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, pH, tulip, *Tulipa gesneriana*.

Abstract

The effect of several media components on the germination percentage of ovules in intraspecific *T. gesneriana* crosses was studied after the application of 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 22%-50% in media for ovary-slice culture, whereas the total concentration of carbohydrates did not decrease remarkable in media for ovule culture.

Abbreviations: WAP - weeks after pollination; MS - Murashige and Skoog (1962) medium; NAA - α-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(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 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 already unique hybrids were obtained using these methods (Van Creij et al., 1997). This is especially important for crosses from which momentarily only few hybrids have been obtained, like *T. gesneriana* x *T. praestans* Hoog (Van Creij et al., 1999). 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. Carbohydrate levels appeared also to regulate expression of a group of genes (Koch, 1996). 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).

Media components have proved to influence the efficiency of embryo rescue techniques in many crops. For this reason, 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 pH of the medium during culture has been determined to get insight into possible changes in pH. 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 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 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. 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

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). 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

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 excised 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.
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 the experiment. The statistical analysis was executed on probit scale (McCullagh and Nelder, 1989).

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, 6 and 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 and pH

The concentration of carbohydrates and the pH have 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 and at 5 WAP. Fourteen ovaries were used per date. Ovules were excised 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 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 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 (F pr. <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 (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, 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.

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.

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 were not found. The germination percentages increased in time from 1.8% to 31% on average at 8 WAP. After maturation on the plant, 20% of the ovules had developed into seed.

The germination percentages were not influenced by placing the ovules on liquid shaken medium (until 16 WAP) as compared to solidified media (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.

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. 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, 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 at 3 WAP and 30% for cultures started at 5 WAP, 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).

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 were slightly lower, fluctuating in time between 3.9-4.5.

Discussion

Carbohydrates

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 22%-50%. Therefore, sucrose seems to have another function, besides being a source of energy, eg. it has 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 high carbohydrate level in medium for ovary-slice culture might also be needed for sugar-regulated gene expression (Koch, 1996). 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 hardly changed 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). The germination

percentage did also not differ for the sucrose concentrations tested in ovule culture of *Pelargonium x hortorum* Bailey (Scemama and Raquin, 1990) and *Helianthus* (Espinasse et al., 1991). Sucrose might be needed for sugar-regulated gene expression during ovule culture of tulip, because the sucrose concentration is not limiting in medium for ovule culture and sucrose does not seem to play a role as osmoticum. Maturation of embryos despite undetectable uptake of sugars was also found in somatic embryogenesis of *Picea mariana* Mill. (Tremblay and Tremblay, 1995).

Other components

The germination percentages were not influenced using different concentrations of MS-medium or agar or BAP. This is in contrast with results obtained in other crops after varying the concentration MS (Kobayashi et al., 1993; Gudin, 1994) and the positive results obtained using media without agar (Savka et al., 1985; Buitendijk et al., 1995). When using cytokinins, positive and negative results, or no effects, were reported, depending on the concentrations and types of cytokinins 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 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; 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, as has also been reported by Skirvin et al. (1986). In ovary-slice culture and ovule culture of tulip the pH of the medium 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 plant species (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). 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

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 allows 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). 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

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 (½MS) 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	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

Table 3. The effect of sucrose concentration (3%, 6%, 9%) of the MS-medium (½MS) on the ovule germination percentages of cultures started 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-benzylamino purine (BAP) (0, 0.01 or 0.1 mg/l) added to MS-medium (½MS+3) on the ovule germination percentages of cultures started 4, 6 and 8 WAP for the cross 'Leen van der Mark' x 'Christmas Marvel'.

	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

Table 5. The ovule germination percentages on MS-media (½MS+3) containing 0.0% (liquid) or 0.7% bacteriological agar (agar) of cultures started 4, 6 and 8 WAP for the cross 'Leen van der Mark' x 'Christmas Marvel'.

	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

Figure 1. The sucrose, glucose and fructose contcentrations 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 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 (see Figure 2).

Figure 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 without explant (C). Ovule cultures were started at 9 WAP (9o). Ovules were dissected from ovary-slices which were pre-cultured from 3 WAP (3s) or from 5 WAP (5s) (see Figure 1).