Sunflower breeding and flow cytometry at Esmeralda Breeding & Biotechnology

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Sunflower breeding and flow cytometry at Esmeralda Breeding & Biotechnology;

“Cytoplasmic male sterility in sunflower,

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Polyploidization in sunflower.”

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Preface

This report consists out of 2 parts, a personal reflection paper and 4 reports/chapters about my research at Esmeralda Breeding & Biotechnology. My main project during my internship was about cytoplasmic male sterility in sunflower. Besides this project I have had several side projects, from which three are included in this report, two about flow cytometry and one about polyploidization. I have enjoyed my stay at Esmeralda and learned a lot during the 6 months I have been doing my internship in Ecuador.

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Reflection paper

During a period of 6 months I have been given the opportunity to get acquainted with Esmeralda Breeding & Biotechnology located in Ecuador. I have walk along with several different breeding programs and was able to get to know the breeding cycle from selection of the parents to the final selection for the commercial variety. Besides selections I also learned different breeding techniques used within these crops.

Company outline

Esmeralda is a flower company located in 10 different countries. In these countries they grow many different crops, bred by themselves but also flowers from other breeders. Most of the flowers produced are exported to either America or the Netherlands. Within the company the quality of the flowers is the most important expect.

The breeding is located in El Quinche, Ecuador (50 km from the capital Quito) and established in 1999. Since 1999 the breeding has grown to a large and influential company with 250 employees and a nice budget. They breed for many different flowers crops (for example *Gypsophila, Hypericum, Roses, Alstroemeria, Zantedeschia, Asters, Solidago, Limonium* and *Trachelium*), but unlike other companies they do not market their bred varieties. The varieties they breed for are only grown on the farms of Esmeralda, making their varieties more exclusive. Furthermore they control the quality and amount of production more intensively by only growing the flowers themselves. The new varieties are carefully selected based on disease resistance, productivity, colour, texture and vase life. Since 1999 they have produced 70 new varieties and every year many new varieties are added to this number.

The structure of the company is very clear. First of all the director is in charge of the company, which is divided into the branches breeding, growing, tissue culture and administration. Every different branch has one or more persons in charge with several, to many employees below them. Besides the director of breeding, the director of Esmeralda Farms, Pieter Ullrich, is very involved with all the breeding of the company and he also decides the direction in which to breed.

Personal experiences within the company

During my stay at Esmeralda Breeding & Biotechnology (EBB) I was able to get to know the company and the people quite good. Because the company was very open I could really decide by myself which subjects I would like to do next to my main project. I could walk along with many different crops and got a broad idea of the flower breeding in total.

The atmosphere at EBB is very open, not only to me but also between the employees and their bosses, which in my opinion promotes a better attitude of the employees. Because the people are much cheaper and the availability of space is much larger in Ecuador than for example here in the Netherlands, these aspects play smaller role in the choices made. Due to this experiments and trials can be conducted which would be way too laborious or would take too much space to grow here in the Netherlands.

In my opinion a weak spot of the company might be that one person decides the direction of the breeding. The varieties are bred for the American but also for the European market. Both these markets have different demands and when one persons, also when he has a lot of knowledge about

both markets, decide the direction of the breeding he might be focused too much in one direction and might lose varieties which could be good for the other market.

Learning goals

When I started my internship I wanted to develop my practical skills in relation to plant breeding. During my study I have had a lot of theory about plant breeding, but never really the practical part of it. For example, during the study you learn which techniques to apply with certain problems, however how to apply these techniques I never learned. Besides developing the practical part of breeding I wanted to get to know a breeding company and how it is to work in a company. For example, on what are the choices based which they make, which problems do they encounter. This subjects have been given attention in several courses, but again I was curious if the practice is the same as the theory.

During my internship I could develop my learning goals. My internship was rather practical and I applied several breeding techniques like polyploidization, flow cytometry, divining crossing barriers, and improving pollen germination media. Besides the breeding techniques I also made part of the crosses myself, which brings in total different problems. Next to developing my practical skills was I also part of the breeding team. For example I made a breeding scheme for a seed propagated crop at EBB. I gave a presentation for the other breeders to learn them what I have learned here at the university about seed propagated crops and discussed my breeding scheme with my supervisor. By discussing this breeding scheme I got acquainted with all the parts of the breeding, as for example setting your breeding goals, making your choice of approach, judging the time needed. With assignments like these I was able to be part of the breeding team and not only walking along.

By walking along with a breeder (in *Limonium* and *Zantedeschia*), I learned to select important varieties in aspect to quality, consumer interest and ease of propagation. Furthermore by making Interspecific crosses with *Eryngium* and *Limonium*, the importance of a precise planning and importance of a procedure within breeding was once more emphasized. This because by making part of the crosses myself it appeared a clear procedure was more important than I expected. Also the planning appeared important in contrast to the time of harvesting the embryo to include it in the embryo rescue program.

With my mean project, cytoplasmic male sterility in sunflower, I worked together with 3 other persons. Because of this I also developed my organising skills, which I will probably also need in a future job. However I noticed that the skill, being a leader, can use more development. I noticed this because in some cases I was not clear enough, which caused in some cases miscommunications. In the beginning there was also a language barrier, but even with this problem I could work good together with the other persons. Furthermore with the project it appeared once more that the theory can be very clear, but that the practice can be quite different and is not always simple to explain.

During my internship I encountered skills which can use further development, like mentioned leadership. Besides this skill it is also important that I don't go to work too fast, but that first I am sure I researched all possible possibilities. This came forward with the project about flow cytometry, where the amount of nuclei might not have been important if I had searched more in literature in advance. Afterwards it became more and more clear about the importance of staining inhibitors, meaning that you should always use the same amount and same source of material (either in vitro or in vivo material).

Besides my learning goals in respect to the university I also wanted to learn how it is to be in another country/ culture. During my stay in Ecuador I got to know a lot of the culture through my guest family and colleagues. They were very open and took me to many different places and I was able to get acquainted with several traditions of Ecuador. I was surprised how easily I got used to the different surrounding and how good I could handle everything in another language, which in the beginning I did not spook. It was a good experience to get to know myself better in respect to my personality. When I for example encountered problems I was able to keep my head clear and so handle the problems correctly. Furthermore the problems didn´t took my whole attention and so I was still able to enjoy my stay in Ecuador.

Cytoplasmic Male Sterility in Sunflower

1.1 Introduction

Sunflower is an economical important crop. It serves as a food and oil crop, but sunflowers are also produced for its ornamental value. Due to its economical value sunflowers are researched intensively, especially for the trait for oil content of the seed. However cytoplasmic male sterility (CMS), due to its importance for producing hybrids, also has been studied thorough. CMS sunflower lines are used to produce hybrids, but within the ornamental market pollen less flowers have a higher value and commonly also have a longer vase life.

"Cytopasmic-Genetic Male Sterility results from the interaction among the cytoplasmic (mitochondrial) and nuclear genes. For a genotype to be male sterile, a proper combination of nuclear background, together with a specific mutation in the mitochondrial DNA (mtDNA) is needed. The nuclear genes, called fertility restoration gens can compensate for the cytoplasmic mutation and normal pollen formation will occur. Therefore the expression of CMS genes is governed by the type (dominant/recessive) of nuclear fertility restoring gens. The fertility restoring alleles have been represented by symbols like Rf (fertility restoring) in sunflower, wheat and corn, and Ms (male fertile) in onions, sorghum and pearl millet. Different combination of sterile (S), normal (N) cytoplasm and fertility restoring genes determining the sterility /fertility of the plant are given in the figure below.

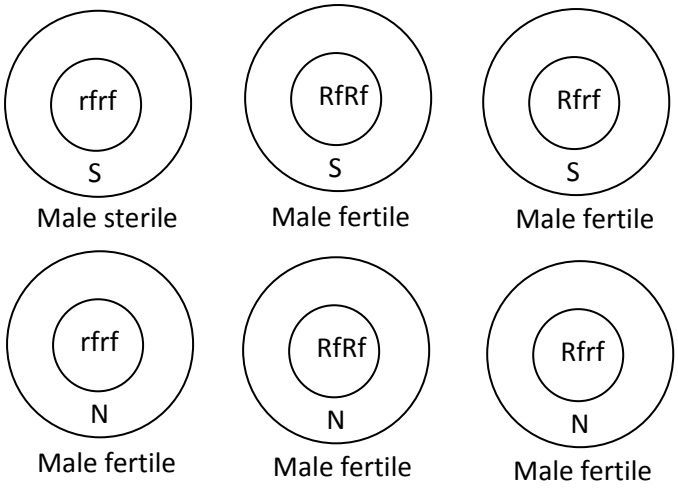

Figure 1.1. Overview of the fertile and sterile genotypes.

Normal (N) cytoplasm, irrespective of the gene in the nucleus results in a fertile plant. Sterile (S) cytoplasm operates only when both the alleles of fertility restoring nuclear gene are recessive (rfrf). Whereas, in the presence of dominant fertility restoring allele, the sterile cytoplasm becomes inoperative and the plant is normal/ fertile" (Chahal and Gosal, 2002).

CMS in Maize, Bean, Rice and Petunia can be caused by several recombination events in the mitochondrial DNA and duplicated regions, which function in other parts of the DNA perfectly normal. Also in *Helianthus* annuus the sterile cytoplasma derived from *H.* petiolaris (PET1) only differs in a small inversion and insertion of a normal DNA sequences. Due to these rearrangements, a new open reading frame is present in the sterile line which produces an additional larger transcript which is translated into a protein causing the sterility (Saumitou-Laprade et al., 1994).

Cytoplasmic male sterile lines in sunflower can be obtained through Interspecific hybridization of *Helianthus* species (i.e. *H*. gigantueus and *H.* annuus). By making backcrosses with a *H.* annuus open pollinating cultivated variety a male sterility was found after the second backcrossing cycle (Whelan, 1980). The sterile genotypes produced white instead of fertile yellow pollen. The same method was also successfully used with crosses between *H.* annuus and *H.* petiolaris, and *H*. maximiliani (Whelan, 1980). In total there are several different cytoplasm on which sterility is based in *Helianthus*, in the study of Crouzillat (Crouzillat, 1991) for example 12 different cytotypes were identified.

CMS can result in three different kinds of sterility, mitochondria signalled Programmed Cell Death (PCD) of premature anthers, homeotic changes and un-development of the pollen. PET1 (CMS derived from *H.* Petiolaris; Leclercq 1969) sterility is an example of a PCD type of sterility and is the most common source of sterility in commercial sunflowers. The mitochondria release cytochrome c, which acts as a signalling compound for the PCD of the premature anthers. The homeotic changes type of sterility can also be found in sunflowers. Namely, in the sunflower CMS line Teddybear (original source unknown), where the male organs are transformed into petals. The last type of sterility, un-development of the pollen was found by Whelan et al. in a sunflower line derived from *H.* gigantueus, CMS GIG1, which produced white pollen (Chase, 2006; Whelan, 1980).

Several studies has been conducted in identification of restoration genes for each source of CMS. However the outcome of these studies indicate that, depending on the source of CMS, male fertility can be restored by either one dominant gene or at least two up to four independent, complementary, dominant genes (Whelan, 1980). However at this moment most of the commercial sunflower varieties grown are based on a single dominant gene controlling the CMS (PET1).

The breeding program with sunflower just started at Esmeralda Breeding. At this moment they have built up a germplasm and are characterizing this. For this it is also important to determine the presence of restorer genes for several types of CMS. With this project the presence of restorer genes for a single source of CMS will be determined and if possible also the type of cytoplasm in contrast to this source of CMS.

1.2 Material and methods

The CMS investigated originated from the sunflower line named Sunrich, however which type of CMS this lines contains is unknown. In total there were three different Sunrich lines available, because these lines originate from the same breeder it was assumed that these lines also contain the same type of CMS. 35 Selected lines were crossed with the CMS Sunrich Gold and the most important lines (13 of the BG lines/ germplasm lines) were crossed with all three types of Sunrich (Gold, Lemon and Orange) to confirm or reject the assumption. From some of the germplasm lines only F_1, F_1S_1 or S_2 progeny were available. With these lines single plants were selected as a father for the crosses with Sunrich Gold. After the crosses all seeds to a maximum of about 50 seeds per cross were planted for evaluation of the progeny.

For each cross the presence of pollen was scored visual, because the CMS evaluated showed the PCD of premature anthers type of sterility, meaning that if the plants show pollen the CMS is not present.

It was assumed that the CMS investigated was controlled by one dominant restorer gene. The selected lines were crossed with a sterile Sunrich line and so the assumed ratio´s sterile : fertile should be either 100% sterile, 100% fertile, or 50% sterile/ 50% fertile. The found segregation was tested against the assumed segregation with a Chi-square test, if a *P* value of lower than 0.05 was found the ratio 3 : 1 was also tested. This ratio was tested because this segregation could be expected when the trait is controlled by two dominant, independent, complement genes instead of one dominant gene.

1.3 Results

In total 35 lines were tested for the presence of restorer genes and if possible to determine the type of cytoplasm in contrast to the cytoplasmic male sterility from the Sunrich type, see appendix I for an overview of the lines and the raw data. An overview of the results for the initial germplasm can be found in Table 1.1. In total 10 genotypes were heterozygous, 7 were homozygous recessive, 1 was homozygous dominant and for 4 genotypes of the germplasm it was unable to determine if the genotype was homozygous or heterozygous for the restorer gene of the Sunrich CMS (because only the progeny of the germplasm was available during the experiment; either a F_1, F_1S_1 or S_2 population). 7 genotypes contained the fertile cytoplasm and for 16 genotypes it was unable to determine the cytoplasm. For 1 line, Moon Walker, it was unable to determine the type of alleles and the cytoplasm.

Table 1.1 Overview of the CMS results

Germplasm	Homozygous Dominant	Heterozygous	Homozygous recessive	Cytoplasm
BG1		**X**		**NA[1]**
BG2		**X**		**NA**
Domino			**X**	**FERTILE**
Earth Walker			**X**	**FERTILE**
Florenza	**X**			**NA**
Garden Statement		**X**		**NA**
Giant Single			**X**	**FERTILE**
M11		**X**		**STERILE[2]**
M1		**X**		**STERILE[2]**
M3		**X[3]**	**X[3]**	**STERILE[2]**
M5		**X**		**STERILE[2]**
M7	**X[3]**	**X[3]**		**STERILE[2]**
Pacino Gold	**X[3]**	**X[3]**		**NA**
Red Sun			**X**	**FERTILE**
Ring of Fire			**X**	**FERTILE**
Russian Giant			**X**	**FERTILE**
SF39		**X**		**STERILE[2]**
SF5		**X**		**STERILE[2]**
SF6		**X**		**STERILE[2]**
Velvet Queen			**X**	**FERTILE**
Bicentenary		**X**		**NA**
Moon Walker	**NA[4]**	**NA[4]**	**NA[4]**	**FERTILE[2]**
Italian White	**X[5]**	**X[5]**		**NA**

[1]NA = Not Available, an evaluation is necessary to determine the cytoplasm.

[2]Assummed sterile or fertile, see discussion.

[3]Unable to determine the difference between a possible hetero- or homo- zygous father plant due to one or two cycles of auto/open pollination.

[4]Unable to determine the genotype due to irregular segregation.

[5]Unsufficient number of plants to determine the difference between a possible hetero- or homo-zygous father plant.

23 of the crosses gave a segregation for the trait of fertility, from which 14 crosses showed a 1 : 1 segregation (Chi square *P* value > 0.05). 9 crosses showed a deviating segregation, which include 5 crosses with Sunrich Gold were up to 13% of the plants showed fertility (3 : 1), while the same father with Sunrich Lemon and Orange gave 100% sterility. 3 crosses, the crosses with Moon Walker showed a 3 : 1 segregation instead of a 1 : 1 segregation (*P* value of 0.44 for the cross with Sunrich Gold; 0.043 (1 : 1 gave a *P* value of $0.49*10^{-6}$) when crossed with Sunrich Lemon; 0.19 when crossed with Sunrich Orange). 1 cross between Sunrich Gold and SF5-5-3 gave rather more fertile than sterile plants and had a *P* value of 0.043, while a 3 : 1 segregation had a *P* value of 0.10, Table 1.2.

Table 1.2 Scoring, segregation and Chi-square of the individual crosses giving segregation

Crossing	**Sterile**	**Fertile**	**Ratio**	**X_2 (in *P* values)**
G - BG2-2[1]	47	7	3 : 1	0.041
G - Earth Walker	47	8	3 : 1	0.073
G – Garden Statement	1	5	1 : 1	0.102
G - Giant Single	34	5	3 : 1	0.079
G - M11-3-1	30	26	1 : 1	0.593
G - M1-4-1	8	5	1 : 1	0.405
G - M1-4-2	10	16	1 : 1	0.239
G - M5-1	14	13	1 : 1	0.847
G - M5-2	27	20	1 : 1	0.307
G - Moon Walker	48	12	3 : 1 (1 : 1)	0.371 (0.000)
L - Moon Walker[1]	38	5	3 : 1 (1 : 1)	0.043 (0.010)
O - Moon Walker[1]	37	18	3 : 1 (1 : 1)	0.186 (0.000)
G - Red Sun	37	1	3 : 1	0.001
G - SF39-6-3	26	26	1 : 1	1
G - SF39-7-2	14	20	1 : 1	0.303
G - SF5-29-4	24	26	1 : 1	0.777
G - SF5-30-2	20	30	1 : 1	0.157
G - SF5-30-3	18	27	1 : 1	0.180
G - SF5-5-3	17	31	1 : 1 (3 : 1)	0.043 (0.096)
G - SF6-15-3	13	9	1 : 1	0.394
G - Velvet Queen	36	5	3 : 1	0.058
O – Bicentenary	1	1	1 : 1	1
G – Bicentenary	1	2	1 : 1	0.564

[1]G means Sunrich Gold, L means Sunrich Lemon and O means Sunrich Orange

1.4 Discussion

From the 23 crosses which gave segregation, 14 crosses could be explained with the principle of one dominant gene. 5 crosses with Sunrich Gold as a mother resulted in a fertility of up to 13% (3 : 1 ratio), while the same fathers with Sunrich Lemon and Orange gave 0% of fertility. A 3 : 1 segregation is expected when the trait is controlled by two dominant, independent and complementary genes. If within Sunrich Gold one of the genes is heterozygous present (Aabb), while for Sunrich Lemon and Orange these genes are homozygous recessive (aabb) a 3 : 1 segregation with Sunrich Gold and not with Lemon and Orange is expected if the father is heterozygous for the other allele and homozygous recessive for the other (aaBb). However this is not likely because the father lines used are fertile lines which most likely are 100% inbreeding lines, that 5 lines used would be heterozygous for the same gene would be very unlikely.

Instability of CMS is reported in Pepper (Shifriss, 1996) and *Brassica* (Singh and Brown, 1991), where the instability of the trait is caused by in interaction of the temperature and the restorer genes. Under warmer growing conditions, for *Brassica*, and colder growing conditions for Pepper the sterile plants produce pollen and is it for Pepper in example possible to reproduce *S rf/rf* genotypes in off season growing conditions. This type of instability of CMS has never been reported for sunflower and is unlikely to be the case in this project. When the CMS would be unstable due to temperature, also fertile progeny in the crosses with Sunrich Lemon and Orange would have been found.

Another explanation, could be a mutation of the cytoplasm. In individual plants it was observed that some parts of the flower head was fertile while 90% was sterile (Fig 1.1) which can be caused by a mutation of the cytoplasm. If this mutation occurs early enough in the development of the flower, the whole flower head becomes fertile.

Figure 1.1 Partly fertile sunflower flower head, pollen present in the red circle;
a. Detailed view with visible pollen; b. flower head overview with fertile part of the flower encircled.

In maize the same problem was observed by Singh and Laughnan (1972). Due to a mutation on the level of the cytoplasm, kernels were found which carried both the male sterile cytoplasm as well as the mutated male fertile cytoplasm, while the type of CMS used was considered very stable. Besides these mutation, they also found a number of additional ear chimeras, which supported the idea of a mutation causing the fertility. Furthermore they indicate that some lines are more sensitive to changes in the cytoplasm than others, which would also explain the observation that only in the crosses with Sunrich Gold as a mother fertile progeny is found due to a mutation and not in the crosses with Sunrich Lemon and Orange as a mother. This same explanation holds true for the cross between Sunrich Gold and SF5-5-3, because of mutated cytoplasm the number of fertile plants is higher than expected. If For example only one more sterile plant was found the ratio 1 : 1 would be more likely according to the Chi-square test than the obtained 3 : 1 ratio.

3 crosses, between the Sunrich lines and Moon Walker, also showed a 3 : 1 segregation for sterility. Because the segregation is found in the crosses with all three Sunrich lines this is not due to an mutation in the cytoplasm. Again this results gives the appearance that the sterility is controlled by two, complementary, independent restorer genes. The Sunrich lines most likely all contain two times the homozygous recessive alleles and if Moon Walker has two times the alleles present in heterozygous form, the segregation found would be 3 : 1. However as mentioned in the introduction, most commercial varieties contain a type of CMS controlled by one dominant gene. Furthermore the segregation found in the other lines confirm that also the Sunrich type of CMS is controlled by one dominant gene. More likely, the Moon Walker line used in this project could be an impure inbreeding line. If this holds true, different genotypes for the trait could have been present. This implies that as well heterozygous as homozygous genotypes for fertility could have been used for collection of pollen. Because the crosses were made as soon as pollen was present, only two or three plants were used to collect pollen. When from these two plants one contained two recessive alleles and the other both the recessive and the dominant allele, a 3 : 1 segregation would be obtained in the progeny. Furthermore this would also imply that the cytoplasm of Moon Walker is fertile. Moon Walker is a sunflower line which is for consumers and not for commercial use. “Moon Walker is a bright multi-branching plant suitable for growing in borders, where they provide a continuous crop of soft yellow blooms with chocolate centers all summer.” (Thompson & Morgan, Ltd.) Because it is for consumer use only, the possibility that Moon Walker is not a 100% pure inbreeding line, is enhanced.

The M and SF genotypes in the germplasm are collected on the growing farms of Esmeralda. This were single sunflowers in a batch of Sunrich Gold, Lemon and Orange which produced pollen and had a deviating phenotype. It appears with this project that these original sunflowers contained one of the restorer genes. Due to this it is likely that the fertile genotypes in the Sunrich batches originate from contamination of the original crosses between the maintainer and sterile line. The collected seeds of the sterile mother line will all have the sterile cytoplasm and so it can be assumed that the fertile genotypes collected from these batches will also contain the sterile cytoplasm with one dominant allele for restoration of fertility (the allele obtained from the contaminated pollen).

1.5 Future research

To determine if the “unexpected” fertile plants really are mutation of the cytoplasm they should be selved and/ or crossed with the Sunrich lines and/ or recurrent crosses with sterile brothers or sisters should be made. By evaluating the progeny of these crosses/ selvings it is possible to determine the cytoplasm and presence of the dominant restorer alleles. When the progeny show a segregation pattern between fertile and sterile plants, it means the fertility of the “unexpected” fertile plants has to do with the genetics of the plant and the answer should for example be sought in 2 independent complementary dominant genes. If however the progeny is 100% fertile (selving) or 100% sterile (crosses with Sunrich and the recurrent crosses), the assumption of a mutation within the cytoplasm is confirmed.

To determine if the CMS of the other available sterile BG’s (germplasm) is the same as the Sunrich type, these BG’s can be crossed with the fertile BG’s (like this project) and/ or fertile F_1 progeny of open pollinated sterile BG’s could be crossed Sunrich Lemon or Orange. When the fertile BG’s are crossed with the sterile BG’s and the found segregation for fertility is the same as obtained with this project, the type of CMS is the same. If this is not the case, the type of CMS is different from the type of Sunrich. The fertile F_1 progeny of open pollinated sterile BG’s can only contain one dominant allele of the source of CMS present in this BG. If the progeny, when crossed with a Sunrich line, show 100% fertility or 100% sterility it is certain the source of the CMS within this BG is different from the source of CMS of Sunrich (i.e. the crosses within this project with F_1 progeny from Pacino).

It would be good to obtain lines with known types of sterility (lines with CMS type PET1, PET2 or GIG1 for example). If these lines are present they could be included within these kinds of projects and besides the presence of the alleles for restoration of the fertility also the type of sterility could be determined. The sterile lines which show the same segregation as the PET1 line when crossed with the fertile BG most likely also contain the PET1 type of CMS.

1.6 References

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1.7 Appendix

Table 1.3. Raw data of the scoring for sterility

Genotype	Sterile	Fertile	Ratio	*P* value[1]
BG1-2 – G	56		1 : 0	1
BG1-3 – G		46	0 : 1	1
BG1-5 – G		48	0 : 1	1
BG2-2 – G	47	7	3 ; 1	0.041
Domino - G	3		1 : 0	1
Domino - L	54		1 : 0	1
Domino - O	44		1 : 0	1
Earth Walker - G	47	8	3 ; 1	0.073
Earth Walker - L	33		1 : 0	1
Earth Walker - O	35		1 : 0	1
Florenza - G		16	0 : 1	1
Florenza - O		9	0 : 1	1
GardenStatement - G	1	5	1 ; 1	0.102
Gardenstatement - L	1		1 : 0	1
GardenStatement - O		13	0 : 1	1
Giant Single - G	34	5	3 ; 1	0.079
Giant Single - L	39		1 : 0	1
Giant Single - O	34		1 : 0	1
M11-3-1 - G	30	26	1 : 1	0.593
M1-4-1 – G	8	5	1 : 1	0.405
M1-4-2 – G	10	16	1 : 1	0.239
M3-A – G		31	0 : 1	1
M5-1 – G	14	13	1 : 1	0.847
M5-2 – G	27	20	1 : 1	0.307
M7-3-1 – G		2	0 : 1	1
M7-3-2 – G		1	0 : 1	1
Moon Walker - G - D	33	6	3 ; 1	0.166
Moon Walker - G - L	15	6	3 ; 1	0.705
Moon Walker - L	38	5	3 ; 1	0.043
Moon Walker - O	37	18	3 ; 1	0.010
P - Domino - G	29		1 : 0	1
P - Florenza - G		11	0 : 1	1
P - Florenza - L		12	0 : 1	1
P - Red Sun – L	41		1 : 0	1
P - Ring of Fire – G	18		1 : 0	1
P - Ring of Fire – O	9		1 : 0	1
Pacino F_1 – G		51	0 : 1	1
Red Sun – G	37	1	0 : 1	0.001
Red Sun – L	20		1 : 0	1
Red Sun – O	61		1 : 0	1
Ring of Fire – L	35		1 : 0	1
Ring of Fire – O	20		1 : 0	1
Russian Giant – L	61		1 : 0	1
Russian Giant – O	26		1 : 0	1
SF39-6-3 – G	26	26	1 : 1	1
SF39-7-1 – G		6	0 : 1	1
SF39-7-2 – G	14	20	1 : 1	0.303
SF5-29-4 – G	24	26	1 : 1	0.777
SF5-30-2 – G	20	30	1 : 1	0.157
SF5-30-3 – G	18	27	1 : 1	0.180
SF5-5-3 – G	17	31	1 : 3	0.100

SF5-6-1 – G		58	0 : 1	1
SF6-15-3 – G	13	9	1 : 1	0.394
Velvet Queen – G	36	5	3 ; 1	0.058
Velvet Queen – L	49		1 : 0	1
Velvet Queen – O	57		1 : 0	1
Bicentenary – O	1	1	1 1	1
Bicentenary – G	1	2	1 : 1	0.564
Italian White – G		1	1 : 0	1
BG2-1 – G		4	0 : 1	1

[1]*P* value obtained when the expected ratio was tested against the obtained ratio with a Chi Square test.

Flow cytometry:

Developing protocols and determining internal standards

With the invention of the flow cytometer and the chopping method of Galbraith (Glabraith et al., 1983) to simply extract plant nuclei, it became quite easy and affordable to determine the DNA content of plant cells. Flow cytometry (FCM) is nowadays frequently used to determine the (relative) DNA content, the ploidy level of plants and for cell cycle analysis. A good definition and short explanation of flow cytometry (FCM) is given by Doležel et al.;

"Measurement of the properties of isolated cells flowing in single file within a liquid sheath as they are intercepted by a high-intensity light source focused in a very small region. Cells are interrogated in a very short time (a few microseconds) during which multiple signals are collected, mainly light scatter and fluorescence emissions in the visible spectrum. Thanks to the progress in electronics and informatics, flow cytometers can readily analyze single particles/ cells at rates of up to 100,000 per second. It is thus possible to discriminate particles/ cells into clusters based on statistical analyses of the set of parameters collected for each particle. Using these statistical analyses, it is possible to electronically separate these populations and identify them using multivariate analytical techniques." (Doležel et al., 2007)

To measure the DNA inside plant cells, leaf samples are chopped together with a extraction buffer. The solution is sieved (30 µM) to filter out large particles. Thereafter the DNA inside the sample is stained with for example DAPI (A-T, base-specific) or PI (propidium iodide, non base-specific) after which the sample can be used for FCM. Fluids inside the flow cytometer brings the sample inside the instrument, but also aligns and separate single particles. For this, in the flow chamber of the flow cytometer, fluid is added to the sample with the particles, creating a stream of single particles. After obtaining single particles, a laser beam with a specific wavelength is lighted on the particles. The light scatter of the laser beam from this particles is thereafter absorbed by filters and directed to photodetectors, which convert the photons into electrons which can be used by computers to created an output. Depending on the type of cytometer, the cytometer, after measuring the sample, can also separate the different particles from each other, based on their optical properties (Doležel et al, 2007).

Chapter 2 and 3 of this report are dedicated to flow cytometry at EBB. These chapters will handle the development of protocols for 7 different crops and the availability and use of internal references to determine the DNA content for 6 crops.

Development of protocols for flow cytometry for 7 crops

2.1 Introduction

Nowadays many plant laboratories have a FCM at their disposal. However before a FCM can be used correctly it is important to have protocols. Without protocols and specialized people who know how the machine works the results are useless and very inconsistent (doležel et al., 2007). Every plant is grown (when grown in vivo) under different growing conditions, and so will contain different proportions of proteins. Due to this every single plant react different to stainings, which is especially true when plants of different crops are used.

During flow cytometry several aspects are important, which could influence the FSC peak position. For example the time of staining because every crop has a different DNA content, from very small (*Arabidopsis* Thaliana 0,3 pg) to very large (*Liliaceae* 127 pg) and so it could take longer before all the DNA of *Liliaceae* is stained compared to *Arabidopsis*. Furthermore, most staining solutions lose their intensity over time due to staining inhibitors and exposure to light, which influences each sample depending on the amount of staining inhibitor. Besides the time of staining, a sample is also stored for a few hours to a day before it is measured and so it is important to know how to store these samples. At last also the amount of DAPI or nuclei can have an influence because if not enough DAPI is present to stain all the DNA, the measurement can be influenced.

Within EBB the following crops are used; Alstroemeria, Anemone, Aster, Gypsophila, Hypericum, Limonium and Zantedeschia. For these crops protocols had to be made to determine the optimal flowing conditions.

2.2 Material and Methods

To determine the optimal conditions for flow cytometry the following aspects were examined:

- The effect of the time of staining
- The effect of the ratio buffer : DAPI
- The effect of storage
- The effect of the amount of nuclei

These aspects were examined by making one master solution with a high amount of nuclei. Experimental samples were obtained by making dilutions of the master solution. To examine the effect of the time of staining on the measurement, the sample was stained with DAPI and the DNA content of this sample was measured at time 0, 3, 15, 30, 45, 60, and 120 or 180 minutes.

For the ratio buffer : DAPI effect, samples with the same amount of nuclei were used, but different amount of DAPI was added to obtain ratio's 1 : 1, 1 : 2, 1 : 3 and 1 : 4.

The storage effect was determined by measuring tubes stored at 5 degrees Celsius in the dark with and without DAPI at sequential days.

In final, the effect of the amount of nuclei was determined by using 4 tubes of the master solution. By removing liquid from the master solution and diluting it again with the buffer, tubes were obtained with the same amount of buffer, but either a concentration of 100% nuclei, 50% nuclei, 25% nuclei or 12,5% nuclei.

The obtained results, the graphs with the FSC peaks, were put after each other in Windows Movie Maker (version 2007) to created a movie in which the effect of each treatment was shown. By evaluating these movies an overview of the results could be made.

2.3 Results

An overview of the final results, the optimum conditions for the different crops, can be found in Table 2.1. The results per variety can be found in the movies.

Table 2.1. The optimal conditions for flow cytometry of 7 crops.

Crop	Time of staining (minutes)	Ratio buffer : DAPI	Storage		Importance of amount of nuclei
			With DAPI[1]	Without DAPI	
Alstroemeria	3 to 60	1 : 2	0 days	4 days	High concentration decreases the Fsc value
Anemone	15 to 60	1 :2	0 days	Min 4 days	High concentration decreases the Fsc value
Aster	0	1 : 2	0 days	Min 7 days	High concentration decreases the Fsc value
Gypsophila	0 to 15	1 : 2	0 days	Up to 5 days	High concentration decreases the Fsc value
Hypericum	0 to 120	1 : 2	0 days	1 day	High concentration decreases the Fsc value
Limonium	3 to days	1 : 2	Min 5 days	Min 5 days	High concentration increases background noise
Zantedeschia	0 to 45	1 : 2	0 days	1 day	High concentration increases background noise

[1]Storage with DAPI decrease the number of intact nuclei.

Besides determining the optimal conditions for the different crops, the difference between in vivo and in vitro material was also determined. It appeared that in vivo material gave a slight to significant lower FSC peak position compared to in vitro material, but in vitro material did gave the same responses on the aspects examined.

2.4 Discussion

The quality of a sample is dependent on the amount of fluorescence inhibitors and coatings of debris. “Coatings and debris are particles of endogenous substances sticking to the nuclei, resulting in a deterioration of the quality of the FCM histogram peaks without necessarily decreasing the overall nuclear florescence” (Doležel et al., 2007). Fluorescence inhibitors, like secondary metabolites, flavonoids, anthocyan and cytosolic compounds can inhibit fluorescence, but the minor particles can also aggregate with the nuclei, leading to an apparent increase in nuclear fluorescence (cf Doležel et al., 2007). Although the auto-fluorescing metabolites is hypothetical and is not proven yet. Cytosolic compounds which are released during nuclei isolation can have a severe effect on the staining of the DNA. In sunflower a variation of 48% was found in different leaves of individual plants, due to the effect of these cytosolic compounds (cf Doležel et al., 2007).

Secondary metabolites are often present in plant cells and can interfere with the staining. The amount of secondary metabolites and other inhibitors present in a plant cell depend per crop or even per leaf of a individual plant and so it is important to always use the same amount of leaf material, as indicated with the results for the flow cytometry protocols. The FSC peak position was slightly to significantly influenced by the amount of leaf material chopped. With a higher amount of leaf material, the amount of inhibitors and debris is also increased, giving the higher background noise and influence on the FSC peak position. In further experiments (results not shown) it appeared that a leaf amount of 0.20 cm^2 was sufficient enough to generate results with a low amount of background material.

When in vitro material was used the FSC peak position was slightly higher in all crops compared to the peak position with in vivo material. This is most likely also due to the effect of inhibitors. The environment in which the plant is grown has a large influence on the presence of inhibitors and in vitro material is 100% free of diseases and are grown under controlled conditions, giving a lower variation and amount in staining inhibitors.

The time of staining, depending on the crop, has a rather large influence on the position of the FSC peak. The samples were kept in the light and at room temperature during the measurements, meaning that the DAPI gradually loses its fluorescence and causing the FSC peak position to decrease. However the amount of decrease differentiated in time and crop, which might be caused again by staining inhibitors.

Development of internal standards for flow cytometry

3.1 Introduction

The Flow Cytometer (PARTEC; CyFlow®) which is used at Esmeralda Breeding & Biotechnology is based on DAPI staining. DAPI only stains the A-T base pairs in the genome. Per crop the amount of A-T base pairs differentiate, for example *Allium* cepa has 34.5% of A-T base pairs and *Zea* mays has 48.9% A-T base pairs (Doležel et al, 2007). Due to this the most common used standards, like *Pisum, Allium, Brassica* and red blood cells of chicken (Doležel et al, 2007, Doležel et al., 2007a), cannot be used to determine the actual DNA content, but could be used to determine the relative DNA content of the different crops growing at Esmeralda Breeding & Biotechnology.

Within literature the actual DNA content of many genotypes of different crops has been determined. This information has been gathered by Benett and Leitch (Bennett and Leitch, 2005) and made available through an open access database (Link: http://data.kew.org/cvalues/).

6 crops were selected for which internal standards had to be found. These were selected based on their importance for Esmeralda Breeding and on the breeding techniques used within these crops. The crops selected were *Alstroemeria*, *Limonium*, *Hypericum*, *Aster*, *Eryngium* and *Gypsophila*. 6 genotypes were available with known DNA content, Table 3.1. All DNA contents mentioned within this report are 2c values.

Table 3.1. Varieties grown at Esmeralda Breeding & Biotechnology with known DNA content.

Genus	Variety	pg content
Alstroemeria	Pelegrina	44.20
Alstroemeria	Aurea (Orange)[1]	53.50
Alstroemeria	Aurea (Yellow)[1]	80.90
Limonium	Perezii	8.70
Hypericum	Perforatum	1.30
Gypsophila	Repens	1.40
Brassica	Napus	2.30

[1]In literature could not be found which Aurea was used when 53.5 pg or 80.9 pg was determined, however the difference was made clear by flow cytometry.

Besides the open accessible database, EBB also has a database with relative DNA contents, obtained by Iribov (Iribov; breeding support laboratory). The relative DNA content determined by this company was based on one internal reference, namely *Ilex* Crenata "Fastigiata". In total Iribov had determined the DNA content of 14 varieties of *Limonium*, 15 varieties of *Hypericum*, 11 of *Aster*, 17 of *Eryngium* and 23 of *Gypsophila*.

During this project it was assumed that the A-T base pair percentage within the same crop does not differentiate significantly because of the close genetic background. Due to this assumption it is possible to determine the actual DNA content of a genotype, when a standard is used from the same crop. In short: find standards to determine the actual DNA content of *Alstroemeria, Limonium, Hypericum, Gypsophila* and the relative DNA content of *Aster* and *Eryngium*.

3.2 Material and Methods

For *Alstroemeria* four varieties with known DNA content were available, which could be used as standards. To confirm that the variety used in literature and the variety grown at EBB was identical, these standards were used to confirm each other. This confirmation was done by determining the DNA content of one standard by another standard. If however only one variety with known DNA content was available (*Limonium, Hypericum* and *Gypsophila*), the Iribov results were used to confirm this standard. For this, the DNA content of the same varieties, as used by Iribov, were determined by the standard. To make the results between the Iribov samples and the obtained samples comparable, the Iribov data was recalculated to correct for the A-T difference between their internal standard and the crop tested. This was done with the varieties with a known DNA content from literature. The data from Iribov was multiplied by a factor X, in which this factor was chosen in such a way that the variety with the known DNA content obtains also this DNA content. For example; Perezii has a DNA content of 8.70 pg, however the pg content determined by Iribov was 3.22. Factor X in this case becomes 8.70 / 3.22 = 2.70 and all the data of Iribov got multiplied by 2.70, making from the 3.22 pg of Perezii 8.70 pg.

For *Aster* and *Eryngium* no varieties with known DNA content were available at EBB. However because of the importance of these crops to EBB, standards from other crops were used to determine the relative DNA content. The correctness of these standards was again checked by making use of the Iribov data. In this case the data were however not calibrated on a known sample from literature, but by selecting one variety and multiplying the DNA contents of the other standards with a factor X, in such a way that the DNA content of this variety is equal for all the different measurements.

Besides the standards of the same crop, *Brassica* Napus was also tested as a standard. *Brassica* was used due to its stability as a standard (Kingman, personal communication). To cancel out the A-T difference between *B.* Napus and the tested crop, the DNA content of *B.* Napus was determined by a variety with a known DNA content from literature of the crop tested and so always deviated from its in literature determined DNA content (2.30 pg).

The stability of the standard was determined by the standard deviation of the FSC peak position, when the standard was run together with a unknown sample. The higher the standard deviation (in percentage to the mean) the lower the stability of the standard.

3.3 Results

3.3.1 Alstroemeria

For Pelegrina there were two species available, namely Pelegrina White and Pelegrina Pink. Unfortunately in literature it was not described which specie was used, and neither could flow cytometry show which species was used due to the small difference in DNA content. Therefore it was decided to use them both.

When a standard was used to calculate the other standard a deviation in DNA content compared to literature of maximum 3.37% was found, see Table 3.2. When these standards were used to calculate the DNA content of four random varieties, the DNA content differentiated to a maximum of 5 pg or 5.6% (Table 3.3). Pelegrina White and Pelegrina Pink showed the lowest standard differentiation in the position of their FSC peak position and Aurea Yellow showed the highest standard differentiation, Table 3.4.

Table 3.2. Calculation of the DNA content of the standards by the standards.

Standard used to calculate the DNA content	Calculated DNA content in pg (% difference with literature)							
	Pelegrina Pink		**Pelegrina White**		**Aurea Orange**		**Aurea Yellow**	
Pelegrina Pink	NA[1]	NA	NA	NA	54,12	(1,16)	78,89	(2,49)
Pelegrina White	NA	NA	NA	NA	55,14	(3,06)	78,27	(3,25)
Aurea Orange	43,73	(1,07)	42,90	(2,95)	NA	NA	78,78	(2,63)
Aurea Yellow	45,33	(2,55)	45,69	(3,37)	54,95	(2,72)	NA	NA

[1]NA = not available

Table 3.3. Calculation of the DNA content of four varieties by the standards.

Standard	**Prima Dona**	**Honey Bell**	**Amor**	**Virginia**
Pelegrina Pink	75,44	75,60	54,36	92,84
Pelegrina White	76,46	77,06	56,75	91,36
Aurea Orange	75,25	75,08	NA[1]	94,22
Aurea Yellow	NA	NA	57,51	96,62

[1]NA = not available

Table 3.4. Standard deviation of the FSC peak positions of the standard when run together with four varieties.

Standard	**Σ[1]**
Pelegrina Pink	5,46
Pelegrina White	7,64
Aurea Orange	11,79
Aurea Yellow	13,51

[1]in percentage to the mean

3.3.2 Limonium

Within *Limonium*, Perezii (8.70 pg) and *Brassica* Napus were used to calculate the DNA content of 19 *Limonium* varieties. From 13 of the 19 varieties the DNA content was also determined by Iribov. The samples of Iribov were calibrated on Perezii and the calculated DNA content of *Brassica* Napus determined by Perezii was used as DNA content of *B.* Napus (2.95 pg).

7 varieties had a calculated DNA content which showed a variation of maximum 2% between the different standards, 8 varieties showed a variation of lower than 5% and 3 varieties showed a variation of up to 15% (0.49 pg). The results of Iribov for China White deviated 80% compared to the DNA content obtained by *B.* Napus and Perezii (Table 3.5).

Table 3.5. Calculation of the DNA content of several *Limonium* varieties.

	DNA content (in pg) according to:		
Variety	**Iribov**	***L.* Perezii**	***B.* Napus**
B. Napus	NA[1]	2,95	NA
Belliofolium	4,38[a2]	4,37[a]	4,41[a]
Beltaard	5,67[b]	5,94[a]	5,87[a]
Caspia	4,40[a]	4,45[a]	4,45[a]
China White	3,01[c]	5,31[b]	5,48[b]
Chorus Magenta	19,02[b]	NA	18,59[b]
Donau	5,77[a]	5,75[a]	5,75[a]
Gemelinii	5,29[a]	5,27[a]	5,19[a]
Latifolium	2,78[c]	3,0[bc]	2,95[bc]
Pink Emely	4,09[b]	4,58[c]	3,90[b]
Pink Lady	21,88[a]	21,44[ab]	21,93[ab]
Safora	5,74[a]	6,41[c]	5,71[a]
S1	NA	6,51[a]	6,46[a]
S2	NA	6,09[b]	5,88[b]
S3	NA	6,19[b]	6,04[b]
S4	NA	6,04[a]	5,94[a]
S5	NA	6,06[a]	6,01[a]
CDB	6,00[ab]	6,11[b]	5,95[a]
Splash Blue	2,83[b]	2,96[ab]	2,95[ab]
White Diamond	NA	5,14[a]	5,16[a]
Perezii	8,70[a3]	8,70[a4]	8,70[a5]

[1]NA; Not Available

[2]Indication of the deviation; a smaller than 2%; b smaller than 5%; c bigger than 5%

[3]DNA content equalled with literature to circumvent the A-T percentage effect of the internal standard (Lactuca sativa 'Capitata')

[4]DNA content determined with literature

[5]DNA content calculated with literature value of Perezii, to circumvent the A-T percentage effect

The stability of *B.* Napus as a standard was higher (standard deviation in percentage of the mean of 8.23) than Perezii (a standard deviation in percentage of the mean of 16.99).

3.3.3 Hypericum

Within *Hypericum*, Perforatum (1.30 pg) and *Brassica* Napus were used to calculate the DNA content of 14 *Hypericum* varieties. The DNA content of these 14 varieties were also determined by Iribov. The samples of Iribov were calibrated on Perforatum and the calculated DNA content of *Brassica* Napus determined by Perforatum was used as DNA content of *B.* Napus (2.12 pg).

3 varieties had a calculated DNA content which showed a variation of maximum 2% between the different standards, 5 varieties showed a variation of lower than 5% and 6 varieties showed a variation of up to 11.7% (0.22 pg) (Table 3.6).

Tab le 3.6. Calculation of the DNA content of several *Hypericum* varieties.

	DNA content (in pg) according to:		
Variety	**Iribov**	***H.* Perforatum**	***B.* Napus**
Ascyron	0,93[ac2]	0,83[c]	0,94[ac]
Bariloche	0,75[ab]	0,73[b]	0,75[ab]
Buckleii	1,37[a]	NA[1]	1,38[a]
Elite Amber	0,78[b]	0,82[ab]	0,81[ab]
ESM H051	1,23[ac]	1,30[c]	1,21[ac]
Flor y Campo	1,89[ab]	1,85[b]	1,91[ab]
Frances	2,28[ac]	2,26[ac]	2,12[c]
Green Condor	0,82[a]	0,82[a]	0,81[a]
Hidcote	2,29[ac]	2,26[ac]	2,12[c]
Monserianum	2,17[b]	2,08[ab]	2,12[ab]
Orientale	0,68[bc]	0,64[ac]	0,65[ab]
Patulum	1,90[ac]	1,91[ac]	2,12[c]
Pink Attraction	0,78[ab]	0,80[ab]	0,79[a]
Red Baron	0,82[a]	0,81[a]	0,80[a]
Perforatum	1,3[3]	1,3[4]	1,3[4]
B. Napus	NA	2,12[5]	NA

[1]NA; Not Available

[2]Indication of the deviation; a smaller than 2%; b smaller than 5%; c bigger than 5%

[3]DNA content calibrated with literature to circumvent the A-T percentage effect of the internal standard (*Ilex* Lactuca sativa 'Capitata')

[4]DNA content determined with literature

[5]DNA content calculated with literature value of Perforatum, to circumvent the A-T percentage effect

The stability of *B.* Napus as a standard was higher (standard deviation in percentage of the mean of 7.62) than Perforatum (a standard deviation in percentage of the mean of 19.07).

3.3.4 Aster

Within *Aster*, *Brassica* Napus (2.30 pg) and *Hypericum* Perforatum (1.30 pg) were used to calculate the relative DNA content of 15 varieties from which 12 varieties the relative DNA content was also determined by Iribov. The different measurements were calibrated on Colon 802 (determined by *B.* Napus). Colon 802 was chosen because half of the sample were measured twice and it appeared Colon 802 gave as well with *B.* Napus as with *H.* Perforatum as a standard the most consistent results.

1 variety had a calculated DNA content which showed a variation of maximum 5% between the different standards, 10 varieties showed a variation of lower than 10% and 6 varieties showed a variation of up to 22.8% (0.19 pg) (Table 3.7).

Table 3.7. Calculation of the relative DNA content of several *Aster* varieties.

	DNA content (in pg) according to:		
Variety	**Iribov**	***B.* Napus**	***H.* Perforatum**
Blue Diamond	4,54[ab1]	4,42[bc]	4,98[bc]
Blue Tri	3,50[ac]	3,06[c]	3,43[ac]
Caespia	NA[2]	2,30[b]	2,15[b]
Celeste	NA	4,39[b]	4,66[b]
Colon 802	2,01[3]	2,01	2,01
Cote Azur	2,25[ab]	2,30[a]	2,37[ab]
Ecuador	4,58[ab]	4,31[ab]	4,48[a]
ESMA002	4,20[a]	4,19[a]	4,38[a]
Florida Marsh 1	2,17[ab]	1,99[ab]	2,07[a]
Florida Marsh 2	3,82[a]	3,78[ab]	3,99[ab]
Florida Marsh 3	3,38[ac]	2,75[c]	3,31[ac]
Florida Marsh	2,01[ab]	2,05[ab]	2,20[b]
Geel	3,92[a]	3,84[ab]	4,05[ab]
Pretty Wendy	4,52[ab]	4,48[ab]	4,12[b]
Ridelii	NA	1,79[b]	1,92[b]

[1]Indication of the deviation; a smaller than 5%; b smaller than 10%; c bigger than 10%

[2]NA; Not Available

[3]DNA contents calibrated with Colon 802 to circumvent the differences in A-T percentage of the internals

The stability of *H.* Perforatum as a standard was higher (standard deviation in percentage of the mean of 10,58) than *B.* Napus (a standard deviation in percentage of the mean of 13,39).

3.3.5 Eryngium

Within *Eryngium*, *Brassica* Napus (2.30 pg) was used to calculate the relative DNA content of 15 varieties from which 9 varieties the relative DNA content was also determined by Iribov. The different measurements were calibrated on Paradize Jack Pot, Blue Glitter and Bourgatti (determined by Iribov).

4 varieties had a calculated DNA content which showed a variation of maximum 5% between the different standards and 5 varieties showed a variation of up to 24% (0.24 pg) (Table 3.8).

Table 3.8. Calculation of the relative DNA content of several *Eryngium* varieties.

	DNA content (in pg) according to:	
Variety	**Iribov**	***B.* Napus**
Alpinium Blue	NA[1]	1
Alpinium	1,24[c2]	1[c]
Paradize Jack Pot[3]	1	1[a]
Amethysium	2,15[c]	1,93[c]
Arabian Dawn	NA	1
Bella Donna	1,06[c]	1[a]
Blue Glitter[3]	1[a]	1[a]
Bourgatti[3]	1[a]	1[a]
Ella Bella	NA	1
Horrydium	NA	2,35
Humile	0,69[c]	0,58[c]
Panniculata	NA	2,31
Peruano Verde	NA	2,79
Tetra Petra	1[c]	0,86[c]
Yuccifolium	4,75[c]	4,25[c]

[1]NA; Not Available

[2]Indication of the deviation; a smaller than 5%; c bigger than 10%

[3]DNA contents calibrated with these varieties to circumvent the differences in A-T percentage of the internals

B. Napus appeared stable with a standard deviation in percentage of the mean of 5.92.

3.3.6 Gypsophila

Due to interaction between the standard *G.* Repens (1.40 pg) *B.* Napus (2.30 pg) and the (other) *Gypsophila* varieties no results could be generated. The samples gave no FSC peaks at all or a maximum of one peak (few samples), while with as well *Gypsophila* as *Brassica* at least two peaks were expected (2n and the mitosis 4n peak).

3.4 Discussion

Due to the low variation in the calculated DNA contents between the different standards it can be concluded that *A.* Pelegrina, *A.* Aurea, *H.* Perforatum, *L.* Perezii and *B.* Napus can be used to calculate the actual DNA content of varieties from the same genus and *B.* Napus can be used for *Hypericum* and *Limonium* varieties. It is better to use *A.* Pelegrina instead of *A.* Aurea because Pelegrina appears to more stable. Furthermore the DNA content of Pelegrina is smaller compared to the commercial varieties, while Aurea sometimes has the same DNA content. If the DNA content is equal or almost equal to the standard, the FSC peak positions overlaps, which influences the precision of the peak position and so influences the calculation of the DNA content. *H.* Perforatum and *L.* Perezii also in some cases have overlapping peaks with commercial varieties, but in most cases have a larger DNA content compared to the commercial varieties of the same crop. Beside these two standards also *B.* Napus could be used within these crops to calculate the actual DNA content (if calibrated to Perforatum or Perezii), but also *B.* Napus showed some overlapping FSC peaks with some commercial varieties. However, *B.* Napus showed a higher stability than the standard of the crop itself.

H. Perforatum can be used as a standard for *Aster* to calculate the relative DNA content. It showed a high stability, higher than *B.* Napus, and the samples showed a rather low variation compared to the other standards. Perforatum proofed to be better than Napus, which is meanly because Napus (2.3 pg) had in several cases overlapping FSC peaks, while Perforatum (1.3 pg) always had a lower FSC peak position.

Although *B.* Napus showed a very high stability when used as a standard within *Eryngium* it would be better to use another standard because many samples (all samples with a DNA content of 1 pg) had a overlapping FSC peak with Napus. Also within *Gypsophila* Napus cannot be used as a standard. It appeared that the interaction between the samples of *Gypsophila* and Napus was so high that no FSC peak at all appeared or only one. The same was the case when *H.* Perforatum was used as a standard.

Overall *Brassica* Napus appeared to be very stable when used as a standard to calculate the DNA content of an unknown sample. Furthermore it appeared that the A-T content of *Limonium* is much lower (28% lower than Napus) and the A-T content of *Hypericum* is higher (9% higher than Napus). This can be concluded because the calculated DNA content of *Brassica* Napus was 2.95 pg when Perezii was used as a standard and 2.12 pg when Perforatum was used as a standard, while in literature *B.* Napus has a DNA content of 2.3 pg.

That one genotype is more stable than another is mainly because of the presence of staining inhibitors and the interaction of proteins present in the different plants. Due to the interaction between the two samples when chopped, stained and run together the FSC peak position can become lower or higher or even one of the two lower and the other higher (Doležel et al., 2007).

3.5 Future Research

At this moment no varieties of *Aster* and *Eryngium* are grown at EBB from which the DNA content is known. If in the future the actual DNA content of these crops has to be calculated it is necessary to have one variety with known DNA content (http://data.kew.org/cvalues/). Besides these varieties it might be good to obtain standard crops which are used a lot in literature and at other laboratories as standard crops. This because they have shown to be very stable and if like in this project the DNA content is determined by a known sample of the crop investigated the A-T difference is cancelled out and the actual DNA content can be calculated.

It appeared that the DNA content of *B.* Napus is very close to the DNA content of *Eryngium* and so not very accurate as a standard. In future experiments it might be good to use *Arabidopsis* Thaliana as a standard, because the DNA content is lower (0.3 pg). Also *B.* Napus appeared to be very stable and *A.* Thaliana is from the same family and so might have the same content of secondary metabolites and other staining inhibitors. Besides *A.* Thaliana also *H.* Perforatum might be a good standard within *Eryngium*. This because the DNA content of *H.* Perforatum is almost half that of *B.* Napus and so like with *Aster* will have no overlap with the FSC peaks of *Eryngium* samples, but still has a DNA content close to the unknown samples.

To find a standard for *Gypsophila* it is important to run several stable crops (like the standard crops used in literature; *Zea* Mays (5.43 pg), *Pisum* Sativum (9.09 pg), *Raphanus* sativus (1.11 pg) (Doležel et al., 2007a) together with *Gypsophila*. When a stable crop is found which doesn't interact with *Gypsophila*, it could be used as a standard to determine the actual DNA content. This because *G.* Repens is available to calibrate the standard (to correct for the A-T difference).

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Polyploidization of Sun Flower

4.1 Introduction

Until now sunflower breeding is conducted in a narrow genetic background (cf Friedt, 1992). The breeding is mainly focused within *Helianthus* Annuus, which is a diploid. Besides diploid species, *Helianthus* also contain tetra and hexaploids (Hu et al., 2010). Species with other ploidylevels are mainly used within research and not within breeding for the commercial market. By making use of polyploidization, crosses with these species should become more successful (smaller difference in genomics of the different species).

Besides crosses with species, polyploidization might give an whole new source of male sterility, namely triploids. If the tetraploids obtained by polyploidization of inbred diploids are fertile, it is possible to have a diploid and a tetraploid with exactly the same genetics. By making the crosses between the tetra and the diploid, possible sterile triploids could be obtained. By making use of triploids, it is for competitive breeders even more difficult to use these new varieties in their breeding programs, especially if they don´t know it is an triploid instead of a sterile plant due to CMS.

In literature many methods for polyploidization are described (Laere, 2008). However regeneration within sunflower is difficult and has not yet been successful at EBB, meaning that not all treatments are available. Due to this, partly polyploidized plants cannot be purified and the whole plant has to be polyploidized, meaning it is necessary to use either the seed , young plantlets or pollen.

Polyploidization experiments in literature make use of mitosis inhibitors like Oryzalin, Colchine or trifluralin (Laere, 2008). These mitosis inhibitors are used to polyploidize the seed or young plantlets with means of submergence, germination of seeds on media enriched with these inhibitors or with the droplet method. The droplet method means that for a number of subsequent days one drop (5 µl) is placed on the growing meristem of the seedling. The concentration of the mitosis inhibitors depend per crop (Przybyla, expert in polyploidization, personal communication).

In literature a submerge treatment of 12h with 0.2; 0.4; 0.6 % of Colchicine had polyploidized sunflower. However also treatments of 5h with 0.015% and 0.025% Colchicine supplemented in the dark without moistening the roots had given polyploidized sunflowers, but the treatment appeared difficult to apply and the experimental conditions were not optimal (Friedt, 1992).

With this project both the submerge and droplet treatment will be applied on sunflower in order to obtain polyploids. In both treatments different amounts of Colchicine and Oryzalin will be used to obtain the optimal conditions for polyploidizing sunflowers.

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4.2 Material and Methods

4 day old plantlets were submerge for 17 hours in 0.05; 0.1; 0.2 % of Oryzalin or 0.2; 0.4; 0.6 % of Colchicine. Each treatment consisted out of 13 plantlets, which after submerging were cleaned with demi-water and planted back in the soil. The cotyledons of the plantlets were either at the point of opening or just opened.

For the droplet method, the cotyledons should just be opened in order to apply the droplet on the meristem. On day 5 all the cotyledons were opened and was the treatment started. From this day the plants received either 3 or 8 days a droplet with 0.05; 0.1; 0.2 % of Oryzalin or 0.2; 0.4; 0.6 % of Colchicine. After 3 days, the treatment stopped for 2 days, after which the treatment was again applied for 5 subsequent days.

In week 5 and 8 the DNA content of the treated plants was compared to the controls in order to identify polyploids.

4.3 Results

4.3.1 Submerge

It appeared that the plantlets on which the treatment was applied were up to two/three times as thick as the control (Fig 4.1a to e). Before planting the mortality of the treated plants was 50% and 5 weeks after planting (in which the plantlets had not grown a single cm) all the plantlets had died.

4.3.2 Droplet

The plantlets treated with the droplet treatment also showed an increase in thickness of the stem. However the difference was bigger with plants treated with Colchicine compared to the plantlets treated with Oryzalin, see Fig. 4.1e to g.

a b c d e f g

Fig. 4.1. a. 0.6% Colchine submerge; b. 0.4% Colchine submerge; c. 0.2% Oryzalin submerge; d. 0.1% Oryzalin submerge; e. Control; f. 0.6% Colchine 8 days; g. 0.2% Oryzalin 3 days.

At planting no plantlets had died. 5 weeks after planting several plants had died or never continued growing after the treatment (the growing point had died). The plants which had survived the treatment showed minor to severe growth irregularities (Fig. 4.2).The DNA content of the survivors was determined. In total 4 diploids, 7 tetraploids, 6 mixoploids and 3 anueploids were found in week 8 (Table 4.1), while in week 5 in total 1 haploid, 3 diploids, 8 mixoploids and 13 anueploids were found (Appendix I).

Fig 4.2. Growth irregularities of the plantlets treated with the droplet method.

Table 4.1. Overview of the results for the droplet treatment after 8 weeks of planting.

Treatment	Living plantlets	Dead plantlets	Ploidylevel
0,05% Oryzalin 3 days	7	6	1 di-; 5 tetra-; 1 mixo- ploid[1]
0,05% Oryzalin 8 days	3	10	1 di-; 2 mixo- ploids
0,10% Oryzalin 3 days	5	8	1 di-; 1 tetra-; 2 mixo-; 1 anue- ploid
0,10% Oryzalin 8 days	2	11	1 di-; 1 mixo- ploid
0,20% Oryzalin 3 days	2	11	1 tetra-; 1 anue- ploid
0,20% Oryzalin 8 days	0	13	-
0,20% Colchine 3 days	4	9	3 mixo-; 1 anue- ploid
0,20% Colchine 8 days	0	13	-
0,20% Colchine 3 days	0	13	-
0,20% Colchine 8 days	0	13	-
0,20% Colchine 3 days	0	13	-
0,20% Colchine 8 days	0	13	-
Control 3 days	13	0	13 diploids
Control 8 days	13	0	13 diploids
Control 0 days	13	0	13 diploids

[1]detailed results can be found in appendix II.

4.4 Discussion

The droplet method is superior to the submerge treatment, because all the plantlets died with the submerge treatment. However this might be caused by the hours the treatment was applied and the percentage of Oryzalin and Colchicine. By lower the percentage of mitosis inhibiter and/ or lowering the hours the plantlets are submerge this treatment might give living plantlets after 5 weeks. The droplet method is like a submerge treatment easy to apply and compared to the submerge treatment the amount of mitosis inhibitor used is much lower. However the droplet treatment is more time consuming than the submerge treatment.

With the droplet treatment as well the amount of mitosis inhibiter as the number of days the treatment lasted had an influence on the amount of surviving plants. It appeared that the “lowest” treatment of 3 days with 0.05% of Oryzalin gave a mortality of 50%. All the treatments gave a high percentage of polyploidy plants, but the anueploids appeared very instable and changed to either a tetra, diploid or mixoploid ploidylevel. Furthermore all the plants treated were malformed (also the plants which were diploids), indicating that the 0.05% of Oryzalin might even be a too high doses. By decreasing the doses of mitosis inhibitor, the side effects of the mitosis inhibiter (severe malformation) is also lowered and so the percentage of survival increased. With a decrease in mitosis inhibiter also the percentage of polyploids will be lowered, but at this moment even the 0.05% of Oryzalin treatment gave a very high percentage of polyploids (85%).

Both the mitosis inhibiters gave polyploids, only the number of survivors for the Oryzalin treatment was higher. However this is caused by the lower percentage used for the Oryzalin treatments compared to the Colchicine treatments. By lowering the doses of Colchicine the number of survivors will increase, but, as mentioned before with Oryzalin, also the number of polyploids will decrease.

At last it appears that a treatment duration of 3 days is sufficient enough to obtain polyploids. When the treatment is applied for 3 days, the number of survivors is higher. Furthermore the treatment of 3 days is less time consuming which becomes important when the method is used within breeding programs.

4.5 Future research

With this project it was found that it is possible to obtain tetraploid out of diploid sunflowers with a simple method for polyploidization. However the anueploids appeared very instable and changed rapidly into di- and tetra-ploids. Unfortunately there was not enough time to test the ploidylevel again in a later growing stage to determine how stable the tetraploids are. Furthermore there was also not enough time to evaluate the tetraploids during flowering. This is important because the diploids used were male sterile and according to Przybyla (personal communication), an expert in polyploidization, it could be possible to restore fertility by polyploidization. If the tetraploid plants are male fertile the pollen can be compared with pollen from diploid plants, which should be half the size compared to pollen of a tetraploid plant.

Within Maize (Birchler, 1993) and Alstroemeria (Heig and Westoby, 1991) it is already proven that triploids are nonviable, but when viable they are highly sterile due to meiotic imbalances. However within sunflower this has never been researched, but might be a good method to obtain male sterile genotypes. With this project it appeared possible to obtain tetraploid plants which are needed to make triploids. The method was simple and successful which increases the usability of triploid sunflower breeding.

It is important that the tetraploid and in the future triploid plants are carefully characterised. This because triploids and tetraploids usually show a different morphology compared to diploids. The change in morphology can be as well positive as negative. It can be expected that the tetraploids will be shorter and have a compact structure.

4.6 Reference

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4.7 Appendices

Appendix I. The flow cytometry results after 5 weeks

Esmeralda Breeding & Biotechnology
Citometría de Flujo EB&B

Etapa/s: _____NA_____ Laboratorio:_____EB&B_____
Fecha de Evaluación: ___1048.4___ Tipo de material:____In Vivo_____
Responsable: ____JvdH_____ Tipo de análisis:____Análisis de ploidía_____
Referencia Interna: Gain & LowerLevel: 432/50

Item	Código o Variedad	Código Provisional	Fcs (C-value)	Cont. relativo de ADN	Observaciones
H. annuus	Control	Sunrich Gold	199	2x	
H. annuus	control 3 days	Sunrich Gold	181	2x	
H. annuus	control 8 days	Sunrich Gold	186	2x	
H. annuus	0.05 oryzalin 3 days plant 1	Sunrich Gold	253	2x	
H. annuus	0.05 oryzalin 3 days plant 2	Sunrich Gold	278	2x	
H. annuus	0.05 oryzalin 3 days plant 3	Sunrich Gold	161 317	mixoploid	32% 2x; 68% 3x
H. annuus	0.05 oryzalin 3 days plant 4	Sunrich Gold	292	Anueploid	
H. annuus	0.05 oryzalin 3 days plant 5	Sunrich Gold	288	Anueploid	
H. annuus	0.05 oryzalin 3 days plant 6	Sunrich Gold	286	Anueploid	
H. annuus	0.05 oryzalin 3 days plant 7	Sunrich Gold	307	Anueploid	
H. annuus	0.05 oryzalin 8 days plant 1	Sunrich Gold	189 380	mixoploid	44% 2x; 56% 4x
H. annuus	0.05 oryzalin 8 days plant 2	Sunrich Gold	174 343	mixoploid	20% 2x; 66% 4x
H. annuus	0.05 oryzalin 8 days plant 3	Sunrich Gold	135	Haploid	
H. annuus	0.1 oryzalin 3 days plant 1	Sunrich Gold	264	Anueploid	
H. annuus	0.1 oryzalin 3 days plant 2	Sunrich Gold			no hay ojas
H. annuus	0.1 oryzalin 3 days plant 3	Sunrich Gold	146 286	mixoploid	84% 2x; 16% 4x
H. annuus	0.1 oryzalin 3 days plant 4	Sunrich Gold	310	Anueploid	
H. annuus	0.1 oryzalin 3 days plant 5	Sunrich Gold	301	Anueploid	
H. annuus	0.1 oryzalin 3 days plant 6	Sunrich Gold	267	Anueploid	
H. annuus	0.1 oryzalin 3 days plant 7	Sunrich Gold	336	Anueploid	
H. annuus	0.1 oryzalin 8 days plant 1	Sunrich Gold	347	Anueploid	
H. annuus	0.1 oryzalin 8 days plant 2	Sunrich Gold	328	Anueploid	
H. annuus	0.2 oryzalin 3 days plant 1	Sunrich Gold	314	Anueploid	
H. annuus	0.2 oryzalin 3 days plant 2	Sunrich Gold	235	2x	
H. annuus	0.2 Colchine 3 days plant 1	Sunrich Gold	173 339	mixoploid	41% 2x; 59% 4x
H. annuus	0.2 Colchine 3 days plant 2	Sunrich Gold	268 543	mixoploid	36% 2x; 39 4x
H. annuus	0.2 Colchine 3 days plant 3	Sunrich Gold	297	Anueploid	
H. annuus	0.2 Colchine 3 days plant 4	Sunrich Gold	320 607	mixoploid	54% 2x; 27% 4x
H. annuus	0.2 Colchine 3 days plant 5	Sunrich Gold	176 340	mixoploid	81% 2x; 19%4x
H. annuus	Control	Sunrich Gold	180	2x	
H. annuus	control 3 days	Sunrich Gold	166	2x	
H. annuus	control 8 days	Sunrich Gold	174	2x	

Appendix II. The flow cytometry results after 8 weeks

Esmeralda Breeding & Biotechnology
Citometría de Flujo EB&B

Etapa/s: _____NA_____
Fecha de Evaluación: ___1050.4_____
Responsable: ____JvdH_____
Referencia Interna:

Laboratorio:_____EB&B_____
Tipo de material:____In Vivo_____
Tipo de análisis:____Análisis de ploidía_____
Gain & LowerLevel: 432/50

Item	Código o Variedad	Código Provisional	Fcs (C-value)	Cont. relativo de ADN	Observaciones
H. annuus	**Control**	Sunrich Gold	**170**	**2x**	
H. annuus	**control 3 days**	Sunrich Gold	**190**	**2x**	
H. annuus	**control 8 days**	Sunrich Gold	**206**	**2x**	
H. annuus	**0.05 oryzalin 3 days plant 1**	Sunrich Gold	**203 377**	**Mixoploid**	**2x 24%; 4x66%**
H. annuus	**0.05 oryzalin 3 days plant 2**	Sunrich Gold	**218**	**2x**	
H. annuus	**0.05 oryzalin 3 days plant 3**	Sunrich Gold	**401**	**4x**	
H. annuus	**0.05 oryzalin 3 days plant 4**	Sunrich Gold	**384**	**4x**	
H. annuus	**0.05 oryzalin 3 days plant 5**	Sunrich Gold	**388**	**4x**	
H. annuus	**0.05 oryzalin 3 days plant 6.1**	Sunrich Gold	**209 402**	**Mixoploid**	**2x 86%; 4x14%**
H. annuus	**0.05 oryzalin 3 days plant 6.2**	Sunrich Gold	**371**	**4x**	
H. annuus	**0.05 oryzalin 3 days plant 6.3**	Sunrich Gold	**202 381**	**Mixoploid**	**2x 67%; 4x14%**
H. annuus	**0.05 oryzalin 3 days plant 7**	Sunrich Gold	**371 722**	**Mixoploid**	**4x78%; 8x10%**
H. annuus	**0.05 oryzalin 8 days plant 1**	Sunrich Gold	**202 303**	**Mixoploid**	**2x75%; 3x25%**
H. annuus	**0.05 oryzalin 8 days plant 2**	Sunrich Gold	**177 340**	**Mixoploid**	**2x 28%; 4x72%**
H. annuus	**0.05 oryzalin 8 days plant 3**	Sunrich Gold	**215**	**2x**	
H. annuus	**0.1 oryzalin 3 days plant 1**	Sunrich Gold	**217 427**	**Mixoploid**	**2x76%; 4x14%**
H. annuus	**0.1 oryzalin 3 days plant 2**	Sunrich Gold	**402**	**4x**	
H. annuus	**0.1 oryzalin 3 days plant 3**	Sunrich Gold	**166 324**	**Mixoploid**	**2x 46%; 4x44%**
H. annuus	**0.1 oryzalin 3 days plant 4,1**	Sunrich Gold	**228 432**	**Mixoploid**	**2x 33%; 4x52%**
H. annuus	**0.1 oryzalin 3 days plant 4,2**	Sunrich Gold	**183**	**2x**	
H. annuus	**0.1 oryzalin 3 days plant 5**	Sunrich Gold	**342**	**Anueploid**	
H. annuus	**0.1 oryzalin 8 days plant 1,1**	Sunrich Gold	**253**	**2x**	
H. annuus	**0.1 oryzalin 8 days plant 1,2**	Sunrich Gold	**330**	**Anueploid**	
H. annuus	**0.1 oryzalin 8 days plant 2**	Sunrich Gold	**207 380**	**Mixoploid**	**2x 62%; 4x29%**
H. annuus	**0.2 oryzalin 3 days plant 1,1**	Sunrich Gold	**411**	**4x**	
H. annuus	**0.2 oryzalin 3 days plant 1,2**	Sunrich Gold	**398**	**4x**	
H. annuus	**0.2 oryzalin 3 days plant 2**	Sunrich Gold	**340**	**Anueploid**	
H. annuus	**0.2 Colchine 3 days plant 1**	Sunrich Gold	**151 293**	**Mixoploid**	**2x61%; 4x39%**
H. annuus	**0.2 Colchine 3 days plant 2**	Sunrich Gold	**278**	**Anueploid**	
H. annuus	**0.2 Colchine 3 days plant 3,1**	Sunrich Gold	**329 617**	**Mixoploid**	**3x28%; 6x50%**
H. annuus	**0.2 Colchine 3 days plant 3,2**	Sunrich Gold	**330 735**	**Mixoploid**	**3x40%; 8x31%**
H. annuus	**0.2 Colchine 3 days plant 4**	Sunrich Gold	**219 426**	**Mixoploid**	**2x 70%; 4x16%**
H. annuus	**Control**	Sunrich Gold	**161**	**2x**	
H. annuus	**control 3 days**	Sunrich Gold	**179**	**2x**	
H. annuus	**control 8 days**	Sunrich Gold	**192**	**2x**	

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