THE EFFECT OF OVULE AGE ON OVARY-SLICE CULTURE AND OVULE CULTURE IN INTRASPECIFIC AND INTERSPECIFIC CROSSES WITH *TULIPA GESNERIANA* L.

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The effect of ovule age on ovary-slice culture and ovule culture in

intraspecific and interspecific crosses with *Tulipa gesneriana* L.

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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. agenensis* DC., *T. praestans* Hoog, *T. kaufmanniana* Regel 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 with 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. 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*.

Abbreviations: WAP: weeks after pollination

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 & Baër 1971, Custers et al. 1995, Van

Creij et al. 1997a). 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* (Van Creij et al. 1997a). In the latter cross, embryos were formed, but these died prematurely or showed a retarded development. The endosperm showed a disturbed development or degenerated in most cases (Van Creij et al. 1997b). 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 several authors (Raghavan & Srivastava 1982, Rangan 1982, Rangan 1984, Collins & Grosser 1984, Williams 1987, Williams et al. 1987). 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 & Nakajima 1975, Scemama & 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, Van Tuyl et al. 1991, Lazaridou et al. 1993).

The application of embryo rescue techniques in tulip breeding has been reported

by Custers et al. (1992, 1995) and Van Tuyl et al. (1990). 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 the 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. agenensis*, *T. praestans*, *T. kaufmanniana* and *T. altaica*. *T. gesneriana* was used as the maternal genotype in interspecific crosses, because the analysis of the percentages of ovules with pollen tube penetration in reciprocal crosses between *T. gesneriana* and other tulip species has proved that mostly the highest percentages with pollen tube penetration in the ovules were obtained when *T. gesneriana* was used as the maternal genotype (Van Creij et al. 1997a). 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. agenensis* DC. (CPRO-DLO number 75145) (former name *T. oculus-solis* St. Amans (Van Raamsdonk & De Vries 1995)), *T. kaufmanniana* Regel (65252-1) and *T. altaica* Pall. ex Sprengel (68596) (former name *T. kolpakowskiana* Regel (Van Raamsdonk & De Vries 1995)) were derived from the CPRO-DLO *Tulipa* collection.

Bulbs were planted in September-October in flats and then stored at 5-9 ^{0}C for 15-18 weeks. The plants were placed in a greenhouse in January-March at a temperature of 15-17 ^{0}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 ^{0}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)) and in February 1993 (experiment II (exp. II)).

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 9 WAP for subsequent ovule culture. Nine to 13 ovaries of 'Christmas Marvel' were used per treatment and 6 ovaries per treatment of 'Leen van der Mark'. Twelve to 28 pods per cross per experiment 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). The LSD is presented for comparison of all different treatments within each experiment.

Four interspecific crosses were studied, using *T. gesneriana* as the maternal genotype and *T. agenensis*, *T. kaufmanniana*, *T. praestans* 'Zwanenburg' 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) 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 ^{0}C until 16-17 WAP. Subsequently they were placed at 5 ^{0}C to induce germination. Twelve weeks later (28-29 WAP), the Petri dishes were transferred to 15 ^{0}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 I-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 ^{0}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 Van Creij et al. (1997a). 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 (Van Creij et al. 1997b), 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 Van Creij et al. (1997b).

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 Van Creij et al. (1997b).

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).

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. In both the compatible cross and the incongruent cross, the germination percentage increased significantly in time. The combination of ovary-slice culture with ovule culture resulted mostly in a germination percentage that was comparable to the

germination percentage obtained after direct ovule culture. Only in the cross 'Christmas Marvel' x 'Leen van de Mark', the germination percentage was higher after the application of ovary-slice culture at 5 WAP followed by ovule culture 4 weeks later as compared to direct ovule culture started at 5 WAP. The length of the period of ovary-slice culture prior to ovule culture mostly did not affect the germination percentage (exp. I, data not shown).

The cross 'Christmas Marvel' x 'Leen van der Mark' and the reciprocal cross have both been made in experiment I. A significantly higer germination percentage was obtained for ovule culture started at 7 WAP and ovary-slice culture started at 5 WAP when 'Christmas Marvel' was used as the mother. The germination percentages did not differ between the two crosses for the other treatments.

Interspecific crosses

The numbers and the percentages of germinated embryos, taken together over all treatments, of the interspecific crosses are presented in Table 2. One flower of *T. gesneriana* has on average 450 ovules. In the cross 'Christmas Marvel' x *T. agenensis*, on average 5.4 ovules germinated per flower for all treatments taken together. 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.

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). No embryo germination was obtained from the cross *T. gesneriana* 'Leen van der Mark' x *T. praestans* 'Zwanenburg' and from the cross *T. gesneriana* 'Prominence' x *T. altaica*. Twelve of the 28 ovaries of the latter cross had died within 3 weeks after pollination.

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. In many of these ovules,

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 seed (179 seeds per flower) of the pods ripened on the plant.

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 (Figs. 2 and 3).

Discussion

Culture method

The results of ovary-slice culture were comparable to or better than the results of direct ovule culture. 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 & Monnier 1985) and *Petunia hybrida* Vilm. (Wakizuka & Nakajima 1975).

Culture at early dates reduces the effiency of both culture methods. This is in accordance with the results obtained by Custers et al. (1995) for ovule culture. The decrease of the germination percentages at younger embryo ages 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. Nevertheless, pro-embryos seem to be able to develop in vitro into spindle shaped embryos. This is suggested by the higher germination percentages (2.3% and 23% for cultures started at 3 WAP and at 5 WAP respectively) in comparison with the percentages of globular embryos one week in culture (cultures started at 3 WAP: of the 13% swollen ovules 5% contained a globular embryo (Table 3A), 5 WAP: 23% and 38%, respectively (Table 3B)). Media composition influenced embryo and endosperm development in several crops (Rangan 1982, Campenot et al. 1992, Comeau et al. 1992, Lippmann & Lippmann 1992). 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 & Bergervoet 1990) and *Alstroemeria* (Buitendijk et al. 1995), or applying embryo culture after ovule culture, like in *Medicago* (McCoy & 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. 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 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 might, therefore, be at 7 WAP (last date studied) or at later dates in this cross.

Less embryos of the cross *T. gesneriana* 'Christmas Marvel' x *T. praestans* germinated in cultures started at the same date 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' (Van Creij et al. 1997a) 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).

Hybrids from the cross *T. gesneriana* x *T. kaufmanniana* can also be produced on the plant (Van Raamsdonk & De Vries 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 & 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 (Van Creij et al. 1997a). 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). In this article, the percentage of germinated embryos using embryo rescue techniques in vitro is reported. 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). After embryo germination it will take at least 5 years of additional growing before tulip bulbs which flower are produced. 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 oryzaline treatment, as done in *Lilium*, may restore fertility (Van Tuyl et al. 1992).

Through the production of unique interspecific hybrids, the ovary-slice culture technique and the ovule culture technique presented in this article proved to be powerful methods for overcoming post-fertilization barriers, which hamper the production of hybrids from many interspecific tulip crosses. Further improvement of culture methods and culture media can still enhance the efficiency of embryo rescue in tulip, resulting in an improvement in the number of embryos rescued at early culture dates.

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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 de Mark' (LvdM), executed in 2 years (exp. I-II) 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 comparison within 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	
Ovule culture:					
3		4.7	2.3	1.6	0.2
5		7.7	13.1	11.1	1.3
7		18.8	26.8	29.1	3.1
9		44.0	41.3	nd	nd
Ovary-slice culture and ovule culture:					
2	7	4.2	2.0	nd	nd
3	6	nd	nd	5.0	0.1
4	5	nd	nd	14.8	nd
5	4	14.1	25.0	20.3	1.2
Plant:		35.4	38.5	41.1	0.0
LSD		7.7	7.7	6.0	0.9

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)	germination 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. altaica*	15	996	0	--	--

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 *T. gesneriana* 'Christmas Marvel' x *T. gesneriana* '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).

WAP	Ovary-slice culture		Ovule culture				
	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).

WAP	Ovary-slice culture		Ovule culture				
	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

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').

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).