	9	0.0 a	-- --
5% sucrose	100	20.8 c	76 bc	78	1.1 ab	38 a
9% sucrose	100	34.3 d	85 bc	80	1.4 ab	40 a
½MS	100	1.5 b	65 bc	80	2.6 ab	55 a
1 mg/l NAA	67	18.5 c	58 bc	89	15.4 c	88 a
10 mg/l NAA	100	0.8 b	nd --	86	17.8 c	79 a
10 mg/l BAP	83	2.2 b	10 a	100	0.2 ab	nd --
plant	71	58.8 e	97 bc	100	179.3 f	81 a

significantly between flowers pollinated with aseptic pollen used for in vitro pollination and flowers pollinated with pollen collected from flowers grown in the greenhouse (mean=140, se=13, averaged for both treatments).

Components added to agar solidified media

The effect of several components added to agar solidified media on the number of seeds produced after in vitro pollination is presented in Tables 1-3. The seed set of several treatments differed between the cross 'Leen van der Mark' x 'Christmas Marvel' and the reciprocal cross (Table 1). The seed set increased significantly at higher sucrose concentrations in crosses with 'Leen van der Mark' as maternal genotype (Table 1). Comparable numbers of seeds were harvested from pollinations on media with 7% sucrose and media with 3.5% fructose and 3.5% glucose (Table 2). In the reciprocal cross, the seed set did not differ significantly between the sucrose concentrations tested, but was extremely low for all concentrations (Table 1). However, 10 of the 11 flowers of the latter cross died during culture on medium with 3% sucrose.

NAA (1 and 10 mg/l) improved the seed set in crosses with 'Christmas Marvel' as maternal genotype as compared to the standard medium, whereas NAA had no (1 mg/l) or a negative (10 mg/l) effect on the seed set in the reciprocal cross (Table 1). NAA (1 mg/l) was added to all media in other experiments (Tables 2-5). The addition of CH increased the seed set significantly as compared to the standard medium (Table 2). The use of MES affected the seed set positively, but not significantly (Table 2). The addition of the following components to the media had no or a negative effect on the seed set: ½MS, BAP, GA_3 and spermine (Tables 1-3). The use of liquid media did not improve the seed set as compared to agar solidified medium (Table 3). However, the lengths and widths reached by ovaries cultured on liquid media (length=46.5 (se=1.4), width=17.5 (se=0.5)) were greater than for ovaries cultured on media with agar (length=34.0 (se=0.5), width=14.6 (se=0.2)) (Fig. 1). The number of seeds harvested per ovary in vitro was in each experiment lower than harvested after pod maturation on the plant.

The germination percentage of seeds produced in vitro was comparable to the germination percentage of seeds harvested after pod maturation on the plant for most treatments (Table 1-3). The addition of BAP to the medium had a negative effect on the germination percentage, compared to most other media (except 3% sucrose) and with seeds formed on the plant (Table 1). Seeds obtained on liquid media germinated significantly less as compared to seeds obtained from media with spermine and seeds obtained after pod maturation on the plant (Table 3).

Table 2. Effect of medium composition on the maturation of ovaries, the number of seeds per ovary and the mean germination percentage after in vitro pollination of 'Leen van der Mark' with 'Christmas Marvel'. MS-medium with 7% sucrose, 1 mg/l NAA and 0.7% bacteriological agar was used as standard medium. Fructose and glucose was added to the media instead of sucrose or the buffer MES or casein hydrolysate (CH) were added to the standard medium. As a control flowers were pollinated and pods matured on the plant in the greenhouse (plant). Means followed by different letters differ significantly at the 1% level.

Media	matured ovaries (%)	seeds per ovary	germination (%)
standard medium (agar)	91	25 ab	95 a
3.5% fructose + 3.5% glucose	88	21 a	88 a
5.3 g/l MES	78	40 bc	95 a
2 g/l CH	91	42 c	89 a
plant	100	133 d	99 a

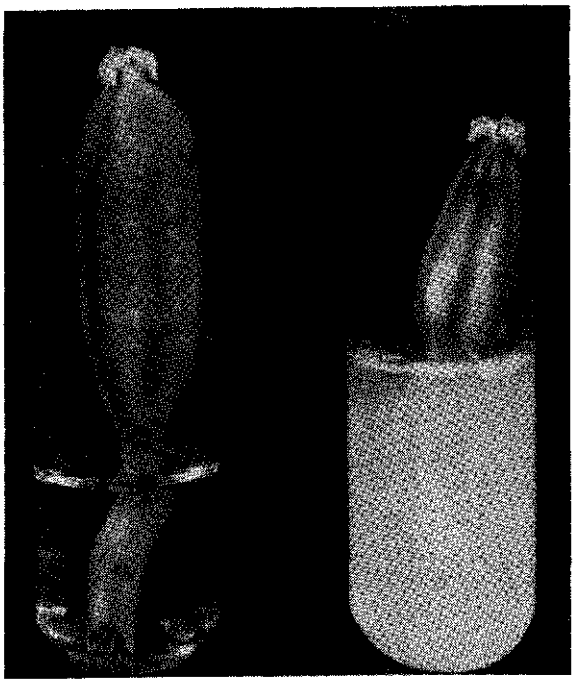


Fig. 1. Pistils of 'Leen van der Mark' pollinated with 'Christmas Marvel' cultured during 13 weeks on liquid media (left) or on media with agar (right).

Table 3. Effect of medium composition on the maturation of ovaries, the number of seeds per ovary and the mean germination percentage after in vitro pollination of 'Leen van der Mark' with 'Prominence'. MS-medium with 7% sucrose, 1 mg/l NAA and 0.7% bacteriological agar was used as standard medium. Agar was omitted from the standard medium (liquid) or GA_3 or spermine were added to the standard medium. As a control flowers were pollinated and pods matured on the plant in the greenhouse (plant). Means followed by different letters differ significantly at the 1% level.

Media	matured ovaries (%)	seeds per ovary		germination (%)	
standard medium (agar)	79	23	a	83	ab
liquid	96	30	a	74	a
1 mg/l GA_3	70	22	a	87	ab
0.2 g/l spermine	78	30	a	95	b
plant	100	187	b	99	b

Components added to liquid media

Some of the components added to agar solidified media were also added to liquid media. The results of the effect of these components in liquid media on the seed set of the cross 'Leen van der Mark' x 'Christmas Marvel' are presented in Table 4. In this experiment, more seeds were harvested per ovary on agar solidified media than on liquid media, in contrast with former results (Table 3). The number of seeds per ovary obtained on media with MES was intermediate to liquid media and media with agar. All other media components tested had no effect on the number of seeds per ovary as compared to standard liquid medium. This experiment (Table 4) was repeated with flowers from a later culture date (data not shown). The number of seeds harvested was much lower than obtained in the experiment presented in Table 4. The number of seeds per ovary was significant higher on medium with MES (mean=9, se=4) than on all other liquid media tested (mean=0.7, se=0.3). The number of seeds produced per ovary was highest on medium with agar (mean=43, se=7). The number of seeds obtained per ovary in a second cross tested, 'Cassini' x 'Prominence', was very low (data not shown). The number of seeds harvested per ovary was highest when medium with agar was used (mean=19, se=6). Addition of MES to liquid medium improved the number of seeds per ovary (mean=4, se=2) as compared to all other liquid media tested (mean=0.5,

se=0.3). In this experiment, pollen tubes had penetrated in only 9% of the ovules of the cross 'Cassini' x 'Prominence'.

The germination percentages of seeds from the cross 'Leen van der Mark' x 'Christmas Marvel' produced on media with 9% sucrose were significant lower than for seeds produced on media with agar and produced after pod maturation on the plant (Table 4). A lower seed germination was also found for seeds produced in the second experiment (data not shown) on medium with 9% sucrose or with casein hydrolysate as compared to all other media. The number of seeds obtained from the cross 'Cassini' x 'Prominence' was too low to compare the germination percentage.

Table 4. Effect of medium composition on the maturation of ovaries, the number of seeds per ovary and the mean germination percentage after in vitro pollination of 'Leen van der Mark' with 'Christmas Marvel'. MS-medium with 7% sucrose and 1 mg/l NAA was used as standard liquid medium. The sucrose concentration was increased, NAA was omitted from the medium or 0.7% bacteriological agar, the buffer MES, casein hydrolysate (CH) or spermine were added to the standard medium. As a control flowers were pollinated and pods matured on the plant in the greenhouse (plant). Means followed by different letters differ significantly at the 1% level. (nd= not determined)

Media	matured ovaries (%)	seeds per ovary		germination (%)	
standard medium (liquid)	91	24	a	78	ab
agar	100	59	b	99	b
9% sucrose	100	19	a	66	a
without NAA	100	22	a	73	ab
5.3 g/l MES	67	34	ab	nd	--
2 g/l CH	75	18	a	73	ab
0.2 g/l spermine	100	21	a	82	ab
plant	100	69	b	94	b

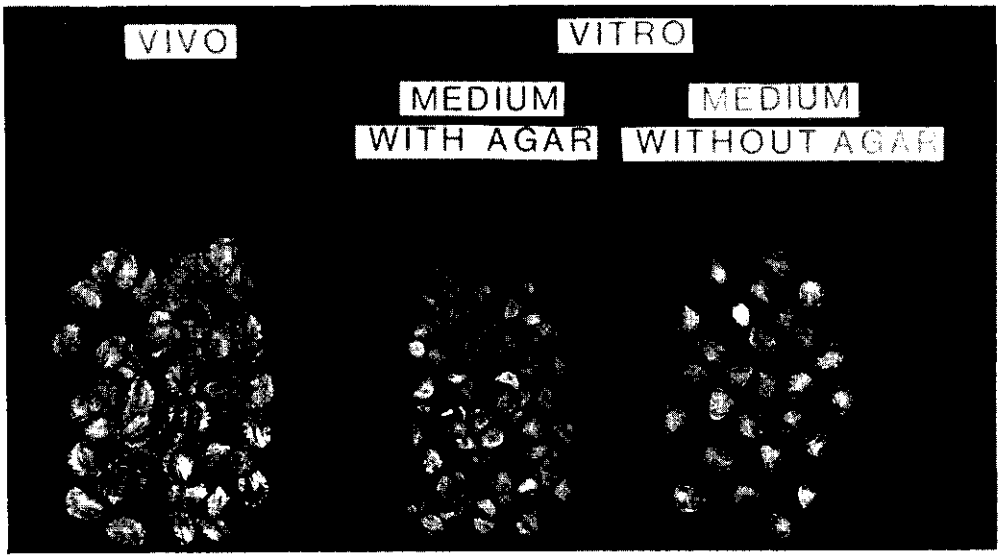


Fig. 2. Seeds from the cross 'Leen van der Mark' x 'Christmas Marvel' obtained after pollination and pod maturation on the plant (vivo) and after in vitro pollination on agar and liquid media.

Combination of liquid media with agar solidified media

The effect of the length of the period during which ovaries were cultured on liquid media and subsequently on media with agar on the number of seeds obtained per ovary is studied in two experiments. The results of both experiments are presented in Table 5. The number of seeds per ovary was affected by the length of the culture period of ovaries on medium with agar. Ovaries cultured during 9-12 weeks on medium with agar produced more seeds per ovary in comparison with shorter culture periods on medium with agar. The use of Daichin agar had a significantly positive effect on the number of seeds obtained per ovary as compared to bacteriological agar (Table 5). The number of seeds obtained after decreasing the sucrose concentration from 7% to 4% at 6 WAP was lower (exp. I) or comparable to (exp. II) the number of seeds per ovary harvested on liquid media.

The lengths and widths reached by ovaries cultured on liquid media (length=51.7 (se=1.2), width=18.8 (se=0.8)) were greater than for ovaries cultured on media with agar (length=45.6 (se=0.9), width=15.7 (se=0.4)), but remained smaller than for ovaries matured on the plant (length=63.4 (se=0.5), width=28.0 (se=0.7)). The size of the seeds were also largest in ovaries matured on the plant and smallest in ovaries matured on media with agar (Fig. 2).

Table 5. Effect of media composition on the maturation of ovaries, the number of seeds per ovary and the mean germination percentage after in vitro pollination of 'Leen van der Mark' with 'Christmas Marvel' in two experiments. MS-medium with 7% sucrose and 1 mg/l NAA was used as standard liquid medium. Ovaries were first cultured during several weeks (0, 3, 6, 9, 12) on standard liquid medium followed by a culture period of some weeks (12, 9, 6, 3, 0, respectively) on medium with 0.7% bacteriological agar. The effect of the addition of 0.7% Daichin agar to the standard medium and of lowering the sucrose concentration at 6 WAP on the seed set was also tested. As a control flowers were pollinated and pods matured on the plant in the greenhouse (plant). Means followed by different letters differ significantly at the 1% level.

Media	Experiment I			Experiment II		
	matured ovaries (%)	seeds per ovary	germination (%)	matured ovaries (%)	seeds per ovary	germination (%)
12 weeks standard (liquid)	91	4 b	91 ab	92	3 b	96 a
9 weeks liquid + 3 weeks agar	100	2 b	53 a	100	0.6 b	89 a
6 weeks liquid + 6 weeks agar	100	15 c	87 ab	100	2 b	77 a
3 weeks liquid + 9 weeks agar	100	46 de	92 ab	100	17 c	92 a
12 weeks agar	91	28 cd	85 ab	73	25 c	96 a
12 weeks Daichin agar	100	55 e	96 b	100	47 e	98 a
sucrose from 7% to 4% at 6 WAP	100	0 a	-- --	100	2 b	100 a
plant	100	180 f	99 b	100	131 f	99 a

The germination percentage in experiment I (Table 5) was significantly lower for seeds harvested from ovaries grown on liquid media during 9 weeks than for seeds obtained on medium with Daichin agar and for seeds obtained after pod maturation on the plant. However, in exp. II, the germination percentages did not differ between seeds produced on the various media and seeds harvested after pod maturation on the plant.

pH and carbohydrates

The pH of the media was adjusted to 5.8 before autoclaving. The pH of the media (liquid and with agar) had values of 5.0-5.3 after autoclaving. The liquid media in test-tubes without explants had a pH varying between 4.4-5.2 (mean=4.9), estimated until 12 WAP. The agar solidified media with explants had a pH of 4.6-4.7 at 1 WAP (2 weeks after starting the culture) and thereafter the pH varied between 3.8-4.8 (mean=4.2). At 1 WAP (two weeks after culture), the pH of liquid media with explants had values of between 3.5-5.0 and during further culturing the pH varied between 3.1-5.2 (mean=4.3).

The effect of the addition of 5.3 g/l MES on pH stability was tested in both media with agar (Table 2) and liquid medium (Table 4). For the standard agar medium, the pH was between 4.7-5.3 (mean=5.0) at two weeks after culturing and the pH was 5.6 when MES was added to the medium (Table 2). For standard liquid medium, the pH varied between 3.9-5.1 (mean=4.6) at two weeks after culturing without MES and between 4.9-5.2 (mean=5.1) for media with MES.

The results from the analysis of the concentration carbohydrates in media with agar are presented in Figure 3 and for liquid media in Figure 4. Other carbohydrates than glucose, fructose and sucrose were not detected in the media. The total concentration carbohydrates in the test-tubes without explants remained relatively constant (se=0.1) during time. The results of these determinations were therefore averaged and taken as reference. About 16% of the sucrose was converted into glucose and fructose after autoclaving the medium. The total concentration carbohydrates decreased significantly in time ($F<0.001$). The total concentration carbohydrates was significantly lower in liquid media from 6 WAP in comparison with media containing agar. The total concentration carbohydrates in liquid media decreased to less than 40% of the concentration of carbohydrates in test-tubes without explants at the end of the culture (12 WAP). The concentrations fructose and glucose in media with explants did not differ significantly ($F<0.001$) between liquid media and media with agar. However, an significant increase ($F<0.001$) in the concentrations fructose and glucose was found during culturing as compared to media without explants. The total concentration of carbohydrates consisted of about 27% glucose and of about 27% fructose (average of 5-8 WAP). The concentration of glucose and fructose in liquid media both decreased from 10 WAP on in

comparison with the concentrations found from 4-9 WAP. The concentration sucrose decreased significantly ($F<0.001$) for both media with agar and liquid media. However, liquid media contained significantly ($F<0.001$) less sucrose from 3 WAP on, compared to media with agar. The concentration sucrose was 20% at 8 WAP in media with agar compared to the carbohydrate concentration of media without explants, whereas it was 6% in liquid media. The concentration sucrose in liquid media from 9 WAP on was less than 1% of the total carbohydrate concentration of media without explants.

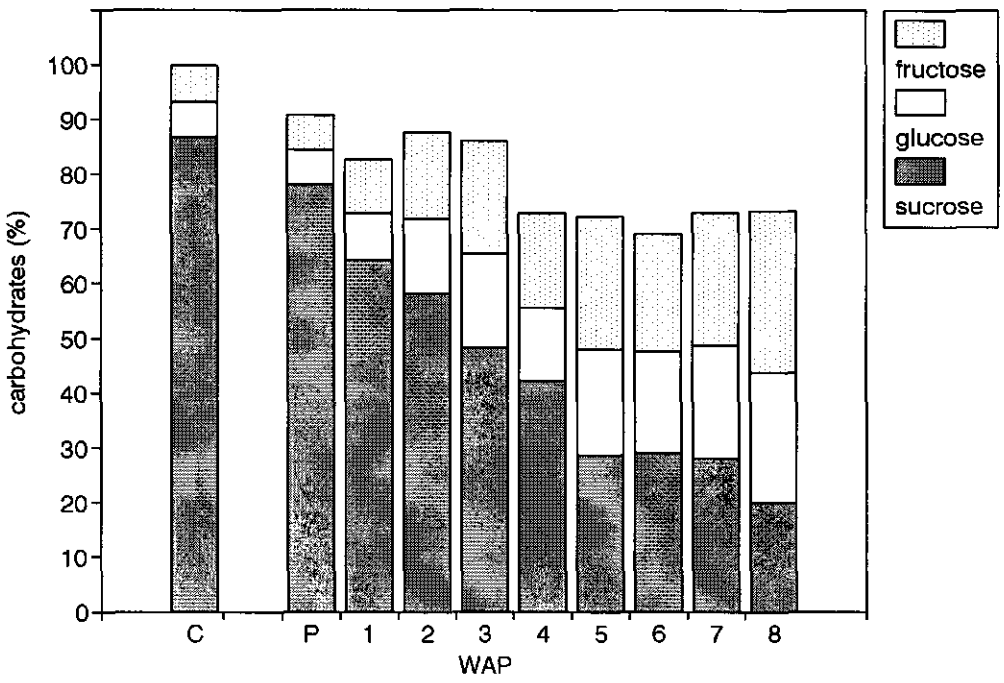


Fig. 3. The sucrose, glucose and fructose concentrations in time (from pollination (P) to 8 weeks after pollination (WAP)) in medium with agar used for in vitro pollination (7% sucrose), related to the concentrations of these carbohydrates in the same autoclaved medium, without explant (C).

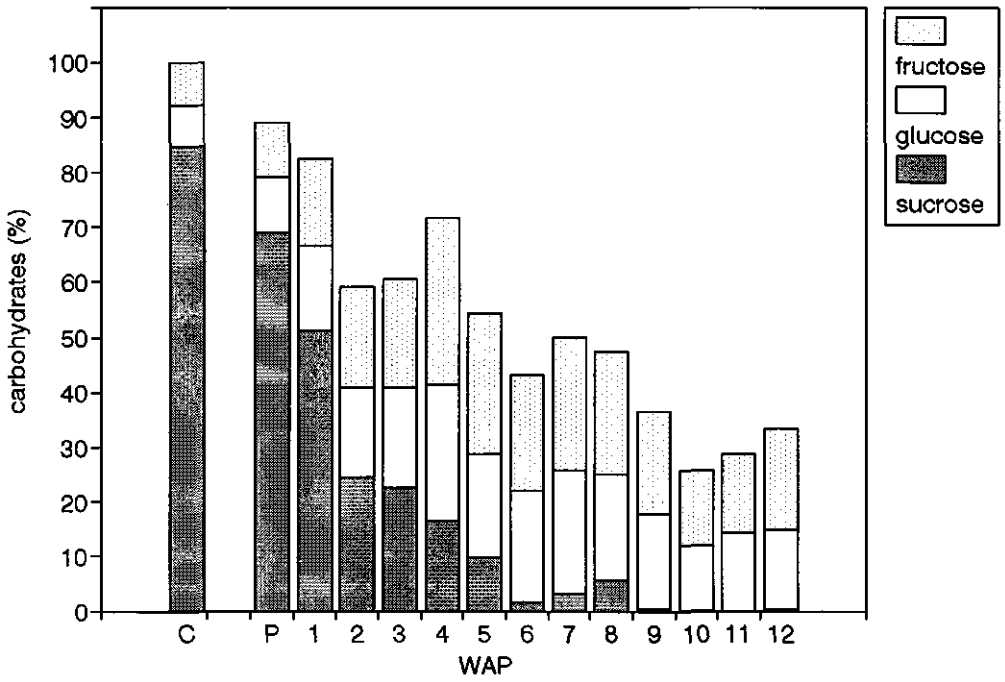


Fig. 4. The sucrose, glucose and fructose concentrations in time (from pollination (P) to 12 weeks after pollination (WAP)) in liquid medium used for in vitro pollination (7% sucrose), related to the concentrations of these carbohydrates in the same autoclaved medium, without explant (C).

Discussion

General

Flowers of *T. gesneriana* cultured from 5-7 days before anthesis and pollinated in vitro produced viable seeds. Addition of 5%-9% sucrose, MES (to liquid media), casein hydrolysate (CH) (to media with agar) and Daichin agar to the culture medium had positive effects on the number of seeds per ovary obtained. However, seeds harvested from liquid media with 9% sucrose or CH showed sometimes a lower germination. The number of seeds per ovary decreased when the media contained 3% sucrose or no agar. The use of ½MS or the addition of BAP had no or a negative effect on the seed set, depending on the cross used. Other components, such as fructose and glucose, GA_3 and spermine did not alter the seed set as compared to the standard media. Differences in the number of seeds per ovary between reciprocal crosses with 'Christmas Marvel' and 'Leen

van der Mark' were found for several media types. However, the seed set was comparable for the reciprocal crosses whenever the media contained 1 mg/l NAA. For various crops, cultivar effects were reported (Gengenbach 1977a, Richards and Rupert 1980).

After in vitro pollination in tulip, often less seeds were obtained than after pod maturation on the plant. This is also reported for other crops (Rao 1965, Usha 1965, Gengenbach 1977b). The number of seeds obtained per ovary after in vitro pollination was found to be varying between the different experiments, ranging from 9% to 86% of the number of seeds produced on the plant for standard agar solidified medium. The lower seed set in vitro may, at least partly, be due to the occurrence of embryo abortion. This is indicated by the high percentage of ovules with pollen tube penetration, which were comparable to those found after pollination on the plant (Chapter 2) and the large differences in seed set between ovaries grown on various media. The seeds produced after in vitro pollination had smaller sizes than those obtained from pods matured on the plants (Fig. 2), like after ovary culture (Nitsch 1951, Mathias and Boyd 1988) or in vitro pollination (Lardon et al. 1993) in other crops. The germination percentages were, nevertheless, as we found, in most cases comparable.

Carbohydrates

Carbohydrates appear to be an important factor for the growth of *T. gesneriana* ovaries in vitro. The total concentration carbohydrates diminished in time, attended with a decrease in the sucrose concentration. The latter was caused by conversion of sucrose into glucose and fructose and to carbohydrate uptake. The higher carbohydrate uptake in liquid media than the uptake in media with agar could explain the larger size of ovaries grown on liquid media. The fact that seed set on liquid media often is lower can not be caused by the higher uptake, because lowering the sucrose concentration (from 7% to 4% at 6 WAP) sometimes had a negative effect on the number of seeds harvested.

The number of seeds produced on media with agar in the cross 'Leen van der Mark' x 'Christmas Marvel' increased with increasing sucrose concentrations. In the reciprocal cross, the seed set remained extremely low at all sucrose concentrations which makes it difficult to detect a possible increase in seed set at higher sucrose concentrations. However, the high number of ovaries which died prematurely when cultured on media with 3% sucrose, suggests a negative effect of this low sucrose concentration. The low number of matured ovaries in the latter cross, the increase in seed set at higher sucrose concentrations on media with agar, the comparable seed set on liquid media with 7% and 9% sucrose, together with the lower germination percentage of seeds produced on liquid media with 9% sucrose indicates that a sucrose concentration of about 7% promotes seed

production and germination optimally in *T. gesneriana*, as also found for *Lilium longiflorum* Thunb. (Van Tuyl et al. 1991).

When sucrose would serve as carbon source only, the addition of 3% sucrose would be sufficient, indicated by the uptake of carbohydrates during 2 weeks. Media were refreshed every 2 weeks. Therefore, the carbohydrates might also be needed as osmoticum. However, seeds can be produced at a wide range of osmotic values, because the osmotic potential of media with fructose and glucose is much higher (doubled when added to water) in comparison with media with the same amount (w/v) of sucrose. The osmotic value of media to which sucrose was added will never attain the high value for media with only fructose and glucose, since media were refreshed every two weeks and not all sucrose is converted into glucose and fructose within two weeks of culture.

Other components

Beside sucrose, the full concentration of MS-medium, CH in media with agar and, in one cross (Table 1), NAA were needed for optimal seed set. However, because CH, added to liquid media, sometimes influenced the germination negatively, further research of the effect of CH is recommended. The ineffectiveness or negative effect of a medium component can have several causes, e.g the tested concentration is not the optimal concentration or the component has no effect on in vitro pollination (or at high concentrations there may be a toxic effect). The concentration of other media components or the culture conditions might also be suboptimal, resulting in the inability to detect an effect or interaction between a medium component and the tested components restrict the uptake. The addition of hormones (Barratt 1986, Varga et al. 1988, Lardon et al. 1993), spermine (Trione and Stockwell 1989), CH (Mohapatra and Bajaj 1988, Takahata and Takeda 1990) or salt mixture (Mathias and Boyd 1988, Lazaridou et al. 1993, Sharma et al. 1995) gave large variations in results after in vitro pollination or ovary culture of several crops.

The addition of agar usually had a positive effect on the seed set as compared to liquid media. An explanation for the comparable number of seeds obtained per ovary for both media in one experiment (Table 3) is not found, nor for the lower germination percentage of seeds produced on liquid media of this experiment as compared to the germination percentage of seeds produced on the plant. Also, no explanation can be given for the lower germination percentage of seeds produced from ovaries grown during 9 weeks on liquid media (Table 5) as compared to those produced on the plant. However, bacteriological agar may have a small toxic effect, because usually more seeds were produced when ovaries were cultured on Daichin agar, which is also used in media for adventitious shoot formation in *T. gesneriana* (Wilmink et al. 1995). Differences in plant

production due to the use of various gelling agents have been reported (Plümper and Odenbach 1991, Simonson and Baenziger 1992).

The carbohydrate uptake on media with agar is lower than on liquid media. Stolz (1971) supposed that agar added to nutrient media used for the growth of excised mature *Iris* embryos reduced water availability by binding of water. A restricted water uptake might enhance seed maturation, which might result in less embryo abortion on media with agar than on liquid media, thus declaring the higher number of seeds.

The pH of media without explants decreased in time, which was also observed in media used for ovary-slice culture and ovule culture (Chapter 6). However, media were never stored for more than a few days before use and media were refreshed every two weeks. Nevertheless, the pH of media with agar was higher (4.6-4.7) after two weeks of culture than of liquid media (3.5-5.0). The significant positive effect of the addition of MES to liquid media on the seed set was not found with media containing agar. This could be caused by compensation of the lower pH-values found in liquid media. The pH decreased more in liquid media than in media with agar, even when 5.3 g/l MES was added. It could be that the buffering capacity was not sufficient for liquid media. Preliminary research (data not shown) has demonstrated that the seed set did not decrease after the addition of 10.6 g/l MES to media with agar. The latter concentration could, therefore, be added to media.

Perspectives

The number of seeds was highest on media with agar. However, seeds produced on media with agar have smaller sizes than those produced on liquid media. This might have a negative effect on the germinal force. The culture of ovaries first during 3-6 weeks on liquid media with MES followed by culture on media with Daichin agar seems to be a good option for the production of many seeds with large sizes. However, the in vitro pollination method might be improved by the addition of other media components. Culture conditions might also be studied and could be optimized for each stage from pollination to seed maturation. In several crops, seed set was improved after in vitro pollination when accessory flower organs remained attached to the pistils (Richards and Rupert 1980, Mathias and Boyd 1988, Higgins and Petolino 1988, Zenkteler 1990a). The culture of ovaries in an 'open in vitro system', in which the peduncle is inserted through an incision in a membrane in the medium, gave good results (Varga et al. 1988, Lardon et al. 1993). According to Varga et al. (1988) this was due to improved transpiration resulting in an increase in nutrient flow as compared to the normal closed in vitro system. The selection of maternal genotypes in relation to media composition also need some further research as shown by the found media genotype interactions. It is recommended to

test as maternal genotypes, those cultivars which proved to be good genitors for interspecific hybridization (Van Eijk et al. 1991, Chapter 2).

Application of in vitro pollination offers good prospects for tulip breeding. Pollination methods, such as intra-ovarian pollination, which might bypass pre-fertilization barriers, can be developed. Interspecific crosses showing post-fertilization barriers can be made in vitro, or ovary culture can be started at early culture dates. After the application of ovary-slice culture and ovule culture at most 5% of the embryos, cultured from 3 WAP, from intraspecific *T. gesneriana* crosses germinated (Chapter 5), whereas 11% (an ovary has on average 490 ovules) germinated after the culture of flowers in vitro prior to pollination. In vitro pollination is therefore an important tool for the rescue of hybrids which abort at an early developmental stage. However, also during ovary culture endosperm still can degenerate and embryos can abort. Ovary-slice culture and/or ovule culture could be applied with ovaries cultured in vitro, resulting in an improved embryo survival rate. For each particular incongruent tulip cross, the most optimal combination of embryo rescue method should be determined, for allowing the production of new interspecific tulip hybrids.

General discussion

8

Introduction

The genus *Tulipa* L. comprises approximately 55 species, of which 49 are described by Van Raamsdonk and De Vries (1992, 1995). Commercial tulips are mainly cultivars of *T. gesneriana* L. and of Darwin hybrids. The present-day cultivars used for cut flower production could be improved by exploiting traits from other *Tulipa* species. In particular, introduction of resistances against Tulip Breaking Virus (TBV), *Botrytis tulipae*, *Fusarium oxysporum* (bulb-rot), but also characteristics such as a short forcing period, good flower longevity and new flower colours and flower shapes are important targets for tulip breeding programs. However, *T. gesneriana* has been crossed successfully with only 12 tulip species by using conventional breeding methods (Van Eijk et al. 1991, Van Raamsdonk et al. 1995). Crossing barriers hinder hybridization between many different tulip species. The main goal of the research presented in this thesis was to bypass crossing barriers between cultivars of the present-day assortment and the other tulip species.

This main goal of the research was divided in two subgoals: (1) the identification of crossing barriers in crosses between *T. gesneriana* and a number of representative tulip species and (2) the development of techniques to bypass these barriers.

To identify crossing barriers, pre-fertilization development and post-fertilization development have been studied. Pollen tube growth and pollen tube penetration in the ovules have been analyzed in crosses between *T. gesneriana* and 13 other tulip species to localize pre-fertilization barriers (Chapter 2). The progamic phase and embryogenesis have been studied in the cross *T. gesneriana* x *T. agenensis* DC. (former name *T. oculus-solis* St. Amans (Van Raamsdonk and De Vries 1995)), resulting in the identification of (post-)fertilization barriers (Chapter 3).

Several techniques were tested on their ability to bypass crossing barriers. The prospects for bypassing pre-fertilization barriers by using the cut-style method, the

grafted-ovary method and placental pollination have been studied (Chapter 4). The possibilities for hormone applications in bypassing barriers in interspecific tulip crosses have been examined (Chapter 4). Embryo rescue techniques are generally applied for bypassing post-fertilization barriers. The influence of the type of embryo rescue technique used (Chapter 5), the effect of the developmental stage at the start of the cultures (Chapter 5) and the influence of media composition (Chapter 6) on the embryo germination percentage in tulip has been studied. Finally, an in vitro pollination system has been developed for tulip (Chapter 7). Crosses were made between *T. gesneriana* and six tulip species in order to try to produce hybrids (Chapters 4 and 5).

Crossing barriers

Pre-fertilization barriers

Pre-fertilization development was studied in crosses between *T. gesneriana* and thirteen other tulip species. The analysis of pollen tube growth in the pistil and penetration in the ovules, by using the combination of callose staining (Kho and Baër 1968) and ovule clearing (Janson et al. 1993), appeared to work accurately. It enabled the recognition of penetrated ovules even after squashing, which often resulted in displacement of the ovules and rupture of adhering pollen tubes. Callose localisation as the only treatment, as used by Kho and Baër (1968), proved to be insufficient.

The analysis of pre-fertilization development showed that, in tulip there is a large variation in timing and nature of pre-fertilization barriers. In some crosses, the pollen tube growth may be stopped in the stigmatic tissue, while in other crosses pollen tube growth is almost not affected. The variation in barriers resulted in pollen tube penetration percentages of the ovules between 0%-79% (Chapter 2). The results on pollen tube growth and pollen tube penetration appeared in most cases to be in agreement with the results of seed set in interspecific tulip crosses on the plant as found by Van Eijk et al. (1991) and Van Raamsdonk et al. (1995) (Chapter 2). Variation in pre-fertilization barriers between various interspecific crosses is also observed in crops such as *Capsicum* (Zijlstra et al. 1991), *Nicotiana* (DeVerna et al. 1987, Kuboyama et al. 1994), *Ipomoea* (Guries 1978, Mont et al. 1993, Kobayashi et al. 1994), *Oryza* (Chang 1978, Sitch and Romero 1990), *Alstroemeria* (De Jeu and Jacobsen 1995) and *Lilium* (Ascher and Peloquin 1968, Dowrick and Brandram 1970, Asano 1980c).

A correlation was found between the results of pollen tube growth and the taxonomy of the genus *Tulipa* (Chapter 2). Therefore, some insight has been gathered on the appearance of pre-fertilization barriers in crosses between *T. gesneriana* and other, not studied, *Tulipa* species. Crosses between *T. gesneriana* and the other species of the same

section proved to be more or less compatible (Van Eijk et al. 1991). Crosses between *T. gesneriana* (maternal genotype) and species of the sections *Eichleres* and *Tulipanum* showed high percentages ovules with pollen tube penetration. This indicates that, although pre-fertilization barriers may reduce the percentages ovules with pollen tube penetration, they do not restrict interspecific hybridization. No or only low numbers of ovules were penetrated by pollen tubes in crosses between *T. gesneriana* (maternal genotype) and species of the sections *Kolpakowskianae* and *Clusianae* and of the subgenus *Eriostemones*. Pre-fertilization barriers prevent interspecific hybridization in these crosses. In case only a limited number of ovules is fertilized, ovaries could possibly abort. The minimum pollen tube penetration percentages needed for subsequent seed set has not been determined in *T. gesneriana*. However, this percentage is at least 10% after cut-style pollination in *Lilium longiflorum* Thunb. (Janson 1992), of which the ovary has the same morphology and number of ovules as compared to *T. gesneriana*.

A relationship between pollen tube growth and the taxonomy of the genus *Tulipa* was not found for the reciprocal crosses, with *T. gesneriana* as paternal genotype. No or only a few ovules were penetrated by a pollen tube in all interspecific crosses studied, except for the cross with *T. didieri* Jord. (section *Tulipa*). It can be concluded in general that *T. gesneriana* can be used best as maternal genotype in crosses with species from the sections *Eichleres* and *Tulipanum*. The percentages of ovules with pollen tube penetration are mostly low in crosses between *T. gesneriana* and species of the sections *Kolpakowskianae* and *Clusianae* and of the subgenus *Eriostemones*, independent if *T. gesneriana* is used as maternal or paternal genotype. However, the difference in cross direction might be the penetration of ovules by pollen tubes in one cross direction and the absence of penetration in the reciprocal cross. Reciprocal differences in pollen tube growth and in embryo formation between interspecific crosses are reported in numerous crops, for example *Capsicum* (Zijlstra et al. 1991), *Brassica* (Quazi 1988), *Ipomoea* (Guries 1978), *Nicotiana* (DeVerna et al. 1987) and *Lilium* (Ascher and Peloquin 1968).

Fertilization barriers

Fertilization barriers are difficult to analyze because fertilization takes place in a short time interval. Indications for the occurence of fertilization barriers are evident in those cases in which pollen tube growth into the embryo sac cavity occurs or pollen tube penetration and pollen tube opening are combined with absence of either the zygote and/or the endosperm. Pollen tube growth in the embryo sac cavity was observed in the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*. The percentage ovules with pollen tube penetration, but without visible nuclei in the embryo sac, was at 12 DAP higher in this cross in comparison with the compatible *T. gesneriana* cross (Chapter 3).

Apparently, fertilization barriers occur in this interspecific cross. However, since many embryos are formed in the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*, fertilization barriers do not hamper interspecific hybridization seriously in this cross.

Five types of embryo sacs have been found in tulip: Fritillaria, Drusa, Adoxa, *Tulipa tetraphylla* and *Eriostemones* (Romanov 1959). Fertilization barriers might occur in crosses between species with different types of embryo sacs due to differences in the arrangement of the nuclei. For example, 3 nuclei are present at the micropylar pole of the embryo sac in the Fritillaria, Drusa and Adoxa type of embryo sac, while in the *T. tetraphylla* and *Eriostemones* type of embryo sacs 5 and 7 nuclei, respectively, are situated at the micropylar pole. The species studied of the sections *Tulipa*, *Eichleres* and *Tulipanum* have all the Fritillaria type of embryo sac. The Adoxa, Drusa and *T. tetraphylla* types of embryo sacs have been found in species of the sections *Kolpakowskianae* and *Clusianae*. The species studied of the subgenus *Eriostemones* showed the *Eriostemones* type of embryo sac (Romanov 1959). Therefore, fertilization barriers in crosses between *T. gesneriana* and species of the sections *Eichleres* and *Tulipanum*, due to differences in types of embryo sac, are not expected, while they might occur in crosses with species of the section *Kolpakowskianae* and *Clusianae* of the subgenus *Tulipa* and with species of the subgenus *Eriostemones*.

Post-fertilization barriers

Endosperm degeneration seems to be the major cause of starvation of the embryo before seed maturity in the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis* (Chapter 3). Embryo abortion preceded by the absence or the retarded or abnormal development of the endosperm is often observed in interspecific crosses (Chapter 1). Crosses between *T. gesneriana* and species of the sections *Eichleres* and *Tulipanum* produce no or low numbers of seeds despite high percentages of ovules showing pollen tube penetration (Van Eijk et al. 1991, Van Raamsdonk et al. 1995, Chapter 2). Hybrids of the crosses *T. gesneriana* x *T. praestans* Hoog (section *Eichleres*) and x *T. agenensis* (section *Tulipanum*) were produced after the application of embryo rescue techniques (Chapter 5). It seems therefore that embryos are formed in most crosses between *T. gesneriana* and species of the sections *Eichleres* and *Tulipanum*, even in those crosses which have never produced hybrids before. Analysis of post-fertilization development will give additional information about the percentages of ovules showing embryogenesis after pollen tube penetration, about the percentages of ovules showing embryo abortion and/or endosperm degeneration and about the time embryos abort. With this knowledge, embryo rescue techniques can be started in each cross at the most optimal developmental stage.

Post-fertilization barriers might be stronger in crosses between species with different

ploidy levels, due to differences in genomic constitution of the embryo and endosperm, caused by the differences in chromosome numbers. Most species of the genus *Tulipa* are diploid, including *T. gesneriana*. Nevertheless, the genus *Tulipa* comprises also the triploid species *T. praecox* and several tetraploid species, such as *T. clusiana* DC., *T. sylvestris* L. and *T. turkestanica* Regel. However, hybrids were in other crops produced of several interploidy crosses with the aid of embryo rescue, such as *Trifolium semipilosum* Fres. (2n=16) x *T. repens* L. (2n=32) (White and Williams 1976) and *Nicotiana trigonophylla* Dun. (2n=24) x *N. tabacum* L. (2n=48) (Chung et al. 1988).

Techniques to bypass incongruity barriers

General

Incongruity barriers can hinder interspecific hybridization during all processes occurring from pollination to the recovery of viable and fertile hybrid plants. Therefore, often a range of techniques must be applied for bypassing the crossing barriers of a specific cross. For example, in interspecific *Lilium* crosses, the cut-style method must first be used for circumventing stylar barriers, then embryo rescue techniques must be applied to recover hybrid embryos (Van Tuyl et al. 1991). Finally, the fertility of the hybrids must often be restored by chromosome doubling (Van Tuyl 1989). In interspecific tulip crosses, both pre-fertilization barriers and post-fertilization barriers are found. Techniques have been studied to bypass crossing barriers occurring in the progamic phase and during embryogenesis. Emphasis has been placed on the development of techniques for bypassing post-fertilization barriers.

Pre-fertilization barriers

The cut-style method did not promote pollen tube growth, even not in the cross *T. gesneriana* 'Christmas Marvel' x *T. turkestanica* (Chapter 4). Most pollen tubes in this latter cross do not reach the ovules. Apparently, crossing barriers occurring in the short stylar part can not be bypassed by using this technique. The grafted-ovary method was applied for permitting the pollen tubes to attain their normal lengths before reaching the ovules. In compatible intraspecific *Lilium longiflorum* crosses, the seed set was low after cut-style pollination, which might be caused by the premature arrival of pollen tubes in the ovary (Janson et al. 1993). However, in tulip the pollen tube penetration percentages were not reduced after cut-style pollination in the compatible cross. The application of the grafted-ovary method offers, therefore, no advantages as compared to the cut-style method.

Intra-ovarian pollination, a type of placental pollination (Kanta and Maheshwari 1963,

Zenkteler 1990b), remains of interest to study on its ability to bypass pre-fertilization barriers. This method comprises the injection of a pollen suspension into the intact ovary or the pollination of ovules through an opening made in the ovary wall. The pollen are thus placed in the proximity of the ovules. Intra-ovarian pollination can be executed on the plant or in vitro, thus allowing pollen tube growth and pollen tube penetration in the ovules under 'natural' conditions in intact flowers. This method can be followed by embryo rescue techniques subsequently.

The pollination of ovules attached to the placenta, another type of placental pollination, offers some prospects for bypassing pre-fertilization barriers, as demonstrated in Chapter 4. Several factors have been proposed to be studied for the optimization of the described procedure for placental pollination, such as the age of the ovaries, the culture media and the type of placental pollination. A time-consuming study seems to be needed for optimization of the described procedure. (Post)-Fertilization barriers might also be more difficult to bypass in crosses between *T. gesneriana* and other species showing pre-fertilization barriers due to greater taxonomical distance between the species, differences in type of embryo sac and differences in ploidy-level. It must therefore first be questioned whether the development of such a technique is necessary.

Fertilization barriers

Fertilization barriers were not studied in the research described in this thesis. If fertilization barriers prevent interspecific hybridization in a cross, the fusion of single gametes offers in the future prospects for bypassing this barrier (Kranz and Lörz 1993, Faure et al. 1994). Fusion of gametes might also be applied for bypassing pre-fertilization barriers, as found in crosses between *T. gesneriana* as maternal genotype and species of the sections *Kolpakowskianae* and *Clusianae* and species of the subgenus *Eriostemones*.

Post-fertilization barriers

Post-fertilization barriers can occur during all developmental stages of the (pro-)embryo. An optimum is expected for the recovery of hybrids in interspecific tulip crosses, caused by an increase in the efficiency of the embryo rescue techniques in time (Chapter 5) and a decrease in the number of viable hybrid embryos in time. This optimum stage will vary between different interspecific tulip crosses, due to differences in the stages at which embryo abortion occurs in a specific tulip cross. This is illustrated by the lower number of embryos recovered from the cross *T. gesneriana* 'Christmas Marvel' x *T. praestans* as compared to the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*, despite comparable percentages ovules with pollen tube penetration. Apparently, less fertilization barriers and/or post-fertilization barriers occur at more advanced developmental stages in

the latter cross.

Seeds of the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis* were harvested in this study for the first time after pollination and pod maturation on the plant after treating the ovaries with 0.1% BAP (Chapter 4). The percentage of germinated embryos obtained in this experiment after embryo rescue was relatively high (6%), in relation with the percentage ovules with pollen tube penetration (13%), in comparison with another experiment (21% penetration, 3% germination (Chapter 5)). The treatment of the ovaries with 0.1% BAP might give a delay in embryo abortion, with as consequence an increase in the percentage embryos rescued and the survival of several embryos after pod maturation on the plant. A delay in pod abscission was found after treating unpollinated ovaries with 1% NAA or 1% BAP. The influence of the application of 0.1% BAP to unpollinated ovaries on pod abscission was not tested. However, the results obtained in this experiment (Chapter 4) must be interpreted with caution, because in other crosses, executed in 1994, seeds have also been harvested after pollination and pod maturation on the plant of the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis* and of the cross *T. gesneriana* 'Leen van der Mark' x *T. agenensis*. Sixteen second-years bulbs have been obtained in total of these crosses. These bulbs are not verified yet on their hybrid character (Eikelboom, personal communication). Additional research is, therefore, needed before definitive conclusions can be drawn on the effect of the application of 0.1% BAP. This was already stated (Chapter 4) for the treatments with 1% NAA and 1% BAP.

Tulip embryos were recovered both after ovary-slice culture followed by ovule culture and after direct ovule culture. The efficiency of these techniques increased with a more advanced developmental stage of the embryos at the start of the culturing. The percentages ovules with germination were comparable to the percentages ovules developed into seeds on the plant at 6-9 WAP, depending on the experiment (Chapters 5 and 6). The lower germination percentages for cultures started at earlier culture dates were caused by a retarded embryo development and/or embryo abortion (Chapter 5). Several aspects have been proposed for further study to achieve an increase of the efficiency at early culturing dates, such as media composition and culture conditions (Chapter 6). The culture of ovules attached to placenta, as done after placental pollination (Chapter 4), also needs further study. A high percentage of ovules with pollen tube penetration showed germination after using this technique (Table 3 Chapter 4). It is therefore interesting to compare the germination percentages of ovules cultured in transversely cut slices (ovary-slice culture) with ovules cultured in longitudinally cut slices (as done in placental pollination). In *Lilium*, it was found that the percentage of germinated embryos was higher in several experiments by using longitudinally cut ovary-slice as compared to transversely cut slices. In other experiments, the percentage of

germinated embryos was lower when ovary-slices were cut longitudinally, because of vitrification of the ovules when they came in contact with the medium (Van Tuyl, personal communications).

The germination percentages for ovary-slice culture followed by ovule culture started at various dates were for some culture dates comparable to direct ovule culture. For other culture dates, they were significant higher (Chapter 5). This means for practical breeding that to save time at a certain moment, ovary-slice culture can be started instead of ovule culture, because the initiation of ovary-slice cultures is less time consuming. The transfer of the ovules from the ovary-slices to ovule culture can be done at a more appropriate time. The time these ovules remained in the ovary-slices did, for the tested cases (2-7 weeks), mostly not influence the germination percentage (Chapter 5), thus allowing to place the ovules individually on medium during a wide range of time.

Integrated system of techniques for bypassing incongruity barriers

Several techniques can be applied to bypass post-fertilization barriers. Ovary-slice culture and/or ovule culture can be used to rescue hybrid embryos. If a defined hormone treatment does delay pod abscission, it is useful to precede an embryo rescue technique by the hormone treatment. This can improve the efficiency of the embryo rescue methods (see above).

Another method which can be applied is in vitro pollination. It allows to optimize the culture conditions which were not completely controlled in the greenhouse, such as light intensity and to optimize the culture medium. The number of embryos which can be obtained after in vitro pollination might be increased by the application of embryo rescue after in vitro pollination. The lower numbers of seeds obtained in vitro in comparison with the seed set after pollination and pod maturation on the plant, despite comparable percentages ovules with pollen tube penetration, indicate the occurence of more embryo abortion after the culture of ovaries in vitro than after pod maturation on the plant (Chapter 7). This is supported by the comparable percentages ovules with pollen tube penetration found for the different media used for in vitro pollination in combination with the significant differences in seed set between the tested media.

The number of embryos which can be rescued by using ovary-slice culture and/or ovule culture at early developmental stages is rather low (Chapter 5), but might be increased by the culture of intact ovaries. From intraspecific *T. gesneriana* crosses, at most 5% of the embryos cultured from 3 WAP germinated after the application of ovary-slice culture and ovule culture (Chapter 5), whereas 11% germinated after the culture of flowers in vitro prior to pollination (Chapter 7). Additional research is needed to determine the effect of ovary culture prior to ovary-slice and/or ovule culture on embryo

survival in interspecific tulip crosses.

Protoplast fusion and genetic transformation

Beside sexual hybridization techniques, techniques for the introduction of new traits into a species by means of asexual hybridization have been developed for many crops. Protoplast fusion (Famelaer et al. 1996) and genetic transformation (Wilmink 1996) are in development for tulip. Interspecific hybridization with the aid of pollination and/or embryo rescue techniques has several advantages over protoplast fusion and genetic transformation. In general, the period needed for the formation of a hybrid embryo is for the latter two techniques longer as compared to sexual hybridization. After protoplast fusion and genetic transformation, embryos are mostly formed after a callus phase. This may result in somaclonal variation. After protoplast fusion of diploid cells, tetraploid cells are formed resulting in tetraploid plants. Backcrossing tetraploid plants with diploid plants may result in the formation of triploid plants, which mostly are sterile. For the application of genetic transformation, protocols need to be developed for both transformation and regeneration, valuable genes should be available, the character introduced must be expressed in the plant and transformed plants must be accepted. To transform plants also more advanced laboratory equipment is required (e.g. C1-facilities) as compared to the equipment used for sexual hybridization.

Factors affecting the rate of success in bypassing crossing barriers

The efficiency of hybrid production of a specific cross is not only determined by the efficiency of the type of technique used for bypassing (a) crossing barrier(s). Several other factors also proved to influence the rate of success for hybrid production:

Flower quality: The culture conditions during forcing influence the quality of the flowers. The most optimal size of the bulbs for flower production may be selected. During forcing, the temperature can be optimized for the production of an optimal quality of flowers. For example, we observed in experiments concerning in vitro pollination in *Lilium* a decrease in the percentage of flowers which survived the transfer from the greenhouse into the test tube at high temperatures during the growth of the flowers.

Tulip forcing was done under relative controlled temperature. Pollinations and subsequent embryogenesis were conducted at the temperature recommended by Kho and Baër (1971) for interspecific tulip hybridization. However, other environmental conditions, like light intensity and relative humidity were not controlled.

Variation between flowers: The percentages of ovules with pollen tube penetration differed largely between the different flowers of the specific crosses (Chapter 2). We

found in the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis* that the percentages of ovules with pollen tube penetration varied between 0%-77% at the first three fixation dates and between 35%-79% at the last three fixation dates. Several ovaries could not be analyzed at the last three culture dates due to pod abscission. It seems, therefore, that there is a correlation between the percentages of ovules with pollen tube penetration and the period ovaries (pods) remain viable. The number of embryos which can be rescued by using ovary-slice culture and ovule culture increases with the age of the ovary at the start of the culture. Therefore, it is recommended to pollinate all flowers available and to select the most viable looking ovaries for subsequent embryo rescue.

Maternal genotype: The maternal genotype affected the percentages with pollen tube penetration (Chapter 2). Van Eijk et al. (1991) reported an influence of the maternal genotype on the seed set in interspecific tulip crosses. The effect of the paternal genotype has not been studied. In several other crops, it was found that also the paternal genotype affected interspecific and intergeneric hybridization (Oettler 1984, Takahata et al. 1993).

Reciprocal differences: Reciprocal differences were found in percentages ovules with pollen tube penetration (Chapter 2) and in seed set on the plant (Van Raamsdonk et al. 1995). In general, *T. gesneriana* can be used best as maternal genotype (Chapter 2).

Media composition: Influence of several media components on the germination percentage after embryo rescue and on the seed set after in vitro pollination was found. Influence of carbohydrates and other media components tested will be discussed subsequently.

* Carbohydrates: Differences in optimal sucrose concentrations of the culture media were found between the different types of cultures. For in vitro pollination, the addition of 5%-7% sucrose proved to be optimal (Chapter 7). The use of media with 9% sucrose in media for ovary-slice culture did improve the germination percentage as compared to 5% sucrose. However, the germination percentage did not differ between media with 3% and 5% sucrose when ovule culture was applied after ovary-slice culture and between media with 3%, 6% and 9% sucrose when used for direct ovule culture (Chapter 6). Apparently, each type of culture has its own requirement for carbohydrates.

The concentrations carbohydrates absorbed in the different cultures can be compared, assuming that the samples analyzed on carbohydrates were representative for the concentrations carbohydrates in the entire Petri dish or test tube. As already calculated (Chapter 6), four ovary-slices absorb 0.63 g carbohydrates in ovary-slice culture. One ovary (eight slices) absorbs therefore 1.3 g carbohydrates in this type of culture. The total concentration carbohydrates decreased with about 10% in ovule culture (Chapter 6). This means that the at most 50 ovules which are placed in a Petri dish absorb 0.075 g carbohydrates (3% sucrose, 25 ml per Petri dish). One ovary has on average 450 ovules, implicating the absorption of 0.68 g carbohydrate by all ovules of an ovary. After in vitro

pollination, 25% (agar solidified media) to 60% (liquid media) of the total carbohydrates was absorbed by the ovaries (Chapter 7). This implies the absorption of 0.44 g to 1.05 g carbohydrates by one ovary (7% sucrose, 25 ml medium). These results indicate that the amount of carbohydrates absorbed by the ovary-slices is in the same magnitude as absorbed by the ovaries after in vitro pollination. However, most carbohydrates are absorbed in ovary-slice culture within 1 to 2 weeks after the start of the cultures, indicating that the carbohydrates are absorbed by the ovary-slices, but not consumed. The concentration carbohydrates decreased during the whole culture period of the ovaries after in vitro pollination. Less carbohydrates were absorbed in ovule culture than in both other culture types, which is not surprising since less tissue (g Fresh weight) is placed in each Petri dish. These results on carbohydrate uptake illustrate the own requirements for media components by each type of in vitro culture. The uptake of a single medium component is, however, also influenced by the other components, as demonstrated by the difference in carbohydrate uptake between agar solidified media and liquid media as used for in vitro pollination (Chapter 7).

* Other components: The differences in medium requirements between ovary-slice culture, ovule culture and in vitro pollination are also reflected by the differences in results after the use of the full or half of the concentration macronutrients and micronutrients of MS and after the use of agar solidified media and liquid media. The germination percentages after ovary-slice culture and ovule cultures did not differ in both cultures between the concentration macronutrients and micronutrients of MS used. However, less seeds were harvested when half of the concentration macronutrients and micronutrients of MS was added to media for in vitro pollination as compared to the full concentration.

The seed set after in vitro pollination was influenced negatively when liquid media were used in comparison with agar solidified media, whereas no difference in germination percentages was observed between liquid media and agar solidified media when ovule culture was applied. After in vitro pollination, the addition of the buffer MES and the use of Daichin agar showed a positive effect on the seed set. The addition of MES to media used for in vitro pollination resulted in an increase of the seed set in comparison with media without MES, however, not always significantly. The pH of the media used for in vitro pollination decreased to 3.5-5.0, which is in the same magnitude as found in media used for ovary-slice culture and ovule culture (3.9-4.5). The addition of MES to media for ovary-slice culture and ovule culture might have, like after in vitro pollination, a positive effect on the number of embryos which can be recovered.

The use of Daichin agar in medium for in vitro pollination experiments did increase the seed set in comparison with the addition of bacteriological agar. Further research will

show if the addition of Daichin agar, which is used as standard in medium for adventitious shoot formation in *T. gesneriana* (Wilmink et al. 1995), to media for ovary-slice culture and ovule culture will result in an improved germination percentage or not. However, the effect of a medium component must be tested for each type of culture, because the above results indicate the differences in media requirements for the applied culture techniques.

Bulblet formation in vitro

The percentage of ovules showing germination was determined in the experiments on the development of embryo rescue techniques. Bulblet formation in vitro and the transfer of the bulblets grown in vitro into the soil is very difficult, both after ovule culture (Custers et al. 1992) and after in vitro propagation of tulip bulbs (Baker et al. 1990, Hulscher et al. 1992, Chanteloube et al. 1995). Improving the bulblet formation in vitro and the transfer of these bulblets into the soil seems to need a rather time consuming research and we therefore decided to study only the germination percentages. However, the formation of bulblets which could be grown to mature bulbs is of great importance for tulip breeders.

Additional research was carried out to get some insight in the percentage germinated embryos developing into first year bulblets. About 1100 ovules which showed germination were further cultured. These ovules were placed on the same medium as used for ovule culture. The ovules came from different experiments and had germinated from June to January. The ovules had attained in March the following stages: 5% had died, 18% showed no development, 59% had formed callus of which more than the half had also formed a bulblet and 18% had only formed a bulblet. The percentage of ovules showing germination on which a bulblet had formed can still increase due to the large difference in time of germination. A part of the ovules with bulblet formation was planted (100 bulblets), of which 36% had formed a second year bulb. The latter results are in agreement with the percentage second year bulb formation as found by Custers et al. (1992) for the cross *T. gesneriana* 'Christmas Marvel' x *T. gesneriana* 'Gander'. Our results show, like the results of Custers et al. (1992), the importance of improving the method for bulblet formation in vitro and the transfer of these bulblets into the soil.

Several problems needs further study to improve bulblet formation in vitro and the transfer of bulblets into the soil. A first topic to be studied is the time the cold treatment must be started and the duration of this cold treatment. Flowers were pollinated in February-March. The cultures were transferred from 15 °C to 5 °C at 16 WAP, which means in June-July. Twelve weeks later, thus in September-October, the ovules were replaced at 15 °C. Most embryos of the ovules cultured germinated spread over the entire

period from August/September-November for the compatible crosses. In general, we found seeds produced on the plant to germinate 6 weeks after the seeds were placed at 5 °C. Thus, the time range in which ovules showed germination was in our in vitro experiments much broader than found for seeds produced on the plant. This wide time range of ovules showing germination in vitro can be explained by the variation in developmental stage of the embryos. All cultures started at different dates after pollination were transferred at the same time to 5 °C. However, the embryo development was retarded in cultures started at 3 WAP in comparison with cultures started at 5 WAP and embryo development on the plant. The developmental stages of the embryos differed also between the ovules of cultures started at the same date (Chapter 5). The optimal time for the transfer of the cultures to 5 °C can, therefore, differ between the cultures started at different dates. Cultures with a retarded development might also need a prolonged cold treatment in comparison with cultures with a normal embryo development in time. Most ovules which showed germination of the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis* were found from October-January. This is at a later stage as observed for the compatible crosses. The optimal period for the transfer of the ovules to 5 °C seems to be at another time than for the compatible crosses.

A second problem is the weight of the bulblets at the time they are planted in the soil. Bulblets must weight about 50 mg for the successful production of second year bulbs (Eikelboom, personal communication). We had planted the bulblets regardless of their weights, which can declare the low recovery percentage of second year bulbs. We have observed that bulblets remained growing, during the whole culture period, despite the fact we never have refreshed the media of the ovules which showed germination. Culturing the bulblets until they have reached a weight of about 50 mg might, therefore, increase the recovery percentage.

The percentage of bulbs of incongruent interspecific crosses could be lower in comparison with compatible crosses due to hybrid breakdown. At this moment, 3 second years bulbs have been obtained of the more than 350 ovules which showed germination of the cross *T. gesneriana* 'Christmas Marvel' x *T. agenensis*. One second year bulb is obtained of the cross *T. gesneriana* 'Christmas Marvel' x *T. praestans*, from 10 ovules showing germination. The lower percentage of bulblet formation in incongruent interspecific tulip crosses makes it even more necessary to improve the bulblet formation in vitro and the transfer of the bulblets into the soil. Research concerning this topic has been started in 1996 at the Bulb Research Centre.

The percentages of seeds obtained after in vitro pollination which germinated were mostly comparable to those obtained from seeds produced on the plant. However, it appeared that less seeds showing germination formed bulblets (25%) from seeds obtained

after in vitro pollination with smaller average weights (8 mg) in comparison with seeds produced on the plant (81%, 50 mg). The low bulblet weight obtained from embryos developed in vitro makes it presumable that further problems can be expected for the production of second years bulbs. An increase in the percentage bulblet formation might be obtained by the germination and bulblet formation in vitro. This underlines the importance of an efficient system for bulblet formation in vitro and transfer of the bulblets into the soil.

Perspectives

Unique hybrids have been recovered from the crosses *T. gesneriana* x *T. praestans* and *T. gesneriana* x *T. agenensis*. The developed methods can thus be used for the production of hybrids from crosses which have never succeeded before. The number of embryos recovered from crosses from which a small number of seeds can be obtained after pollination and pod maturation on the plant might be increased by using embryo rescue techniques. Traits from several *Tulipa* species can be introduced in the present-day cultivars.

The introduction of resistance against pathogens is an important target in breeding of flower bulbs. High levels of resistance to Fusarium are found in cultivars of *T. gesneriana* (Van Eijk et al. 1985). Several cultivars of *T. fosteriana* Hoog ex W. Irving, which can be crossed with *T. gesneriana*, showed high levels of resistance to Tulip Breaking Virus (TBV). If other tulip species possess absolute resistance to Fusarium, to TBV or to other pathogens is yet not exactly known. Additional research is therefore needed to detect all possible resistances against pathogens in tulip species. The occurrence of resistance to Botrytis in the tulip species is at this moment studied at CPRO-DLO.

Another factors which can hamper interspecific hybridization in tulip is the formation of triploids. The use of triploid hybrids for further breeding is mostly impossible due to F1-sterility. Bypassing this crossing barrier is necessary for backcrossing the F1-hybrids to one of the parents. Additional research is needed on this topic. A reason for backcrossing the F1-hybrids to *T. gesneriana* are the better forcing quality and the longer flower longevity of *T. gesneriana* as compared to the other tulip species (Eikelboom personal communication).

New flower colours, flower shapes and other morphological characteristics of several tulip species can already be introduced in the present-day assortment with the aid of the described embryo rescue techniques. This gives a new impulse to the breeding of tulips. Several Dutch breeding companies already use the embryo rescue techniques developed.

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Summary

The genus *Tulipa* L. comprises about 55 species. The tulip species are classified in two subgenera, *Tulipa* and *Eriostemones*, which are subdivided into five and three sections respectively. Commercial tulips are mainly cultivars of *T. gesneriana* L. and of Darwin hybrids, the latter of which are obtained by interspecific hybridization between *T. gesneriana* and *T. fosteriana* Hoog ex W. Irving. The present-day tulip assortment could be improved considerably by introducing traits from other tulip species, such as resistance for diseases and short forcing period. With conventional breeding methods, hybrids can only be obtained from a limited number of interspecific crosses. Most interspecific crosses are hampered by crossing barriers. The main goal of the research on crossing barriers in tulip was to bypass crossing barriers between cultivars of the present-day assortment and the other tulip species. This main goal was divided into two subgoals: (1) the identification of crossing barriers in crosses between *T. gesneriana* and a number of representative tulip species and (2) the development of techniques to bypass these barriers.

Pre-fertilization development and post-fertilization development have both been studied in a number of crosses to identify crossing barriers. Pre-fertilization development was analyzed in crosses between cultivars of *T. gesneriana* and 13 tulip species from all eight sections of the genus *Tulipa* (Chapter 2). Pollen tube growth in the pistil and pollen tube penetration in the ovules showed much variation between the crosses in progamic development. Depending on the cross, pollen tubes grew as far as the stigma or the style or continued growing down into the ovary. This resulted in percentages of ovules with pollen tube penetration varying between 0%-79%. The percentages ovules with pollen tube penetration differed between the flowers of a specific cross, between the different years and between the maternal genotypes used.

The progamic phase and embryo and endosperm development were studied in the incongruent cross *T. gesneriana* x *T. agenensis* DC. (former name *T. oculus-solis* St. Amans) (Chapter 3) and in a compatible *T. gesneriana* cross. Less pollen tubes penetrated the ovules in the interspecific cross in comparison with the compatible cross. In the interspecific cross, the embryo development was more often retarded and higher percentages of ovules with aberrant embryogenesis (from 32 days after pollination) were found than in the compatible cross. In most ovules with aberrations in embryo development, also the endosperm showed abnormalities. Endosperm degeneration seems to be the major cause of starvation of the embryo before seed maturity in the cross *T. gesneriana* x *T. agenensis*.

Several techniques have been studied for bypassing pre-fertilization barriers in interspecific tulip crosses: the cut-style method, the grafted-ovary method and placental

pollination (Chapter 4). The application of the cut-style method or the grafted-ovary method in the interspecific crosses did not improve the pollen tube growth and pollen tube penetration in the ovules as compared to ordinary stigmatic pollination. The percentage of ovules with pollen tube penetration was also not increased after placental pollination. However, the pollination procedure applied for placental pollination might still be optimized to improve the percentage of ovules penetrated by pollen tubes.

The effect of hormone treatments and embryo rescue techniques on the ability of bypassing post-fertilization barriers was studied. Ovaries were treated with the cytokinin BAP (0.1% or 1%) or the auxin NAA (1%) at 12 days after pollination (Chapter 4). Seeds of the cross *T. gesneriana* x *T. agenensis* were obtained on the plant after treating ovaries with 0.1% BAP. Seed production on the plant from this cross has not been reported previously. Additional research is needed before definite conclusions can be drawn about the effect of hormone treatments for bypassing crossing barriers in a wider range of interspecific tulip crosses.

Two embryo rescue techniques were studied: ovary-slice culture and ovule culture. Compatible *T. gesneriana* crosses were used as model system. The efficiency of direct ovule culture and ovary-slice culture followed by ovule culture was compared for cultures started at 2-9 weeks after pollination (Chapter 5). The influence of media composition on the percentage of ovules showing germination was also studied (Chapter 6). In most cases, the percentage of embryos that germinated increased significantly with a more advanced developmental stage of the embryos at the start of the culture. The lower efficiency at early culture dates is due to embryo abortion and retarded embryo development. The germination percentages for ovary-slice culture followed by ovule culture, started at various dates, were for some culture dates comparable to direct ovule culture, but for other culture dates significantly higher. The length of the period of ovary-slice culture prior to ovule culture mostly did not affect the germination percentage.

Media composition influenced the percentage of ovules showing germination after embryo rescue. The germination percentage was influenced by the sucrose concentration used for ovary-slice culture. 9% Sucrose resulted in higher germination percentages as compared to 5% sucrose. The sucrose concentrations in media used for subsequent ovule culture (3%, 5%) or in media used for direct ovule culture (3%, 6%, 9%) did not influence the germination percentages. Analysis of the carbohydrates concentrations revealed that the total concentration of carbohydrates decreased with 22%-50% in media for ovary-slice culture (9% sucrose), whereas the total concentration of carbohydrates remained rather constant in ovule culture (3% sucrose) applied after ovary-slice culture. Comparable germination percentages were obtained by using media with the full or half of the concentrations micronutrients and macronutrients of the MS-medium during ovary-slice culture and

subsequent ovule culture. For direct ovule culture, started at 4, 6 and 8 weeks after pollination, the germination was not improved by the addition of the cytokinin BAP (0.01 and 0.1 mg/l), nor by the use of liquid shaken culture.

An in vitro pollination procedure has been developed in order to perform an integrated system of pollination, fertilization and embryo rescue techniques under optimal controlled environmental conditions. Once an in vitro pollination procedure has been developed, it can also be used for the post-fertilization culture of whole ovaries. By using compatible intraspecific *T. gesneriana* crosses as model, the effect of the following media components on seed set and seed germination could be studied (Chapter 7): concentration of macronutrients and micronutrients of MS-medium, concentration sucrose, fructose and glucose, the auxin NAA, the cytokinin BAP, the gibberellin GA_3, spermine, casein hydrolysate, the buffer MES and the use and type of agar. The application of MS-medium at full strength, with 5%-7% sucrose and 1 mg/l NAA proved to be suitable. Analysis of carbohydrate uptake revealed that on liquid media significantly more carbohydrates were absorbed by the ovaries than on agar solidified media. However, the numbers of seeds produced were higher on media with agar than on liquid media. The use of Daichin agar improved the seed set as compared to bacteriological agar. The addition of the buffer MES to liquid media, to control the pH, affected the seed set positively. The effect of the use of casein hydrolysate needs additional research. Other components tested had no or negative effects on the seed set and/or seed germination.

Unique hybrids have been obtained from the crosses *T. gesneriana* x *T. praestans* Hoog and *T. gesneriana* x *T. agenensis* by using hormone treatments and/or ovary-slice culture and ovule culture. These techniques proved to be suitable for rescuing embryos of incongruent interspecific tulip crosses. Improvement of culture conditions and culture media can still increase the efficiency, especially at early culture dates. Bulblet formation in vitro after embryo germination and the transfer of the bulblets grown in vitro into the soil were not the subject of our research, but proved to be problematic. For hybrid plant production, it is therefore of great importance to improve the methods of hybrid plant recovery after embryo germination in vitro. For the introduction of genes for resistance in the cultivar assortment of tulip by means of the in this study developed methods, it is of importance to locate possible resistance genitors in the tulip species. Bypassing F1-sterility, due to the formation of triploids and sterile diploids in interspecific tulip hybridization, also needs additional research. Traits from a number of tulip species such as forcing time, flower colours, flower shapes and other morphological characteristics can already be introduced in the present-day assortment with the aid of the described embryo rescue techniques. This can give a new impulse to the breeding of tulips.

Samenvatting

Het geslacht *Tulipa* L. omvat circa 55 soorten. De tulpensoorten zijn in 2 subgeslachten ingedeeld, *Tulipa* en *Eriostemones*, die onderverdeeld zijn in respectievelijk 5 en 3 secties. Het sortiment commerciële tulpen bestaat hoofdzakelijk uit cultivars van *T. gesneriana* L. en de Darwin hybriden, die verkregen zijn uit kruisingen tussen *T. gesneriana* en *T. fosteriana* Hoog ex W. Irving. Het huidige sortiment snijtulpen zou aanzienlijk verbeterd kunnen worden door het inbrengen van eigenschappen vanuit andere tulpensoorten, zoals ziekteresistentie en een korte forceerperiode. Met het gebruik van traditionele veredelingsmethoden kunnen echter slechts hybriden verkregen worden van een beperkt aantal combinaties tussen verschillende tulpensoorten. De meeste combinaties leveren geen hybriden op door het optreden van kruisingsbarrières. Het hoofddoel van het onderzoek aan kruisingsbarrières bij tulp was het omzeilen van deze barrières bij combinaties tussen cultivars van het huidige sortiment en de overige tulpensoorten. Dit hoofddoel is onderverdeeld in 2 subdoelen: (1) de lokalisatie van kruisingsbarrières in combinaties tussen *T. gesneriana* en een aantal representatieve tulpensoorten en (2) de ontwikkeling van technieken om deze barrières te omzeilen.

De progame fase en embryo en endosperm ontwikkeling zijn bestudeerd met als doel kruisingsbarrières te lokaliseren. De progame fase is bestudeerd in combinaties tussen cultivars van *T. gesneriana* en 13 tulpensoorten van alle 8 secties van het geslacht *Tulipa* (hoofdstuk 2). Het onderzoek van de groei van pollenbuizen in de stamper en de ingroei van pollenbuizen in de zaadknoppen toonde aan dat er een grote diversiteit bestaat tussen de verschillende kruisingen in de ontwikkeling gedurende de progame fase. Afhankelijk van de combinatie stopten de pollenbuizen met groeien in de stempel of in de stijl of groeiden ze door tot in het vruchtbeginsel. Dit resulteerde in percentages zaadknoppen met pollenbuisingroei tussen 0%-79%. De percentages zaadknoppen met pollenbuisingroei varieerden tussen de bloemen van een specifieke combinatie, tussen de verschillende jaren en tussen de genotypes gebruikt als moeder.

De progame fase en embryo en endosperm ontwikkeling zijn bestudeerd in de incongruente combinatie *T. gesneriana* x *T. agenensis* DC. (vroegere naam *T. oculus-solis* St. Amans) en in een compatibele *T. gesneriana* kruising (Hoofdstuk 3). In de interspecifieke combinatie waren minder pollenbuizen een zaadknop ingegroeid dan in de compatibele combinatie. In vergelijking met de compatibele combinaties was de embryo ontwikkeling in de interspecifieke combinatie vaker vertraagd en vertoonden zaadknoppen met embryo- en endospermontwikkeling meer afwijkingen (vanaf 32 dagen na bestuiving). In de meeste zaadknoppen met afwijkingen in de embryo ontwikkeling vertoonde ook het endosperm abnormaliteiten. Endosperm degeneratie lijkt de hoofdoorzaak te zijn van embryo abortie

voor de afrijping van het zaad in de combinatie *T. gesneriana* x *T. agenensis*.

Een aantal methoden is bestudeerd voor het omzeilen van kruisingsbarrières die voor de bevruchting optreden in interspecifieke tulpencombinaties: de afgesneden-stijl methode, de geënte-vruchtbeginsel methode en placentale bestuiving. De pollenbuisgroei en de ingroei van de pollenbuizen in de zaadknoppen was, in vergelijking met bestuiving op de stempel, niet toegenomen na gebruik van de afgesneden-stijl methode en de geënte-vruchtbeginsel methode. Ook na placentale bestuiving was het percentage zaadknoppen met pollenbuisingroei niet toegenomen. De bestuivingsmethode gebruikt voor placentale bestuiving kan echter nog geoptimaliseerd worden.

Hormoonbehandelingen en embryo-reddingstechnieken zijn bestudeerd om te bepalen of ze gebruikt kunnen worden om kruisingsbarrières na de bevruchting te omzeilen. Voor de hormoonbehandelingen zijn vruchtbeginsels 12 dagen na bestuiving behandeld met lanoline met daarin BAP (0.1% of 1%) of NAA (1%) (Hoofdstuk 4). Zaden van de kruising *T. gesneriana* x *T. agenensis* werden op de plant geoogst na behandeling van de vruchtbeginsels met 0.1% BAP. De oogst van zaden van deze combinatie op de plant is niet eerder gerapporteerd. Aanvullend onderzoek is nodig voordat eenduidige conclusies getrokken kunnen worden over het effect van hormoonbehandelingen op het omzeilen van kruisingsbarrières die na de bevruchting optreden in een groot aantal combinaties tussen tulpensoorten.

Twee embryo-reddingstechnieken zijn bestudeerd: ovariumplakcultuur en zaadknopcultuur. Vruchtbeginsels van compatibele kruisingen met *T. gesneriana* zijn als modelsysteem gebruikt. De efficiëntie van directe zaadknopcultuur is vergeleken met de efficiëntie van ovariumplakcultuur gevolgd door zaadknopcultuur bij cultures die 2 tot 9 weken na bestuiving zijn ingezet (Hoofdstuk 5). De invloed van de samenstelling van het medium op het kiemingspercentage is bestudeerd (Hoofdstuk 6). Het percentage gekiemde embryo's nam in het algemeen significant toe met een meer gevorderd ontwikkelingsstadium van het embryo. De lagere kiemingspercentages voor cultures die in een vroeg ontwikkelingsstadium gestart zijn, worden veroorzaakt door embryo abortie en door een vertraagde embryo-ontwikkeling. De kiemingspercentages verkregen na ovariumplakcultuur gevolgd door zaadknopcultuur waren voor een aantal tijdstippen van inzetten vergelijkbaar met die verkregen na het direct uitvoeren van zaadknopcultuur. De kiemingspercentages waren voor een aantal andere tijdstippen van inzet hoger na toepassing van ovariumplakcultuur. Het kiemingspercentage werd niet beïnvloed door de lengte van de periode dat de zaadknoppen in de ovariumplakjes zaten voordat zaadknopcultuur gestart werd.

De samenstelling van het medium heeft invloed op het kiemingspercentage na toepassing van embryo-reddingstechnieken. Het kiemingspercentage werd beïnvloed door de concentratie

sucrose gebruikt in media voor ovariumplakcultuur. Bij gebruik van media voor ovariumplakcultuur met 9% sucrose kiemden meer embryo's dan bij gebruik van 5% sucrose. Het kiemingspercentage verschilde echter niet tussen de media met verschillende sucrose concentraties gebruikt voor zaadknopcultuur, uitgevoerd na ovariumplakcultuur (3%, 5%), of gebruikt voor directe zaadknopcultuur (3%, 6%, 9%). De analyse van de suikerconcentratie toonde aan dat de totale suikerconcentratie met 22%-50% afnam in media gebruikt voor ovariumplakcultuur (9% sucrose), terwijl de totale suikerconcentratie relatief constant bleef in media gebruikt voor de daaropvolgende zaadknopcultuur (3% sucrose). Vergelijkbare kiemingspercentages zijn verkregen bij gebruik van de hele of de helft van de concentratie macro- en micronutriënten van het MS-medium voor zowel ovariumplakcultuur als de daaropvolgende zaadknopcultuur. Bij directe zaadknopcultuur, gestart 4, 6 en 8 weken na bestuiving, nam het kiemingspercentage niet toe na toevoeging van de cytokine BAP (0.01 en 0.1 mg/l) aan het medium en ook niet door gebruik van vloeibaar medium, geplaatst op een schudder.

Een procedure voor in vitro bestuiving is ontwikkeld. Dit biedt de mogelijkheid om het gehele proces van bestuiving tot en met de toepassing van embryo-reddingstechnieken uit te voeren onder optimale, gecontroleerde milieucondities. De methode toegepast voor in vitro bestuiving kan ook gebruikt worden voor in vitro cultuur van hele vruchtbeginsels na bevruchting. Als model zijn compatibele combinaties tussen cultivars van *T. gesneriana* gemaakt. De invloed van de volgende mediacomponenten op de zaadzetting en de kieming van het zaad is onderzocht (Hoofdstuk 7): concentratie macro- en micronutriënten van het MS-medium, concentratie sucrose, fructose en glucose, de auxine NAA, de cytokinine BAP, de gibberelline GA_3, spermine, caseïne hydrolysaat, de buffer MES en het gebruik van agar en het type agar. Een geschikt medium bleek het MS-medium te zijn, bestaande uit de normale concentratie macro- en micronutriënten, waaraan 5%-7% sucrose en 1 mg/l NAA was toegevoegd. De analyse van de concentratie suikers in het medium toonde aan dat vruchtbeginsels op vloeibaar medium significant meer suikers opnamen dan wanneer ze op medium met agar stonden. Bij gebruik van medium met agar werden echter meer zaden verkregen dan bij gebruik van vloeibare medium. De zaadzetting was hoger indien Daichin agar gebruikt werd in plaats van bacteriologische agar. De toevoeging van de buffer MES aan vloeibare media, om de pH op een constant niveau te houden, had een positief effect op de zaadzetting. Om het effect van het gebruik van caseïne hydrolysaat te knnen beoordelen is verder onderzoek noodzakelijk. De andere onderzochte mediacomponenten hadden geen of een negatief effect op de zaadzetting en/of kieming van het zaad.

Unieke hybriden zijn verkregen van de combinaties *T. gesneriana* x *T. praestans* Hoog en *T. gesneriana* x *T. agenensis* na toepassing van hormoonbehandelingen en/of ovariumplakcultuur en zaadknopcultuur. Deze embryo-reddingstechnieken bleken gebruikt

te kunnen worden voor het laten overleven van embryo's van incongruente interspecifieke tulpenkruisingen. De efficiëntie van beide methoden kan verhoogd worden door verbetering van de kweekcondities en van de gebruikte media, met name voor die cultures die in een vroeg ontwikkelingsstadium van het embryo ingezet worden. De bolvorming in vitro na embryo kieming en de uitplantbaarheid van deze bolletjes in de grond was geen onderzoeksdoel maar bleek erg moeilijk te zijn. Om hybride planten te verkrijgen is het daarom van groot belang om de methode vanaf de kieming van het embryo tot het verkrijgen van hybride planten te verbeteren. Voor het inkruisen van resistentie in het huidige assortiment snijtulpen met behulp van de in dit onderzoek ontwikkelde methoden, moet nog onderzoek gedaan worden naar het voorkomen van alle mogelijke resistenties bij tulpensoorten. Het omzeilen van F1-steriliteit, veroorzaakt door het verkrijgen van triploïden en steriele diploïden na interspecifieke hybridisatie in tulp, moet eveneens verder onderzocht worden. Op dit moment kunnen eigenschappen zoals forceerbaarheid, bloemkleur en bloemvorm en andere morfologische eigenschappen van de verschillende tulpensoorten reeds in het huidige sortiment tulpen ingekruist worden met behulp van de beschreven embryo-reddingstechnieken. Dit kan een nieuwe impuls geven aan de veredeling van tulpen.

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Curriculum vitae

Maria Gerarda Martina (Marjan) van Creij werd op 12 december 1965 geboren te 's-Hertogenbosch. In 1984 behaalde zij haar VWO-diploma op het Mgr. Zwijsen College te Veghel. In hetzelfde jaar begon zij de studie Plantenveredeling aan de huidige Landbouwuniversiteit te Wageningen. Als onderdeel van deze studie liep zij 6 maanden stage op het INRA, domaine St. Maurice te Montfavet in Frankrijk. Een afstudeervak Plantenveredeling is uitgevoerd bij de afdeling Siergewassen van CPRO-DLO (toen IVT). Een afstudeervak Bedrijfskunde is gedaan in Verpleeghuis Houtwijk te 's-Gravenhage. In juni 1990 studeerde zij af.

Van juli 1990 tot april 1994 was zij als onderzoeker werkzaam op het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek aan het project "Toepassing van bloembiologische technieken in soortkruisingsonderzoek bij lelie en tulp" in het kader van het door de overheid en bloembollenbedrijfsleven gefinancierde Urgentieprogramma Bollenziekte- en veredelingsonderzoek. Na april 1994 heeft zij grotendeels part-time gewerkt aan het afronden van dit onderzoek en het beschrijven van de resultaten van het onderzoek aan tulp, resulterend in dit proefschrift.