

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

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*Key-words:* carbohydrates, embryo rescue, media composition, tulip, *Tulipa gesneriana*.

### Abstract

The effect of several media components on the germination percentage of ovules in intraspecific *T. gesneriana* crosses was studied by using two embryo rescue techniques viz. ovary-slice culture followed by ovule culture and direct ovule culture. The addition of 9% sucrose to 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 with ovules excised from the ovary-slices (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, at 6, and at 8 WAP, the germination percentages did not differ between ovules cultured on media with 3%, 6% or 9% sucrose. The addition of the cytokinin BAP (0.01 or 0.1 mg/l) had no effect on the germination percentage. The use of liquid-shaken culture resulted in germination percentages which were similar 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 19%-48% in media for ovary-slice culture, whereas the total concentration of carbohydrates did not decrease remarkably in media for ovule culture.

*Abbreviations:* WAP - weeks after pollination; MS - Murashige and Skoog (1962) medium; NAA -  $\alpha$ -naphthalenacetic acid; BAP - 6-benzylamino purine

### 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 (Van Creij et al., 1999). The efficiency of seed/seedling 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 percentage of recovered embryos after ovary-slice culture and ovule culture increased in tulip with increasing embryo age (Custers et al., 1995; Van Creij et al., 1999). However, in many incongruent interspecific crosses, embryo rescue techniques 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 momentarily only few hybrids have been obtained, like *T. gesneriana* x *T. praestans* Hoog (Van Creij et al., 1999) or from crosses which will not succeed with the currently available methods.

The media used for embryo rescue of different crops vary in composition and are more complex for young embryonal stages. Sucrose is often applied as carbon source. 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 salt mixture and vitamin mixture (Kobayashi et al., 1993; Gudin, 1994), type and concentration of hormones (Cohen et al. 1984; Campenot et al., 1992), agar (Savka et al., 1985; Buitendijk et al., 1995) and amino acids have been reported (for review see Williams et al., 1987; Sharma et al., 1996).

Media components have been proved to influence the efficiency of embryo rescue techniques in many crops. In this study, media components have been studied in ovary-slice culture and ovule culture in tulip. Results are presented on the influence of sucrose, macronutrients and micronutrients, 6-benzylamino purine (BAP) and agar on the germination percentage of embryos of intraspecific *T. gesneriana* crosses, cultured from different dates after pollination. The concentration of sugars in the medium during culture has been studied in order to determine the role of carbohydrates during ovary-slice culture and ovule culture in tulip.

## **Material and methods**

### *Plant material and pollination method*

Bulbs of *T. gesneriana* L. 'Christmas Marvel' and *T. gesneriana* 'Leen van der Mark' were obtained from commercial stocks. 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. Pollen was rehydrated before pollination at a 100% relative humidity for two hours at 15 °C.

### *Plant treatments*

Pods (henceforth called ovaries when used for *in vitro* culture) were collected 3-8 weeks after pollination (WAP). Ovaries were surface sterilized by soaking them in 70% ethanol for

1 minute, 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 from the ovaries or from the ovary-slices. 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 and then 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 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*

The standard medium for ovary-slice culture was composed of half of the concentration macronutrients and micronutrients and the normal concentration of 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  $\alpha$ -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). The pH was adjusted before the addition of agar and before autoclaving the medium during 20 minutes at 120 °C. Nystatin was dissolved in dimethyl sulphoxide (DMSO). NAA, Nystatin and antibiotics were filter sterilized and added after autoclaving.

### *Combination of ovary-slice culture and ovule culture*

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 at 5 weeks after pollination (WAP). The slices were placed on medium which consisted of ½MS or MS, both with 5% or 9% sucrose. Four weeks later, at 9 WAP, ovules were excised from the ovary-slices and placed individually on medium. The media consisted of ½MS or MS, both with 3% or 5% sucrose. Approximately the same number of slices or of ovules from each position in the ovary were placed on each medium. Results were analyzed statistically by means of the t-test (Payne et al., 1993). The statistical analysis was executed on probit scale (McCullagh and Nelder, 1989).

### *Direct ovule culture*

The influence of the sucrose concentration (3%, 6%, 9%) and of bacteriological agar (liquid (0.0%) and 0.7%) and of the cytokinin 6-benzylamino purine (BAP: 0, 0.01 and 0.1 mg/l) on the percentage of germinated ovules were investigated in three different experiments. Liquid media were placed on a shaker at 15 rpm.

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, at 6 and at 8 WAP, the ovules of 6 ovaries were excised and placed on medium. Each of the three carpels of an ovary was used for a different treatment within an experiment. Ovules cultured in liquid media were placed on agar-solidified medium at 16 WAP when all cultures were transferred from 15 °C to 5 °C.

Results were analyzed statistically by means of the t-test (Payne et al., 1993). The LSD is presented for comparison of all different treatments within each experiment.

## *Carbohydrates*

The concentration of carbohydrates has been measured during culture 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 or at 5 WAP. Fourteen ovaries were used per date. Ovules were excised from the ovary-slices at 9 WAP and placed individually on medium.

Samples for carbohydrate analysis were taken weekly from 4 to 10 WAP and at 12, 16, 24, 32 and 42 WAP. 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. 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 as described by Lipavska and Vreugdenhil (1996). 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 (ovary-slice, cultivar, medium) 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 germination percentage of ovules from slice 7 was significantly ( $S < 0.001$ ) lower than those from ovary-slices 2-6.

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 ( $S < 0.001$ ) 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 ( $S < 0.001$ ) 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, 21% with 'Leen van der Mark' as mother. 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.

### *Direct ovule culture*

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

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. The germination percentages were also not influenced by placing the ovules on liquid-shaken medium (until 16 WAP) as compared to solidified media. Interactions between the application time of ovule culture and the medium used were not found in both experiments. The germination percentages increased with increasing the ages of ovules, comparable to the germination percentages shown in Table 3.

### *Carbohydrates*

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. Carbohydrates other than glucose, fructose and sucrose were not detected 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 and before inoculation, 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. The total carbohydrate concentrations had diminished between 6-9 WAP with 23%-31% for cultures started at 3 WAP (average 26%) and with 19%-48% for cultures started at 5 WAP (average 30%), as compared with 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 (Figure 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).

### **Discussion**

The addition of 9% sucrose to media for ovary-slice culture resulted in a higher germination percentage than the addition of 5% sucrose. If sucrose is freely 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 diminished only by 19%-48%. Therefore, sucrose seems to have also a role as osmoticum.

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 (Van Creij et al., 1999). 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.

The concentrations of carbohydrates in the medium for ovule culture changed little 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 from 5 WAP, respectively, germinated (Van Creij et al., 1999). For continuing embryogenesis, it seems that the ovules must have absorbed carbohydrates from the ovule culture medium. An osmotic effect of the sucrose concentrations used in medium for ovule culture is not expected, in contrast to media for ovary-slice culture, because the germination percentage did not differ for the sucrose concentrations tested in direct ovule culture of tulip (Table 3). Maturation of embryos despite undetectable uptake of sugars was also found in somatic embryogenesis of *Picea mariana* Mill. (Tremblay and Tremblay, 1995).

The germination percentages were not influenced using different concentrations of MS-medium or agar or BAP. This can be explained by several factors. Firstly, the tested components really do not influence the germination percentage. Secondly, the concentration of other media components or the culture conditions might be suboptimal and as a consequence 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; Neal and Topoleski, 1983; Schmitz and Lörz, 1990).

With the methods developed for ovary-slice culture and ovule culture in tulip, germinating embryos have been obtained of compatible tulip crosses. Although by using these methods less embryos of compatible crosses were recovered in several cases than after pod maturation on the plant, these methods allow us to produce embryos of incongruent interspecific crosses which do not produce seeds after pollination and pod maturation on the plant. In fact, unique hybrids have been produced from the crosses *T. gesneriana* x *T. agenensis* and *T. gesneriana* x *T. praestans* after using ovary-slice culture and ovule culture (Van Creij et al., 1999). The efficiency of the embryo rescue methods proved to be dependent on the sucrose concentration in the media for ovary-slice culture. As already stated in the introduction many different media components are used in media for embryo rescue. Thus the efficiency of the available embryo rescue methods for tulip might still be raised by the addition of other components to the media. However, it appeared that the efficiency of the production of germinating embryos was also influenced by the specific cross made. An 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). Therefore, to improve this powerful method for the production of new unique hybrid tulip embryos not only media components but also genetic effects might be studied.

## Acknowledgements

We thank H.M.C. van Holsteijn, L.H.W. van der Plas, J.M. Sandbrink and J.L. van Went for critical reading this manuscript, J. Janssen for the statistical analysis, W. Eikelboom for his technical assistance and W.A. van Dijk and J.P. van Empel for their constant care of the plant material. Part of the work was supported by the Urgency Programme for Research on

Diseases and Breeding of Flower Bulbs. The analysis of the carbohydrate contents was partly funded by the European Communities' BIOTECH Programme, as part of the Project of Technological Priority 1993-1996.

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*Table 1.* The effect of ovary-slice origin on the ovule germination percentages for the cross 'Christmas Marvel' x 'Leen van der Mark' (CM) and the reciprocal cross (LvdM). The mean germination percentages per ovary/pod after ovule culture and after pod maturation on the plant are also presented.

Crosses	ovary-slice*									plant
	1	2	3	4	5	6	7	8	Mean	Mean
CM	5.6%	39.9%	41.4%	36.3%	36.4%	29.9%	24.5%	5.7%	33%	56%
LvdM	8.5%	22.5%	19.5%	24.0%	31.0%	23.6%	13.7%	1.7%	21%	33%

\*Ovary-slices 1 to 8 are successively cut from the top towards the bottom of the ovary.

*Table 2.* The ovule germination percentages 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 whole concentration macronutrients and micronutrients (MS) or half of these concentrations ( $\frac{1}{2}$ MS) and either 5% or 9% sucrose for ovary-slice culture and 3% or 5% sucrose for ovule culture. The results from ovules of the ovary-slices 2-7 were analyzed.

ovary-slice culture	ovule culture			
Media	$\frac{1}{2}$ MS + 3% sucrose	MS + 3% sucrose	$\frac{1}{2}$ MS + 5% sucrose	MS + 5% sucrose
$\frac{1}{2}$ MS+5% sucrose	34.7%	21.0%	26.8%	27.4%
MS+5% sucrose	31.3%	26.9%	24.2%	31.3%
$\frac{1}{2}$ MS+9% sucrose	37.4%	40.0%	39.6%	31.0%
MS+9% sucrose	47.9%	49.5%	38.0%	46.7%

*Table 3.* The effect of sucrose concentration of the medium ( $\frac{1}{2}$ MS) on the ovule germination percentages of cultures started 4, 6 and 8 weeks after pollination (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<sub>WAP</sub> : 6.8  
LSD<sub>sucrose</sub> : 6.8  
LSD<sub>WAPx sucrose</sub> : 11.7