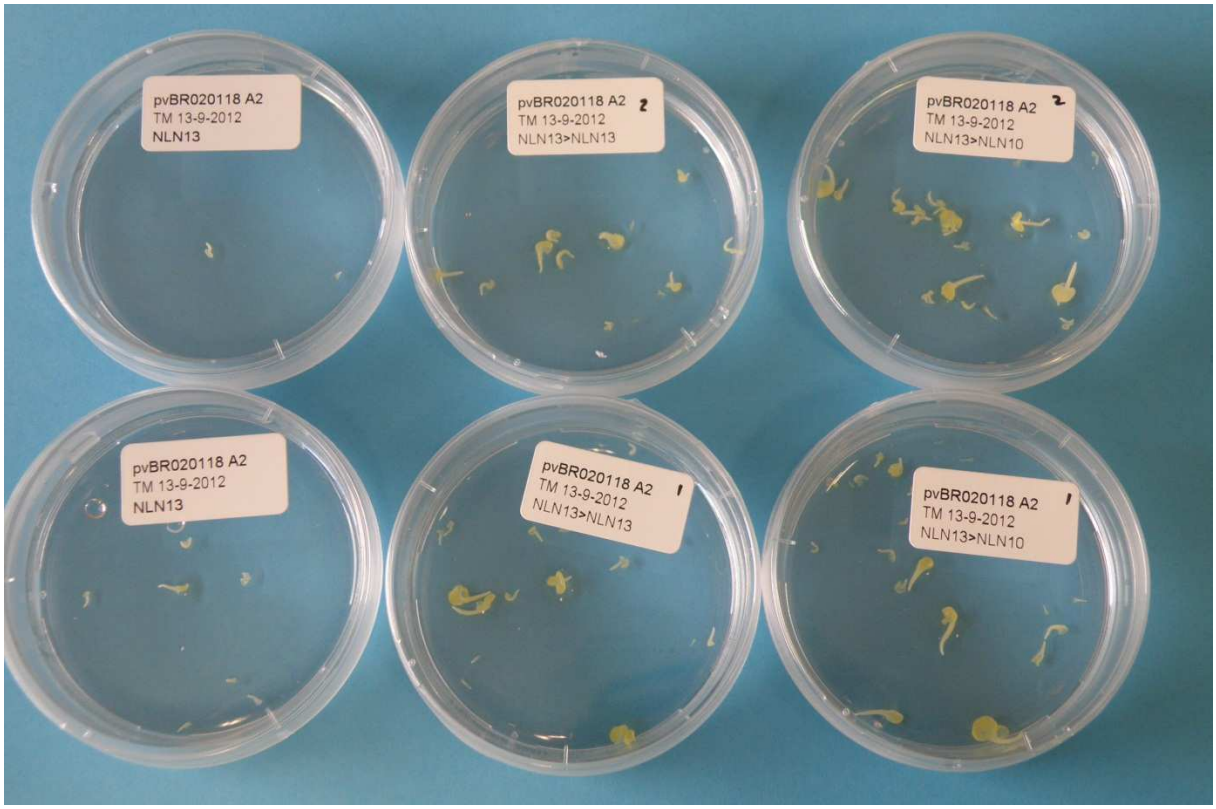
Optimization of Doubled Haploid protocol for *Brassica rapa* ssp.

The effect of sucrose reduction in the culture medium on embryo formation.



Thesis for Master of Science in Plant Biotechnology

Performed by: Tim Minkhorst

Supervisors: Dr. Ir. Guusje A.B. Bonnema

Mr. Johan Bucher

Department of Plant Breeding

Wageningen University

November 2012

Optimization of Doubled Haploid protocol for *Brassica rapa* ssp.

The effect of sucrose reduction in the culture medium on embryo formation.

Tim Minkhorst

Registration number: 801105 573060

MSc Thesis - PBR-80436

Plant Biotechnology (MPB)

Specialization: Functional Plant Genomics

Supervisors: Dr. Ir. Guusje Bonnema

Johan Bucher

Examiners: Dr. Ir. Guusje Bonnema

Dr. Frans Krens

Laboratory of Plant Breeding

Wageningen University, The Netherlands

January 2012 – November 2012

Acknowledgement

This is it.

Three simple words that can mean a lot. It can be applied to the results in this report that describes the optimization of the DH protocol. The sucrose drop might be it.
It can also be applied to my time here at Wageningen UR. With this report I close another chapter in my life of being a student for the second time.
For this period I have to acknowledge several people. But first I have to thank my company for giving me the opportunity to obtain my Master degree.

Next I like to thank Charlotte, who became my wife during my study, for all your support during those years, keeping me motivated and reaching for the max.

I like to thank Dr. Guusje Bonnema for the three roles in which she supported me. Thank you as study coordinator for guiding me through these years as a master student. Thank you as head of the brassica group to let me do my thesis within the plant breeding department even though I was a plant biotechnology student. Thank you as supervisor for your input on my research and report.

I like to thank Johan Bucher for his supervision on my practical work at the lab and climate cells and his input and ideas for my research project.

Dr. Rients Niks for his help with the UV microscope.

And finally everybody that is part of the brassica group. You are all a great team together and it was a pleasure to work with all of you. You gave me a lot of joyful memories!

After 27 months of being a student and 10 months a brassica group member it is hard to say but:

This was it!

Table of Contents

Abbreviations

DH	Double haploid
MCS	Multi cellular structure
MSC	Microspore culture
QTL	Quantitative trait locus
BRO	Broccoletto
CC	Chinese cabbage
FT	Fodder turnip
MIZ	Mizuna
NG	Neep greens
O	Oil seed type
OR	Oil rape
PC	Pak choi
RC	Rapid cycling (oil type)
RO	Rape seed oil
SO	spring seed oil
T	Turnip
TG	Turnip Greens
VT	vegetable turnip
WO	Winter turnip oilseed rape
WU	Wu ta chai
YS	Yellow sarson, oil seed morphotype

Abstract

Brassica rapa is a species that has been intensively studied for several reasons. Its wide genomic diversity (various morphotypes), its use as a model crop for studying polyploidy in plants and their worldwide use as vegetables, oil source, animal feeds and condiments. The brassica research group exploits genetic variation within the *B. rapa* genus. They are interested in the genes that cause variation in the observed traits. This variation is studied by QTL mapping or association mapping. For both studies Doubled Haploid populations are used.
The major goal of my master thesis was to optimize the existing microspore culture protocol in order to obtain Doubled Haploid embryos from all *B. rapa* accessions representing all the different morphotypes including recalcitrant lines. Based on a literature review a sucrose reduction was selected and investigated in order to improve the existing protocol. A total of 18 accessions that represent the different morphotypes of *B. rapa* were selected based on different criteria.
Most of the morphotypes showed to be responsive to microspore culture, 17 out of 18 accessions resulted in one or more embryos, although variation in response between different accessions was large.

The results showed that a reduction of sucrose in the culture media from 17% or 13% to 10% leads to an increase in the number of embryos produced and reduces the time before the embryos become visible in the microspore culture for the tested accessions. Also divisions occurred earlier and more frequently when a reduction of sucrose in the culture media was applied. Furthermore the stage of the isolated microspores are likely having an effect on the embryo response of the culture and should contain no more that 30% bi-nucleates.

Parameters as the optimal culture media for the different morphotypes and the timing of the sucrose reduction treatments can be explored further.
Overall an improved Doubled Haploid protocol is available for the Brassica group.

1 Introduction

1.1 *Brassica rapa*

The Brassica genus comprises six crop species, each with considerable morphological variation. Through interspecific hybridizations in all possible combinations, three basic diploid plant species *B. rapa* (A genome, n=10), *B. oleracea* (C genome, n=9) and *B. nigra* (B genome, n=8) gave rise to three amphidiploid species *B. napus* (AC genome, n=19), *B. juncea* (AB genome, n=18) and *B. carinata* (BC genome, n=17) (U N 1935) which shown in figure 1.

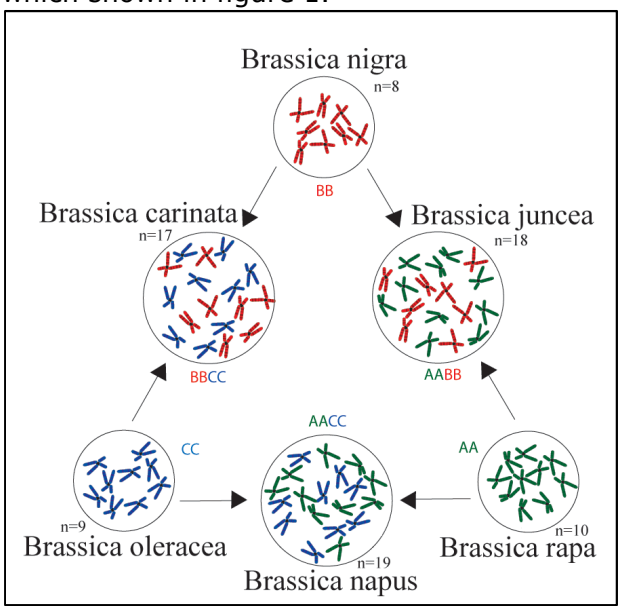


Fig.1 the triangle of U which describes the six crop species of the Brassica genus. Interspecific hybridization of the diploid species (corners) resulted in the amphidiploid species (centres). (U N 1935)

The genus Brassica has a long history of worldwide cultivation, and cultivated Brassica species comprise a large and diverse group of important vegetable, oil, fodder, and condiment crops (Zhao, Artemyeva, et al. 2010). In 2010 the production of cabbage and other brassicas was close to 58 billion tonnes (FAO 2012).

Within the Brassicaceae family *B. rapa* (syn. Campestris) is one of the most diverse species with the longest cultivation history. *B. rapa* comprises leafy vegetables, turnips, and oils. The leafy vegetables include heading Chinese cabbage, pak choi, mizuna, mibuna, komatsuna, and neep greens where the turnips include vegetable and fodder types and the oil types include both the annual and
biannual oil types (Zhao, Wang, et al. 2005).

The large variation in cultivated subspecies of is present in two different centres of diversity; Europe and Asia. Turnip and oleiferous turnip rape, and also broccoletto in Italy, are the dominating forms in Europe. Leafy vegetables such as Chinese cabbage, Pak choi and Narinosa are likely domesticated first in China. China is also the centre of diversity of Chinese turnip rape (ssp. oleifera), which is a unique turnip rape (oil type). Other accessions of *B. rapa* most likely evolved from different morphotypes in the two centres of origin (Zhao, Wang, et al. 2005). *B. rapa* is cultivated in India as an oilseed crop including Yellow sarson and Brown sarson, but no wild forms are known in India (Rakow 2004). Takuno et al. (2007) propose that turnip, ssp. rapa was the primitive type of cultivated *B. rapa*, which originated in Central Asia or in Europe and diffused both to East Asia and to Europe and India.

1.2 Doubled Haploid principle

Normal pollen development takes place in the anthers. This anther contains four anther wall layers (epidermis, endothecium, middle layer and tapeteum) which enclose the fluid filled locule that contains meiocytes (Bedinger 1992). Pollen development follows a tightly controlled sequence of events that can be divided into two major processes: microsporogenesis and microgametogenesis (Touraev, Vicente and Heberle-Bors 1997). Both processes are illustrated below (fig.2) for Arabidopsis (Twell, Park and Lalanne 1998). During microsporogenesis a meiocyte or pollen mother cell, surrounded by a callose wall to isolate the meiocyte from other cells, undergoes two meiotic divisions resulting in a tetrad containing 4 haploid microspores. After dissolution of the callose wall the released young microspores grow and an outer pollen wall, or exine is synthesized. Further development of the pollen is characterized by the migration of the centralized nucleus towards the periphery and the growth of multiple vacuoles that will fuse into one large vacuole. Approximately 5 days after meiosis the microgametogenesis takes place where the microspore undergoes an asymmetrical division, pollen mitosis I. This division results in a bicellular pollen containing a vegetative cell and a generative cell (Bedinger 1992). The vegetative cell exits the cell cycle and further microgametogenesis is completed by a second symmetrical division of the generative cell forming the two sperm cells (Twell, Park and Lalanne 1998).

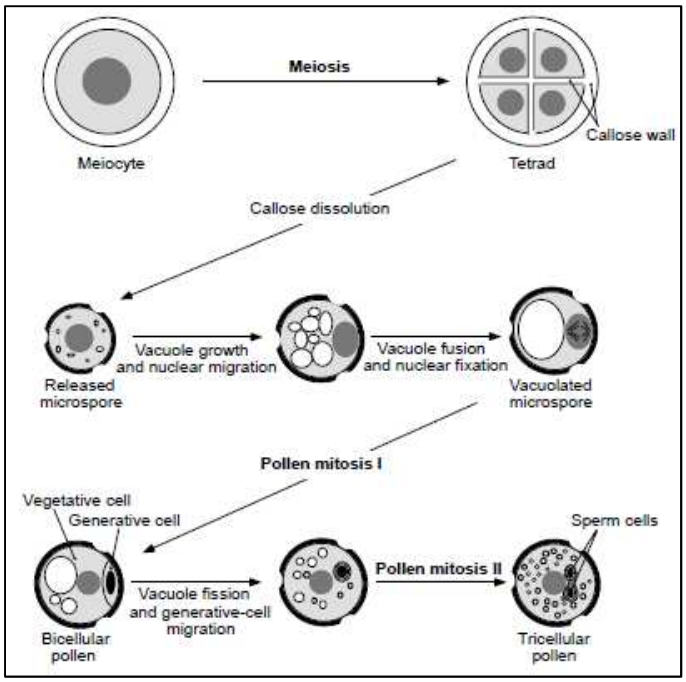


Fig.2 The pathway of male gametophyte development in Arabidopsis showing the processes of microsporogenesis and microgametogenesis (Twell, Park and Lalanne 1998).

The production of Haploids can be achieved by gynogenesis or androgenesis. Both use the gametes as starting material for haploid production. In gynogenesis haploids are proceed from the embryo sack (megagametophyte) and in androgenesis microspores, by anther culture or isolated microspores, are used as target tissue (Friedt and Zarhloul 2005). In order to obtain haploid embryos as shown in fig. 3 via microspore culture, microspores should be in the correct stage to switch from the gametophetic to the sporophytic pathway (embryogenesis pathway) when subjected to stress treatments (Touraev, et al. 1996). As described in the paper of Shariatpanahi (2006) many different stress treatments can be applied including hot and cold temperatures, colchicine and carbon starvation.

Anionesei (2005) composed a list of subcellular changes that occur in different species during the conversion of a gametophytically programmed cell to an embryo founder cell. These changes are: (1) fragmentation of the vacuole by formation of cytoplasmic strands from the perinuclear to the subcortical cytoplasm, (2) movement of the nucleus to the center of the microspore resulting in a central phragmosome, (3) increase in size of the cell, (4) formation of a new cell wall below the exine, (5) size reduction of the nucleolus, (6) compaction of chromatin, (7) structural simplification of the plastids, (8) size reduction of the starch grains, and (9) no marked structural change of mitochondria.

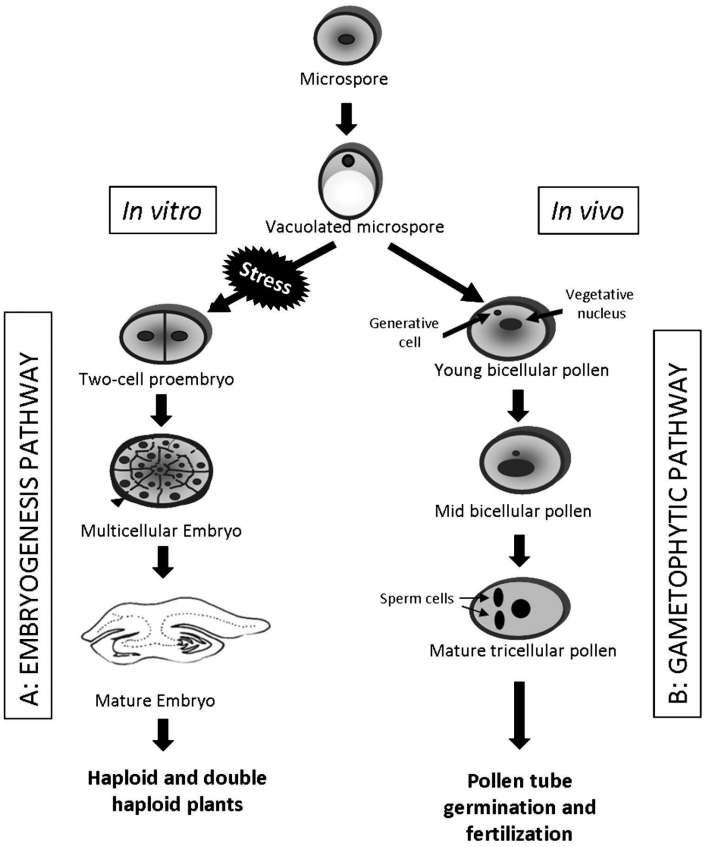


Fig.3 Embryogenic and gametophytic developmental pathways of a barley microspore. Scheme representing the main stages of the two developmental pathways that the microspore can undergo (Rodríguez-Serrano, et al. 2011)

1.3 DH production in Brassica

Haploids and DHs have been produced in *Brassica ssp*. using anther culture or isolated microspores. The first successes using Brassica anthers cultures were reported by (Keller, Rajhathy and Lacapra 1975) and Thomas and Wenzel (1975) and in 1982 Lichter developed an alternative culture system for isolated *B. napus* microspores. Since then remarkable progress has been made (Gil-Humanes and Barro 2009).

B. napus microspores in the late unicellular or early bicellular stages are competent for embryo formation. Gametophytic development is maintained at 18°C and can be changed to sporophytic development by elevating the temperature to 32°C (Custers, et al. 1994). The heat shock treatment influences microtubule distribution leading to a more symmetrical division in the microspores and blocks the further gametophetic development (Babbar, et al. 2004).

Touraev et al (1997) observed that upon stress treatment, isolated *B. napus* microspores swell and
their cytoplasm undergoes structural reorganization. The nucleus moves to a more central position and cytoplasmic strands are formed that pass through the vacuole and connect the perinuclear cytoplasm with the subcortical cytoplasm.

Furthermore, Simmonds and Keller (1999) reported that in heat-stressed Brassica the first structural change seen after the transfer of in vitro cultured microspores to an elevated temperature is, along with the movement of the nucleus to the center inside the microspore, the appearance of a preprophase band (PPB) of microtubules, which does not form during the first haploid mitosis in normal pollen development. The appearance of these PPBs in heat-induced microspores marks sporophytic development, and that continuous PPBs are required for cell wall consolidation and embryogenesis.

All structural changes mentioned lead to symmetric division of the microspore. Zaki and Dickinson (1991) state that it is a key factor that the first division of cultured microspores to become embryos is symmetrical.

1.4 Use of DH in breeding

Plant breeding is focused on continuously increasing crop production to meet the needs of an ever growing world population. The breeders' search for novel genetic combinations, with which to select plants with improved traits to satisfy both farmers and consumers, is endless. About half of the dramatic increase in crop yield obtained in the second half of the last century has been achieved thanks to the results of genetic improvement, while the residual advance has been due to the enhanced management techniques (pest and disease control, fertilization, and irrigation). Biotechnologies provide powerful tools for plant breeding, particularly doubled haploid (DH) technology, can effectively help to select superior plants from segregating populations and represent a particularly attractive biotechnological method to accelerate plant breeding by creating homozygous lines in one step (Germana 2011).

Most *B. rapa* accessions are self-incompatible and much time and cost are required to breed inbred lines by self-pollination. Microspore culture is an efficient way of producing DH Brassica plants (Zhang, Wang, et al. 2012). For example the use of doubled haploids in breeding vegetable Brassicas changed the timescale for producing inbred lines to use as parents of hybrid cultivars from 6+ years to approximately 2 years (Dias 2001). Furthermore DH are very useful to create segregating populations between morphotypes which could be used for (association) mapping or (e)QTL analyses (Ferrie and Möllers 2011).

1.5 Current application of DH technology in the Brassica research group.

The brassica research group exploits genetic variation within the *B. rapa* genus. They are interested in the genes that cause variation in the observed traits, like heading, turnip formation etc. Part of this variation is studied by QTL mapping in a segregating Doubled Haploid population created from microsporogenesis of a single F1 plant. An example of a population created this way is the DH38 population which is used to study flowering time (Zhao, Kulkarni, et al. 2010). Another way to study genetic variation is by association mapping. Association mapping is complicated by the fact that most *B. rapa* accessions are self-incompatible and as a consequence accessions retain a high degree of heterozygosity and heterogeneity. This complication hampers assessment of quantitative traits and genotyping in multi-environmental trials (Zhao, Artemyeva, et al. 2010). Therefor the *B. rapa* core collection of 239 accessions described by Zhao et al. (2010) and 17 additional accessions from Russian and Korean co-workers are subjected to microspore culture in order to create a *B. rapa* Diversity Fixed Foundation Set (DFFS) representing a structured sampling of the genetic diversity across the global *B. rapa* gene pool. Using the current protocol approximately 120 lines are fixed remaining 136 lines to be fixed.

For all this genetic research, it is essential to have a DH protocol that is applicable to all morphotypes. The current protocol is based on Custers et al (1994), which was primarily developed for *B. napus*, with some modifications. In the *B. rapa* protocol NLN13 (modified Nitch medium by Lichter supplemented with 13 % sucrose) was replaced with Gamborg B5 medium supplemented with 13% sucrose and 5 % manitol as wash medium and the cultures are incubated at 32 C for 24 hours instead of 72 hours. However, it turned out that this protocol is not responsive for all accessions.
Within the accessions that are already fixed with the current protocol we could not detect whether certain morphotypes were more responsive to the DH procedure than other morphotypes.
Also when looking at the genetic relationship among accessions, as described by the Phenogram A of Zhao (2005), we could not see a pattern that certain clustered accessions had better responses.

1.6 Scope of the thesis

For the brassica group the production of *B. rapa* Double Haploid lines through microspore culture is mainly used to develop a Diversity Fixed Foundation set. Since there are several crucial steps in this process to create a DFFS, and in this thesis I could not assess all steps, the focus will be on the initiation of embryogenesis.

1.6.1 Research goals

The major goal of my master thesis is to optimize the existing microspore culture protocol in order to obtain embryos from all *B. rapa* accessions representing all the different morphotypes including recalcitrant lines.
Furthermore I like to gain more inside knowledge on the developmental process of microspore embryogenesis and the response of the different *B. rapa ssp.* Finally I would like to combine all knowledge to predict the embryogenic response of the different accessions.

1.6.2 Approach

In order to improve the existing protocol that was primary designed for *B. napus* a literature review will be conducted on papers that describe *B. rapa* DH production. From this literature the critical parameters will be selected and based on these findings a basic protocol will be designed together with test parameters . Accessions will be selected to cover all morphotypes and will be tested with the basic protocol and/or the test parameters. Data will be collected and the results discussed.

2 Material and methods

2.1 Literature review.

For the literature review papers were selected where the primary focus was on *B. rapa* DH production. In case the papers referred to other protocols that were designed for other brassica genera these were also included since *B. rapa* gave response to that protocol. From these papers the key criteria were selected, which resulted in the production or improvement of DH production.

2.2 Selection of accessions.

For the experiments two batches of *B. rapa* accessions were selected from the *B. rapa* core collection which consists of 239 accessions described by Zhao et al. (2010) and 17 additional accessions from Russian and Korean co-workers, according to different selection criteria. The first group consisted of 18 accessions and was used to screen the different accessions for the response to the different culture media used during the experiments. For this group the main criterion was coverage of all morphotypes of *B. rapa*. The next criterion was DH response, where the ideal response was low to moderate. These responses show that an accession was responsive to microspore culture but effects by the tested treatments, both positive and negative, remain visible. The selected accessions showed variable responses from no to moderate embryogenesis according to previous isolations. The last criterion was the availability of seed. Data from earlier sowings on time to flower determined the number of batches sown for each accession since some accessions/ morphotypes require more 200 days to flower.
For the second group of *B. rapa* one morphotype, Broccoletto was chosen. Availability of seed determined the total number of accessions in this group. Only two broccoletto accessions have been tested for their DH response. These broccoletto accessions had low embryogenic response.

An overview of the selected accessions is presented in table 1. In this table also the morphotype and DH response is given.

Table 1: Selected accessions for microspore isolation with their morphotype and DH response.

pvBR number	Genotype name	Collection resource	Originating organisation accession name	Species	Sub taxa in originating gene bank	DH response
pvBR020122	BRO-025	WUR	Natalino	*Brassica rapa*	Broccoletto	n.a.
pvBr020119	BRO-026	WUR		*B. rapa*	Broccoletto	n.a.
pvBR020117	BRO-027	WUR	Quarantina	*B. rapa*	Broccoletto	n.a.
pvBR020121	BRO-028	WUR	Tardivo	*B. rapa*	Broccoletto	n.a.
pvBR020118	BRO-029	WUR	Norantino	*B. rapa*	Broccoletto	n.a.
pvBR020123	BRO-030	WUR	Sessantina	*B. rapa*	Broccoletto	intermediate-good
pvBR020061	BRO-103	WUR	Tsja Sin; No.P1R5T5	*B. rapa*	Broccoletto	n.a.
pvBR020092	BRO-127	WUR	Edible Flower	*B. rapa*	Broccoletto	n.a.
pvBR080028	CC-53V	VIR	Local	*B. rapa*	pekinensis	low
pvBR020134	FT-097	WUR	Buko; Bladraap	*B. rapa*	Fodder Turnip	low
pvBR020155	MIZ-128	WUR	Round Leaved Mibuna	*B. rapa*	Mizuna	none
pvBR080041	NG-215V	VIR	Uzuki Komatsuna	*B. rapa*	Komatsuna	poor
pvBR080090	O-218V	VIR	Nabo silvestre	*B. rapa*	sylvestris	low
pvBR020080	OR-216	WUR	Xi Qiu Bai Cai	*B. rapa*	Chinese turnip rape	n.a.
pvBR080072	PC-Vr.930V	VIR	May 8	*B. rapa*	Pak choi	low
pvBR020158	RC-144	WUR	FIL501	*B. rapa*	Rapid cycling	n.a.
pvBR020235	RO18	WUR		*B. rapa*	Rape seed oil	n.a.
pvBR020104	SO-032	WUR	Pusa Kalyani	*B. rapa*	Spring Turnip Oilseed Rape	n.a.
pvBR080086	T-1283V	VIR	Zolotoj shar	*B. rapa*	rapa	n.a.
pvBR020100	TG-129	WUR	Vitamin Na	*B. rapa*	Turnip Greens	n.a.
pvBR020096	VT-115	WUR	Kairyou Hakata	*B. rapa*	Vegetable Turnip	n.a.
pvBR020108	WO-080	WUR		*B. rapa*	Winter Turnip Oilseed Rape	low
pvBR080095	WU-391V	WUR	Xing Yang	*B. rapa*	Narinosa/ purpuraia	n.a.
pvBR020159	YS-033	WUR	Dys 1	*B. rapa*	Yellow Sarson	n.a.
pvBR020160	YS-143	WUR	FIL500	*B. rapa*	spring yellow sarson	low
	DH4079		topaz	*B. napus*		high

2.3 Sowing and growth conditions

Seed were sown in different batches according to the days to flowering with a maximum of 3 sowings per accession in the period between 9th of March and the 20th of July (see appendix for work scheme). A maximum of 4 seeds were sown in small ø 5 cm pots filled with commercial soil and placed in a climate cell (B3). Settings of this climate cell were 18°C, 60% humidity and a light intensity of 19500 lux with a 15/9 hours day/night rhythm.
After 2 weeks seedlings were transplanted into 2 large ø 17 cm pots filled with commercial soil with a maximum of 2 seedlings per pot. Plants were replaced into the same growing conditions. After acclimating one of the seedlings was removed resulting in 2 plants per batch. Plants were kept in these growing conditions until the plants start flowering (first flower buds start to open). When flowering the plants were placed into climate cell B4 with 10°C, 70% humidity and a light intensity of 8000 lux with a 18/8 hour day/night rhythm.

2.4 Microspore isolation

Microspore isolation was performed according to a protocol based on Custers et al (1994), which was primarily developed for *B. napus*, with some modifications. Inflorescences were collected from the plants when the first buds start to flower. Buds were selected with a size of approximately 2.9 mm, measured with a marking gauge. The selected buds were sterilized in a 2 % sodium hypochlorite solution for 10 minutes and washed 3 times in sterile water for resp. 1,4 and 10 minutes. Sterile bud were gently squeezed in cold wash media (3.16 g/L Gamborg B5 medium including vitamins, 13% sucrose and 5% manitol, pH 5.8, sterilized by autoclaving) The suspension was filtered over 2 layers of 50 µm nylon filter and collected into a sterile 10 ml centrifuge tube with a final volume of 9 ml. Subsequently the suspension was centrifuged in a cooled centrifuge (4°C) for 3 min. at 750 rpm. The supernatant was poured of and the pellet was resuspended in 10 ml wash media and centrifuged again. This step was repeated and after the last wash the pellet was resuspended in 1 ml NLN medium (see appendix 3, composition depended on experiment). Samples of 8 and 20 µl were collected for stage and density determination. The last was done using a Fuchs Rosenthal counting chamber (0.2 mm depth). Four squares in a diagonal were counted and the average was multiplied by 5000 in order to obtain the microspore density per ml. The microspore suspension was diluted to a final concentration of 20.000 msp/ml and 3 ml was plated in 6 cm ø petri dishes. Plates were sealed with 2 layers of parafilm and incubated at 33 °C for 48 hours in complete darkness and afterwards transferred to 25°C again in the dark.

2.5 Sucrose reduction and media refreshment.

In some of the experiments the effect of a sucrose reduction after 24 hours was tested. For this experiment a maximum of three parameter could be tested, depending of the number of plates obtained after microspore isolation, including the untreated control. For the sucrose refreshment the culture media (NLN17 or NLN13) was replaced by NLN10. Since this refreshment is realized by fresh medium the control should be refreshed as well. For both refreshments with or without sucrose reduction the following procedure was followed. The plates were collected from the incubator. From each plate the 3 ml of culture media was transferred into a sterile centrifuge tube and the tubes were centrifuged at 700 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 3 ml of fresh culture media depending on the parameter. The suspension was then transferred back into the original petri dish. After sealing with parafilm the plates were placed again into the incubator for the remaining 24 hours.

2.6 Stage determination and monitoring observations

An eight µl sample collected after the last washing step was diluted with the same amount of dapi staining (1.25 mg/ml 4,6-diamidino-2-phenylindole) and incubated overnight in the fridge. The next day 14 µl was mounted on a microscope slide and covered with a cover slip. The samples were observed under the Olympus UV microscope with appropriate filter blocks at an 40x2 magnification. Stage was determined by giving an overall score in percentage of the present stages. The stages that were scored were mid- and late uni-nucleate, bi-nucleate and tri-nucleate resulting is a final score like M/L 20/80 which correspond to 20% mid and 80% late microspores.

For monitoring of the cultures 100 µl samples were collected in an eppendorf tube for the first 8 days of culturing. The samples were centrifuged for 3 min. at 4000 rpm. From the supernatant 90 µl was removed and the pellet was resuspended in the remaining 10 µl. An equal amount of dapi was added and observations took place as described above.

2.7 Embryo harvest and maintaining

Embryos, which were derived from microspore culture, were counted and or harvested 4 to 6 weeks after the cultures were initiated. Time of harvest depended on the development of the embryo and harvest intervals. The embryos were placed on 9 cm ø petri dishes with solid Gamborg B5 (3.16 g/L Gamborg B5 medium including vitamins, 2.5% sucrose and 1% phyto blend agar, pH 5.8, sterilized by autoclaving) with a maximum of 10 embryos per dish. The dishes were placed in a culture room with 16 photoperiod at 25°. To prevent direct light the plates were covered with a single layer of cheesecloth.

2.8 Data analyses

For each isolation all parameter were recorded in an excel file (see appendix 3). All data was sorted and analysed using GenStat (15th Edition).

3 Results

In this chapter the results of the literature review and microspore culture experiments will be presented in several paragraphs. The first paragraph will cover the literature review and the derived experimental setup . In the second paragraph the tested accessions are listed followed by the stage of the microspores in paragraph 3. Paragraph 4 shows the results of embryogenesis per media and treatment. Finally, the results of the monitoring experiment are presented in paragraph 5.

The amount of available buds for microspore isolation varied per accession and also due to various reasons like the number of flowering stalks, amount of buds per stalk and plant age. This resulted in large variations in the number of isolations per plant. Furthermore, variation in the amount of microspores present after isolation had a huge influence on the number of petri dishes per isolation and therefor also an effect on the number of plates that could be tested for the different treatments per accession. These variations caused unbalanced results, which makes it more difficult to interpreted the results. For this reason for each experiment the number of plates are given besides the mean embryo production per plate for the test parameters.

For each isolation date *B. napus* was used as a control genotype. The embryogenesis of *B. napus* is in general very high (more than 100 embryos per perti dish) and therefore not shown in the results.

3.1 Literature review on *Brassica rapa*

A literature screen on *B. rapa* protocols resulted in the overview shown in table 1. This overview was used to review and improve our existing protocol.

As table 2 shows most protocols also use Gamborg B5 medium supplemented with 13 % sucrose as wash medium (B5-13). Lou et al.(2008) and Wong et al.(1996) use NLN13 both as wash as well as induction medium. Since the use of NLN13 as wash medium of the original protocol of Custers (1994) was replaced by B5 in the existing protocol this will not be tested. Baillie et al. (1992) and Ferrie et al. (1995) use half strength B5-13 as wash medium but there is no evidence that this reduction of salts is the major contributor to improved embryogenesis. Both Guo and Pulli (1996) and Burnett et al.(1992)used a wash medium without iron. Burnett describes that the presence of iron in B5 medium was influencing the colour of the microspore pellet which is directly correlated to the embryogenesis potential. Yellow pellets were indicative of good embryogenesis whereas microspores from green pellets failed to produce embryos. In case green microspore pellets are observed with the current procedure this modification is interesting to test. Since there are no major differences the wash media used in the current protocol and the literature, this parameter will not be the primary focus.

In contrary to the wash medium, many variations in induction (culture) medium as well as in or combination with additional stress treatments are seen in the different publications.
Interestingly is that for subspecies pekinensis and chinensis in general the same protocol is used as our current protocol. However minor differences can be the trigger to their success.

Sato et al. (1989), Wang et al. (2009) and Zhang et al. (2001) reduced the salt concentration of their macro nutrients in the induction medium. Sato et al. (1989) reports that 1/2 macro salts increase embryo formation while Wang et al (2009) observed significantly more embryo production than full strength NLN medium for several purple flowering stalk genotypes and moreover, some non-responsive genotypes also produced embryos when the nutrient concentration was halved. Dias et al (2001) also reported that embryogenesis was significantly increased in some Broccoli varieties (*B. oleracea*) by the reduction of macro salts and that particularly the reduced concentration of NO_3 promoted embryogenesis.

Sato et al. (2002) describes the promoting effect of a cold pre-treatment of bud in liquid B5-13 medium for 3 up to 20 days. They propose that this cold pre-treatment might destroy microtubules which result in symmetrical cell division of late uni-nucleate microspore. Symmetrical divisions lead to embryogenesis where a-symmetrically divisions lead to normal pollen development.

According to Zhang et al. (2011) addition of 40 µM PCIB (p-Chlorophenoxyisobutyric acid; an auxin inhibitor) increase embryogenesis up to 6 fold compared to control treatments and also a quicker response in embryogenesis. These observations were similar to those of Agerwal et al. (2006) in B. *juncea* who postulate that PCIB is probably involved in promoting embryo development in multicellular grains due to overcoming the inhibitory effect of high auxin concentration.
The last major difference in induction medium and additional stress treatment is the use of NLN17 as induction medium reported by Ferrie et al. (2005) or combined with refreshment of the culture with NLN10, described by Baillie et al. (1992), Ferrie and Keller (1995) and Gu et al (2003). Refreshment of the culture has two benefits. Firstly, different concentrations of sucrose are required at different stages of microspore development. Baillie et al. (1992) and Lionneton (2001) refer to a paper of Dunwell and Thurling (1985) where they discovered that a high level of sucrose in beneficial for the initial microspore survival and divisions because a medium containing 17% sucrose generates an osmotic potential similar to that of the anther homogenate, but a lower level is required for the continuation of this division. Secondly, as observed by Kott et al. (1988), changing induction medium after 24 hours, at the initial stage of microspore culture, is essential to get rid toxic elements released from the older bi-nucleate microspores, resulting in normal embryo initiation and development.

Table 2: Overview of literature describing Brassica campestris (Rapa) microspore isolation. Highlighted in bold are factors that contribute to the success according to the authors.

publication	Specie	ssp.	wash medium	Induction medium	Temperature /duration	additional stress	density msp/ml	yield
Custers et al. 1994	Napus		NLN-13	NLN-13	32C/72h		40000	Not mentioned
Ajisaka et al. 1999	Rapa						200000	
Baillie et al. 1992	Campestris	oleifera	1/2 B5-13	NLN-17 + 0.83 mg/l KI	32C/48h	**cultures refreshed after 48 H with NLN 10**	100000	46.3 E/100 buds
Burnett et al. 1992	Rapa	oleifera	**B5-Fe0**	**NLN 0.5 Fe--> NLN13**	33C/over night		25000-100000	2 fold and more
Cao et al. 1994	Rapa	chinensis		NLN 10 + 0.5 mg/l NAA and 0.05mg/l BA	33C/24h		100000 - 200000	57E/bud
Ferrie and Keller 1995	Rapa	oleifera	1/2 B5-13	NLN17 +0.83 mg/l KI	32C/48H	**cultures refreshed after 48 H with NLN 10**	100000	up to 70 E/100 buds
Ferrie et al. 2005	Rapa			NLN-17 + 0.44uM BA no glutamine	32C / 48h	**brassinolide (BL) 10-7 EBR ;10-6, 10-7**		-
Gu et al. 2003	Rapa	chinensis	B5-13	(NLN17; NLN10) + 0.83 mg/L KI	32C/48h	2% AC W/V **refreshed to NLN10 after 48h**	20000	3.8- 42.2 E/bud
Guo & Pulli 1996	Campestris	oleifera	B5-13 Fe0	NLN13+ 0.83 mg/L KI	32C/72 h	150 mg/l AC	50000	0-5930 E/100 buds
Lou et al. 2008	Rapa		NLN13	NLN13	31C/72H		40000	
Na et al. 2010	Rapa			NLN 13 1x macro 0.5x micro		Active charcoal 20 mg/10 ml		
Nishioka et al. 2005	Rapa							
Parihar et al. 1999	Rapa		B5-13	NLN13	25C/10-15d		20000	up to 0.12% of microspore
Sato et al. 1989	Campestris	pekinensis	B5-13	1/2 macro + vit NN; Micro MS -10	33C/24h		200000	0.1-0.25 % of microspore
Sato et al. 2002	Campestris	pekinensis	mB5-13	NLN13 +0.3mg/L BAP	33C/24h	**3 or 10 days cold pre treatment**		increase
Sato et al. 2002	Campestris	pekinensis	mB5-13	NLN13 +0.3mg/L BAP	33C/24h	**7-20 days cold Pre-treatment of buds in mB5-13**	50000	up to 2 fold vs. control
Wang et al. 2009	Campestris	chinensis	B5-13	**1/2NLN macro**	32C/18h	Ac 0.015%	50000	0-70 E/ dish
Wong et al. 1996	Campestris	parachinensis	NLN13	NLN13+ 0.05mg/l BA	32C/48h		100000	2-5 E/10buds
Zhang et al. 2001	Campestris	pekinensis	B5-13	1/2 NLN 10	32.5 C/24h		100000	0-800 E
Zhang et al. 2012	Campestris		B5-13	NLN13	32C/24h	Ac 0.015%	50000	
Zhang et al. 2011	Campestris	pekinensis x chinensis	B5-13	NLN13	32C/24h	Ac 0.015% **+ 40 µM PCIB**	50000	up to 6 fold
Zhang et al. 2011	Campestris	pekinensis x chinensis	B5-13	NLN13	**35C/24h**	Ac 0.015%	50000	up to 2 fold

Experimental design

Based on the literature screen the focus of this thesis project will be on culture media, and more specific on the amount of sucrose present in the media at specific time points. Therefore the hypothesis is that a sucrose drop from 17% to 10 % will lead to an increase of embryos in our *B. rapa* accessions and might overcome recalcitrance.

To test this hypothesis I will uses the following settings as standard protocol.

- B5-13 as wash medium
- NLN13 as induction medium
- 0.015% active charcoal
- 32ºC for 48 hours as stress treatment.

To test the effect of sucrose reduction of NLN17 to NLN10, first the effect of changing NLN13 to NLN17 will be tested. When there are no differences in response to NLN13 or NLN17 the last will be used to test the effect of the sucrose drop to NLN10, otherwise the sucrose reduction from NLN13 to NLN10 will be tested. Furthermore as an second control treatment the cultures will be refreshed with the same medium.
During the culture period of the isolated microspores, the cultures will be monitored in order to see differences in embryo numbers and quality of embryos between treatments (sugar concentration and refreshing) of the same accession as well as differences between the accessions for the same treatment.

3.2 Overview of accessions tested for microsporogenesis

For the optimization of the doubled haploid protocol 25 accessions (18 accessions from the first and 7 accessions of the second batch shown in table 2) were selected. From these 25 accession two accessions did not germinate (BRO-026 and OR-216) and four accessions did not flower in time in order to isolate microspores(FT-097, NG-215V, PC-Vr.930V and T-1283V). Accession RC-144 flowered relatively fast resulting in too low number of buds to harvest which contained to old microspores. This accession was therefore excluded from the results. The remaining total number of tested accessions was reduced to 18 (12 accessions of the first batch and 6 of the 2nd batch).

Since the number of plants used for microspore isolation varies, data from all isolations was combined to get an average number of embryos per accession per petri dish. Data of which plant is used for each isolation can be seen in the overview table in appendix 3.

The response of the different accessions to microspore culture is shown in figure 4 and table 3. Figure 4 shows the mean number of embryos of each accession per petri dish over all isolations, with varying treatments and or /protocols while table 3 also shows the total number of isolated petri dishes and the total number of embryos produced per accession.

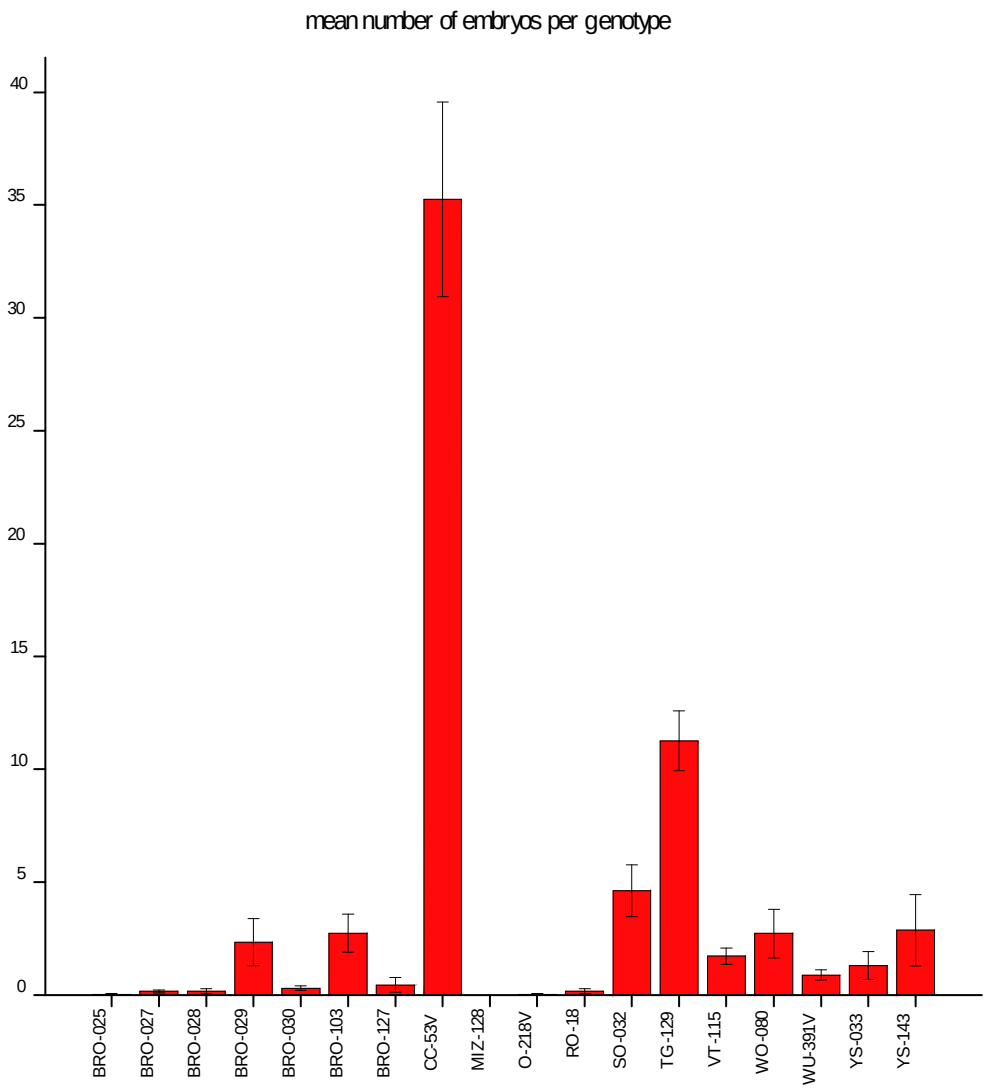


Figure 4: Mean number of embryos per accession (genotype) per petri dish over all isolations with their standard error. Total no. of petri dishes , the total and mean no. of embryos produced are showed in table 3.

From figure 4 is can be seen that that most accession respond to microspore culture (17 out of 18) with different success. Chinese cabbage accession CC-53V shows the highest average number of embryos (35.25) per petri dish while accessions MIZ-128 give no response at al. Furthermore the accessions BRO-25, BRO-27, BRO-28; O-218V and RO-18 showed a very low response (below 0.167 embryos/perti dish, see table 3)

Besides variation in the response per accession as shown in figure 4 and table 3, variation was seen in the embryo response of different plants within an accession. For example accession BRO-127 was isolated 4 times, three times plant A1 and 1 time plant B1 and only the last resulted in the production of embryos (see appendix 3).

Table 3: Summary of the results per accession over all isolations. Shown are the no. of observed plates, and the total and mean number of embryos per accession together with the corresponding standard deviation (s.d.) and standard error (s.e.) of mean.

accession_nr	No.observed plates	embryos			
		Total	Mean	s.d.	s.e.mean
BRO-025	32	1	0.031	0.177	0.0312
BRO-027	36	6	0.167	0.378	0.063
BRO-028	12	2	0.167	0.389	0.1124
BRO-029	38	89	2.342	6.364	1.0324
BRO-030	47	14	0.298	0.657	0.0958
BRO-103	26	71	2.731	4.295	0.8423
BRO-127	27	12	0.444	1.739	0.3348
CC-53V	32	1128	35.25	24.417	4.3164
MIZ-128	9	0	0	0	0
O-218V	35	1	0.029	0.169	0.0286
RO-18	12	2	0.167	0.389	0.1124
SO-032	47	217	4.617	7.803	1.1382
TG-129	23	259	11.261	6.362	1.3266
VT-115	29	50	1.724	1.925	0.3575
WO-080	36	98	2.722	6.435	1.0725
WU-391V	18	16	0.889	0.963	0.2271
YS-033	13	17	1.308	2.213	0.6138
YS-143	15	43	2.867	6.14	1.5852

3.3 Stage of microspores

The developmental stage of the microspores is essential for the response to microspore culture. The optimal stage of the microspores for *B. rapa* is late uni-nucleate with a maximum of 20% of bi-nucleate microspores. In previous studies stage was correlated to bud size, and this this correlation was rather similar for the different morphotypes that were tested.

The stage of the microspores was determined for each accession after microspore isolation by an overnight staining with dapi. Therefor the measured bud size was an estimation of the stage present in the microspores. During the observations under the UV microscope the different stages of microspores were scored in percentages. These percentages were converted into classes from young to old in order to get an representative overview in number of isolations and produced embryos as shown in fig 5 and 6. For Example stage 1 is 80% mid- 20 % late uni-nucleate and stage 18 is 10% late uni-nucleate and 90% bi-nucleate. A complete of the stage groups overview is given in table 4.

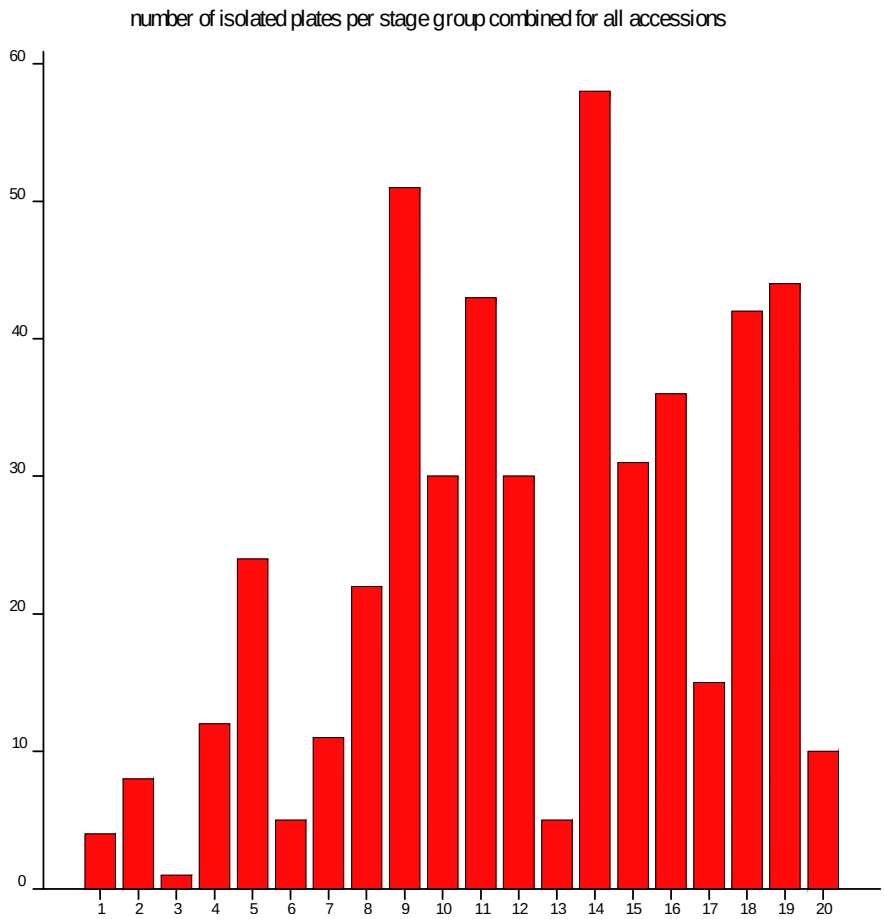


Figure 5:The total number of petri dishes with isolated microspores over all isolations divided over the microspore stage groups. Microspore stage groups go from young (1 is 80% mid- 20 % late uni-nucleate) to old (18 is 10%:late uni-nucleate and 90% bi-nucleate). The optimal stage are represented by the stage groups 9 to 11.

Figure 5 illustrates which stages were ended up in the microspore culture after the dapi staining. While the optimal stage is between groups 9 to 11 a large variation is present for the stage groups of the isolated microspores. From this figure and table 4 you can see that only 124 out of the 482 cultured petri dishes are in the optimal range and that more than 50% of the petri dishes (271) contain microspores that are older than the optimal range.

In figure 6 the mean number of embryos per petri arranged per microspore stage group is shown together with the standard error. The majority of embryos produced are in the range of microspore stage group 4 till 14 (50% mid- 50% late uni-nucleate to 50% late uni-nucleate- 50% bi-nucleate) with the highest mean number of embryos (58.4) at stage group 13. A remarkable observation which can be seen in figure 6 and table 4 is that most embryos a produced around the optimal stage of microspores (stage group 9 to 11), according to the literature, instead of within that range.

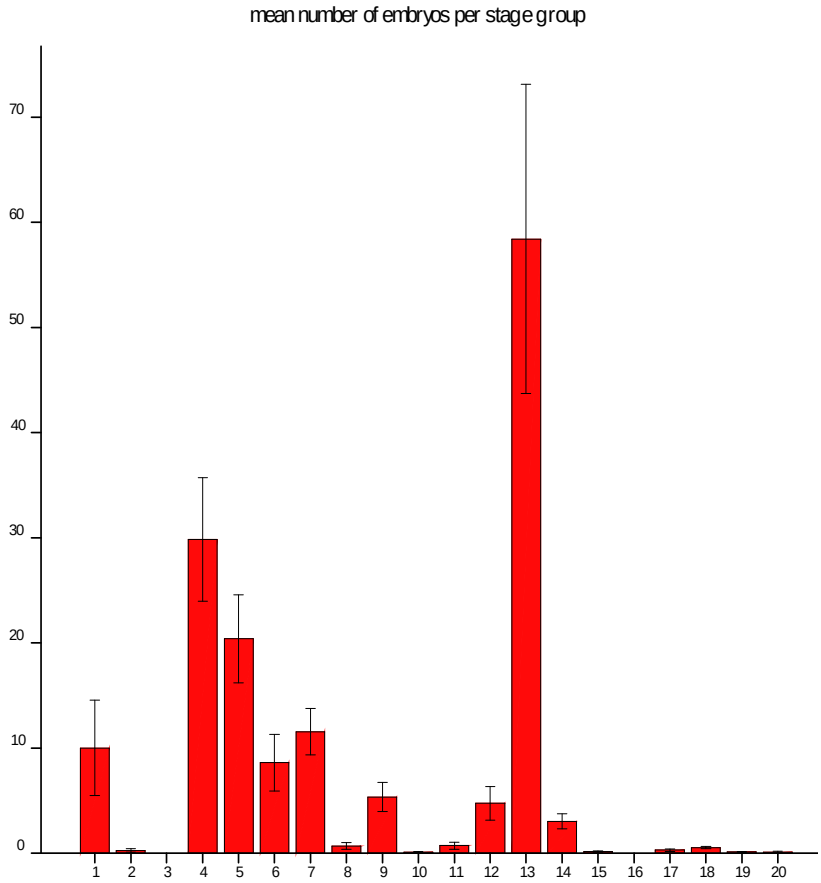


Figure 6: The mean number of embryos over all isolations per petri dish divided per microspore stage group with their standard error. Microspore stage groups go from young (1 is 80% mid- 20 % late uni-nucleate) to old (18 is 10%:late uni-nucleate and 90% bi-nucleate). The optimal stage are represented by the stage groups 9 to11.

An overview of all combined data is shown in table 4 including an standard deviation and standard error of mean. In this table as well as figure 6 it is clearly seen that the higher microspore stage groups hardly result in embryos but some are formed. Furthermore the youngest stage used still resulted in embryo formation.

Table 4: Summary of the results per microspore stage. Shown are the no. of observed plates, and the total and mean number of embryos per stage together with the corresponding standard deviation (s.d.) and standard error (s.e.) of mean. The optimal microspore stage groups are highlighted.

Microspore stage group	stage of isolated microspores	No.observed plates	embryos			
			Total	Mean	s.d.	s.e.mean
1	80% mid- 20% late uni-nucleate	4	40	10	9.092	4.546
2	70% mid- 30% late uni-nucleate	8	2	0.25	0.463	0.164
3	60% mid- 40% late uni-nucleate	1	0	0	*	*
4	50% mid- 50% late uni-nucleate	12	358	29.833	20.36	5.877
5	40% mid- 60% late uni-nucleate	24	489	20.375	20.46	4.176
6	30% mid- 70% late uni-nucleate	5	43	8.6	6.066	2.713
7	20% mid- 80% late uni-nucleate	11	127	11.545	7.381	2.225
8	10% mid- 90% late uni-nucleate	22	15	0.682	1.585	0.338
9	100% late uni-nucleate	51	272	5.333	9.826	1.376
10	90% late uni-nucleate 10% bi-nucleate	30	3	0.1	0.305	0.056
11	80% late uni-nucleate 20% bi-nucleate	43	30	0.698	2.242	0.342
12	70% late uni-nucleate 30% bi-nucleate	30	142	4.733	8.706	1.589
13	60% late uni-nucleate 40% bi-nucleate	5	292	58.4	32.898	14.713
14	50% late uni-nucleate 50% bi-nucleate	58	175	3.017	5.286	0.694
15	40% late uni-nucleate 60% bi-nucleate	31	5	0.161	0.374	0.067
16	30% late uni-nucleate 70% bi-nucleate	36	0	0	0	0
17	20% late uni-nucleate 80% bi-nucleate	15	4	0.267	0.458	0.118
18	10% late uni-nucleate 90% bi-nucleate	42	22	0.524	0.671	0.104
19	100% bi-nucleate	44	5	0.114	0.321	0.048
20	bi-nucleate + tri-nucleate	10	1	0.1	0.316	0.1

3.4 Culture media and treatment effects

Based on the literature screen the focus of this thesis project was on culture media, and more specific on the amount of sucrose present in the media at specific time points. Therefore the hypothesis was that a sucrose reduction from 17% to 10% would result in a higher embryo yield.
In order to test the effect of the sucrose reduction from NLN17 to NLN10 first the effect of changing the standard culture medium NLN13 to the higher sucrose NLN17 was tested.

The results of this comparison are shown in figures 7 and 8. Figure 7 illustrate the total number of isolated petri dishes per accession per media, while in figure 8 the mean number of embryos per accession per media is shown together with the standard error of means. Data of microspore isolations treated as control (no other test parameter) are shown. For BRO-103 no control experiments cultured in media NLN13 were performed (see figure 7). Other accessions that show no data in figure 8 had no embryo production.

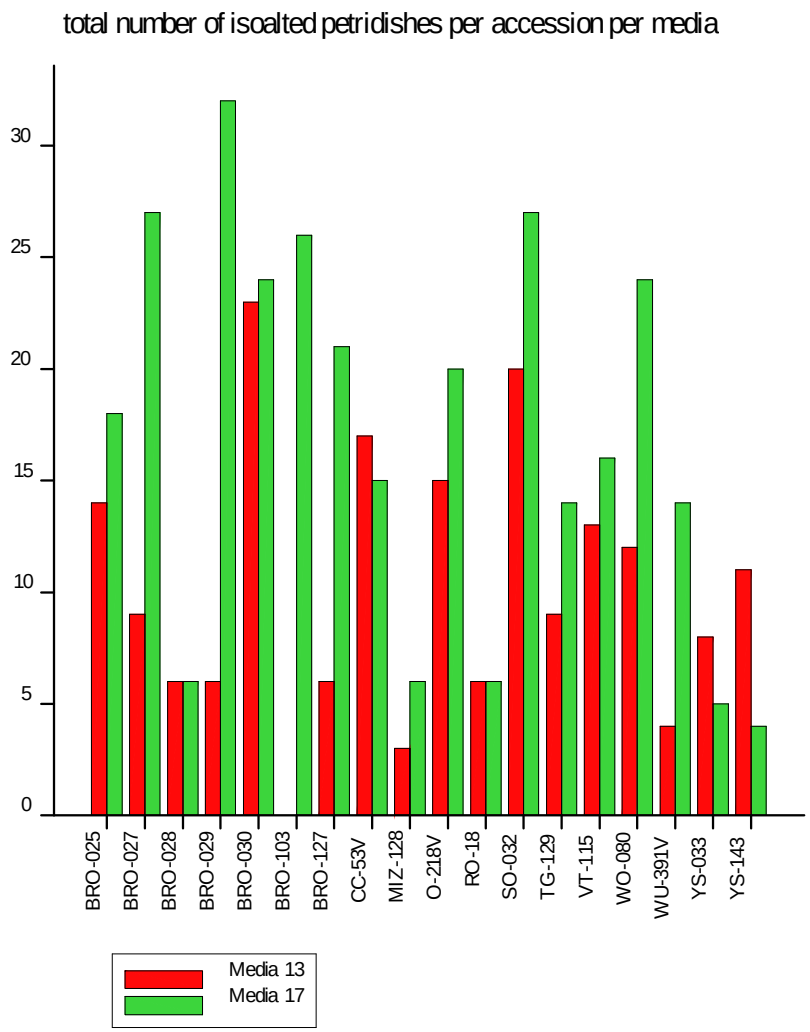


Figure 7: The total number of petri dishes with isolated microspores per accession per media (NLN13 or NLN17) that were treated according to the control parameters.

As you can see in figure 8 most of the accessions show no difference between the mean number of embryos per accession whether cultured in NLN13 or NLN17, except for BRO-029 which showed a higher mean number of embryos for media NLN13 and SO-032, WU-391-V and WO-080 which showed a higher mean number of embryos when cultured in NLN17.

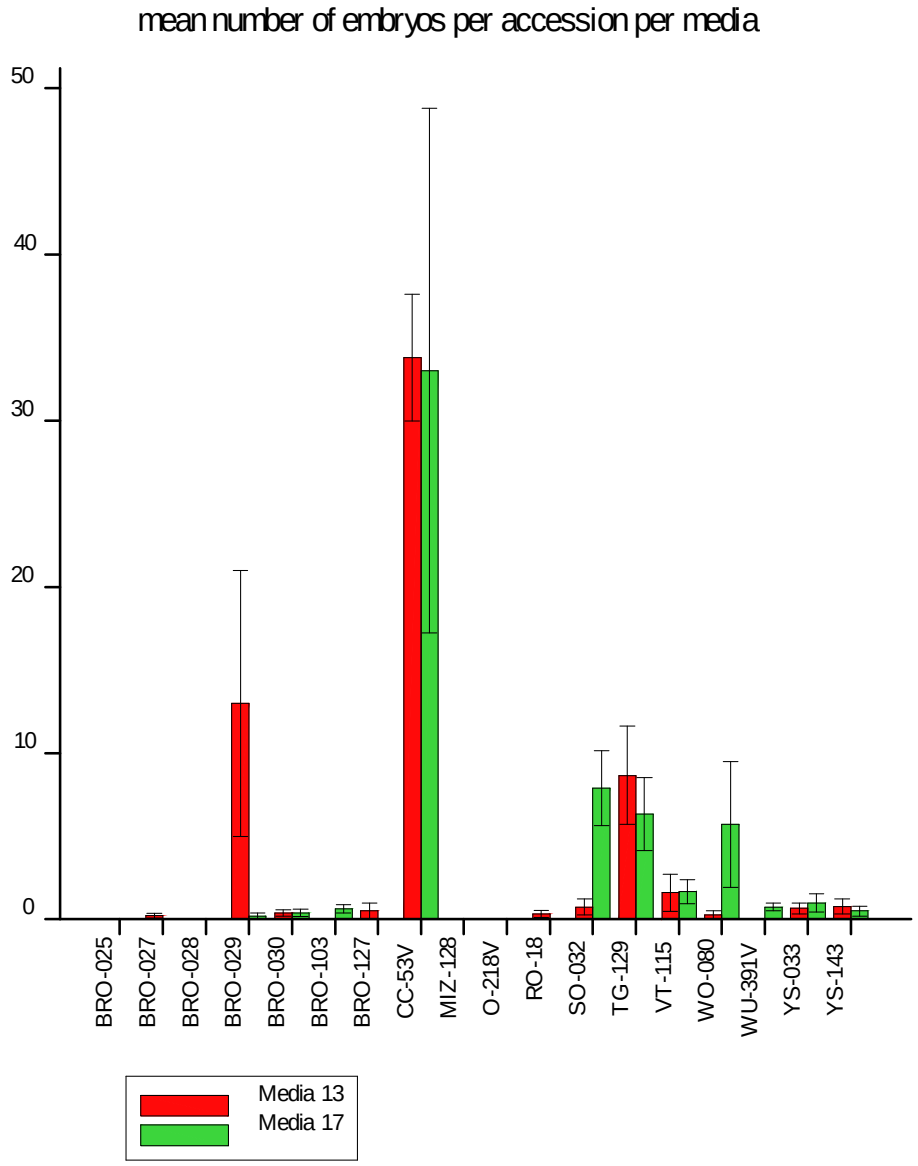


Figure 8: The mean number of embryos per accession per media (NLN13 or NLN17) that were treated according to the control parameters together with the corresponding standard error of mean.

At the time that the experiments were conducted the results of the comparison of the two culture media were not available. Therefore the effect of the sucrose reduction on the different accessions was tested with both culture media NLN13 and NLN17.
In order to see the effect of a sucrose reduction accessions were cultured for 24 hours in NLN17 or NLN13 in an 33ºC incubator. After 24 hours the plates were divided into 3 treatments (when sufficient plates were available) shown below. After the treatment the cultures were placed back for another 24 hours at 33ºC followed by continuous culturing at 25 ºC.

- Control; no change of media
- Drop; change of culture media with NLN10 (reduced level of sucrose)
- Refresh; change of culture media with the same fresh media

The results of the different treatments are shown in the figures 9, 10 11 and table 5. Figure 9 shows the total number of isolated petri-dishes per accession per treatment. In figure 10 the mean number of embryos per accession (genotype) per treatment is shown while in figure 11 the mean number of embryos per treatment per culture media averaged over all accessions is given. In both figures 10 and 11 the standard error of means shown by the error bars. The graphs in figures 9 and 10 are composed from data from both culture media (NLN13 and NLN17) and the exact numbers are given in table 5.

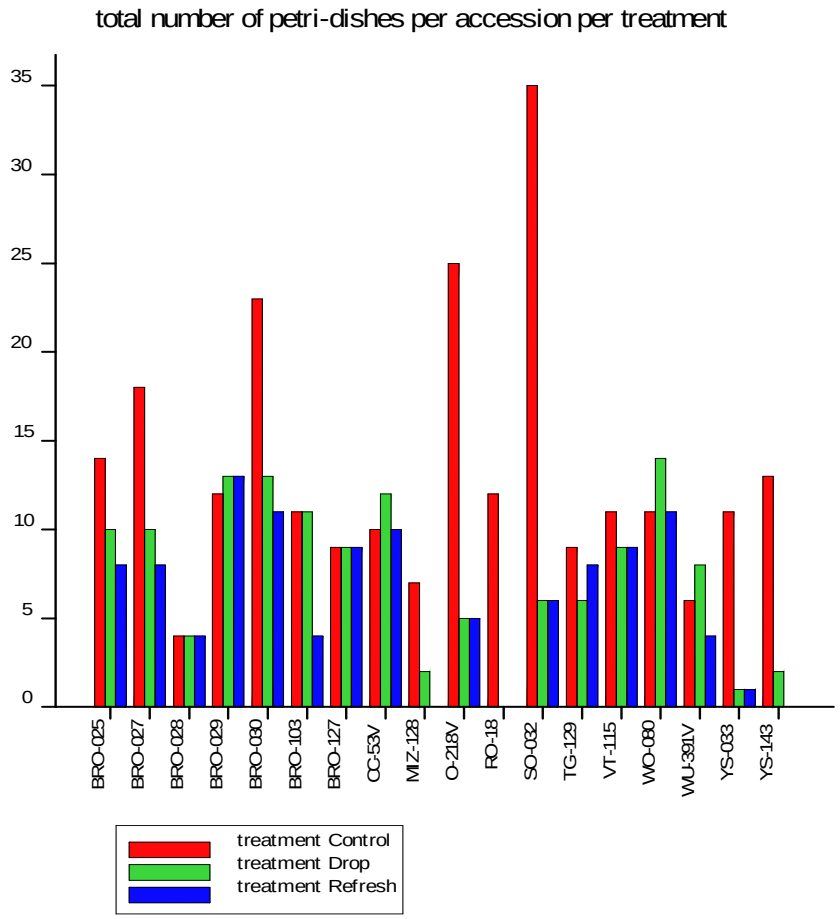


Figure 9: The total number of petri-dishes with isolated microspores per accession per treatment. Data is combined from both culture media (NLN13 or NLN17).

From figure 9 and table 5 it can be seen that most accessions were isolated at least once with each treatment resulting in at least one petri-dish. The exceptions are MIZ128 and YS-143 which had no refreshment treatment and RO-18 which was only treated as control. These missing values are indicated by an "-" in table 5. For accession YS-033 only one petri dish was observed for the sucrose drop and the refreshment treatments and show therefore no error bar in figure 10 and are marked by an "*"in table 5.

In figure 10 it is clearly shown that there is a large variation in response between the different accessions, which was already observed in figures 4 and 8. More interestingly is that accessions that have a moderate to good response (more than an average of two embryos per petri dish) in the control treatment, like BRO-029 and CC-53V, show less increase in embryos compared to the sucrose drop and refreshment treatment . While other accessions which have a low embryo response in the control treatment, like BRO-103 and both YS, show a strong increase in embryo response for the sucrose drop treatment. Furthermore, from figure 10 and table 5 you can see that most of the accessions showed a higher average number of embryos per petri dish in the sucrose drop treatment compared to the control treatment. Refreshment of the culture resulted in similar embryo responses compared to the sucrose reduction treatment for accessions TG-129 BRO-029 and WU-391V. A lower embryo responses is observed for the refreshment treatment when compared to the sucrose reduction treatments for the accessions BRO-103, SO-032, VT-115, WO-080 and CC-53V.

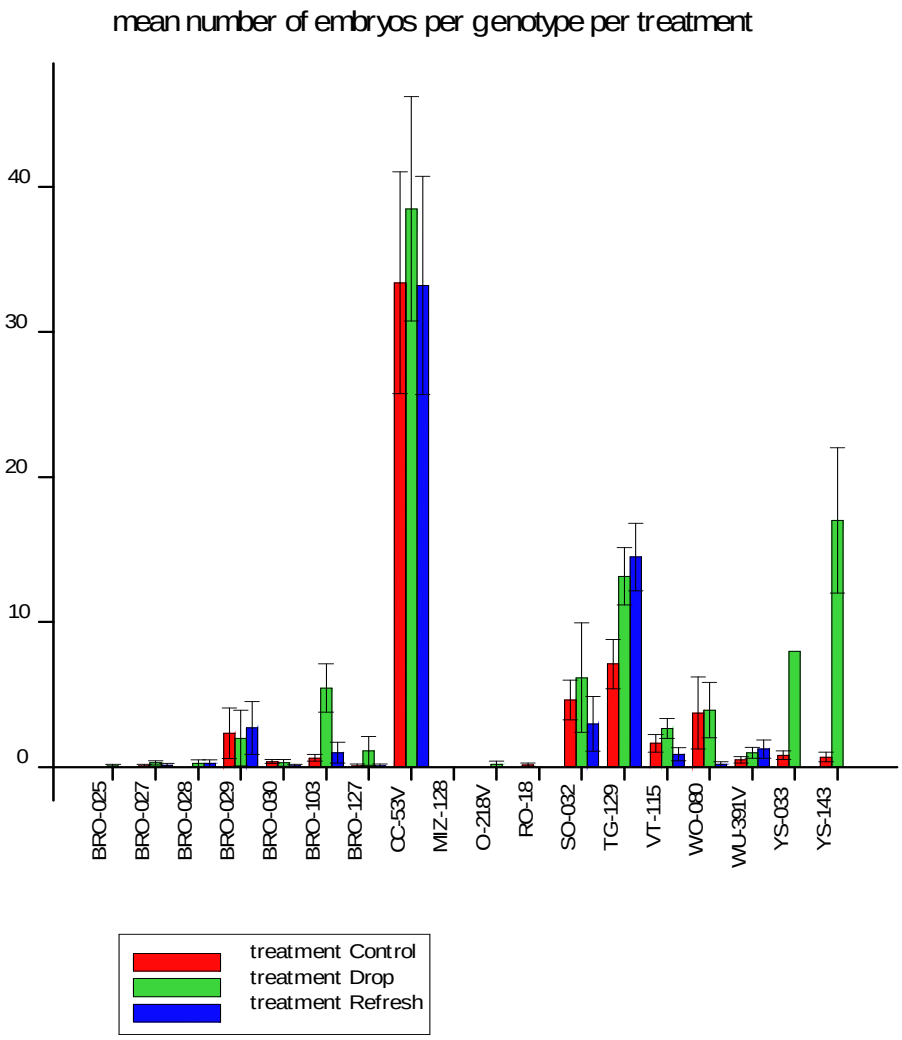


Figure 10: The mean number of embryos per accession per treatment with the corresponding s.e. of means. Data is combined from both culture media (NLN13 or NLN17).

Table 5: Summary of the results per accession per treatment combined for both media (NLN13 and NLN17). Shown are the no. of observed plates, and the total and mean number of embryos per stage together with the corresponding standard error (s.e.) of mean.

	Control (continuous NLN)				sucrose drop (NLN -->NLN10)				sucrose drop (NLN -->NLN)			
	#	embryos			#	embryos			#	embryos		
accession	observed	Total	Mean	s.e.mean	observed	Total	Mean	s.e.mean	observed	Total	Mean	s.e.mean
BRO-025	14	0	0.00	0.00	10	1	0.10	0.10	8	0	0.00	0.00
BRO-027	18	2	0.11	0.08	10	3	0.30	0.15	8	1	0.13	0.13
BRO-028	4	0	0.00	0.00	4	1	0.25	0.25	4	1	0.25	0.25
BRO-029	12	28	2.33	1.75	13	26	2.00	1.92	13	35	2.69	1.82
BRO-030	23	9	0.39	0.14	13	4	0.31	0.24	11	1	0.09	0.09
BRO-103	11	7	0.64	0.24	11	60	5.46	1.67	4	4	1.00	0.71
BRO-127	9	1	0.11	0.11	9	10	1.11	0.99	9	1	0.11	0.11
CC-53V	10	334	33.40	7.66	12	462	38.50	7.74	10	332	33.20	7.53
MIZ-128	7	0	0.00	0.00	2	0	0.00	0.00	-	-	-	-
O-218V	25	0	0.00	0.00	5	1	0.20	0.20	5	0	0.00	0.00
RO-18	12	2	0.17	0.11	-	-	-	-	-	-	-	-
SO-032	35	162	4.63	1.37	6	37	6.17	3.78	6	18	3.00	1.90
TG-129	9	64	7.11	1.70	6	79	13.17	1.97	8	116	14.50	2.33
VT-115	11	18	1.64	0.61	9	24	2.67	0.69	9	8	0.89	0.46
WO-080	11	41	3.73	2.49	14	55	3.93	1.91	11	2	0.18	0.18
WU-391V	6	3	0.50	0.22	8	8	1.00	0.38	4	5	1.25	0.63
YS-033	11	9	0.82	0.30	1	8	8.00	*	1	0	0.00	*
YS-143	13	9	0.69	0.33	2	34	17.00	5.00	-	-	-	-

-: no data obtained for that particular treatment for the corresponding accession
*: no standerd error of means available

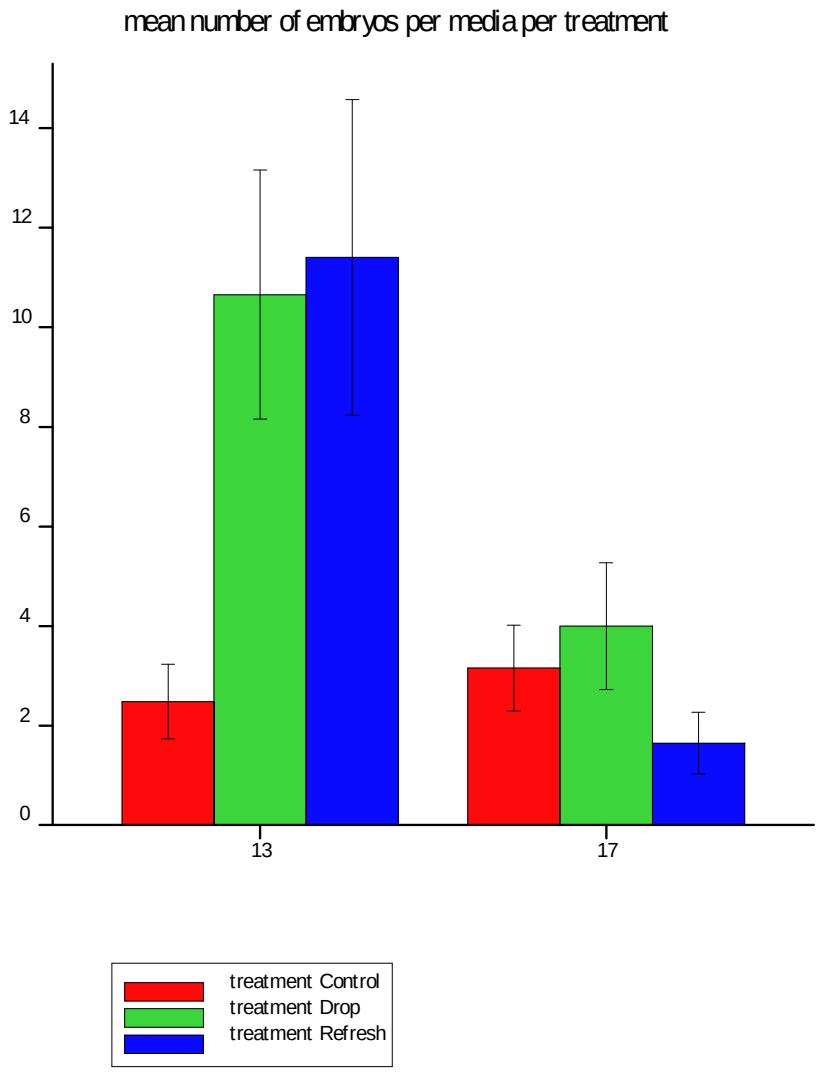


Figure 11: The mean number of embryos per treatment per culture media with the corresponding s.e. of means. Data is combined over all accessions.

As figure 11 shows a large increase in number of embryos when comparing the sucrose drop and refreshment treatment with the control, using NLN13 as starting culture media. For NLN17 effects of a sucrose reduction and a refreshment are non-significant. as starting culture media. Number of embryos in control NLN13 and NLN17 are comparable. Also the mean number of embryos of the sucrose drop treatments is higher for both media compared to the control treatments although for NLN17 this difference is less strong.
Besides the difference in the number of embryos that are produced per treatment also the difference in time before the embryos are visible (Time of Flight) is affected by the different treatments which can be seen in the figures 12, 13 and 14. Embryos do not only appear faster, also they are more developed in the similar time period .

Figure 12 shows the clearly the difference in development of accession CC-53V (pvBR080028) between the plates treated with a sucrose drop from NLN17 to NLN10 (left) and the control treatment (right). This difference was not visible any more at the time of harvest. The picture was taken at 17th of august, 2 weeks after microspores isolation.

Figure 12: developed embryos after 14 days of culturing of accession CC_53V treated with sucrose drop (left) and control NLN17 treatment (right). Plates with sucrose drop show more developed embryos compared to the control treatment

Figure 13 shows clearly the difference in Time of Fight. *B. napus* used as control isolation was treated with a sucrose drop (top), refreshment (right) and control NLN17 treatment (left). The sucrose drop shows much faster visible embryos. A few weeks later no difference was observed in the total number of produced embryos or in the morphology of the embryos. The picture was also taken at 17th of august, 2 weeks after the microspores were isolated.

Figure 13: Developed embryos after 14 days of culturing of *B. napus* treated with sucrose drop (top) refreshment (right) and control treatment (left). Plates with sucrose drop show more and earlier embryos compared to the control- and the refreshment treatment.

Figure 14: developed embryos after 14 days of culturing of accession YS-033 treated with sucrose drop (right) and control NLN13 treatment (left). Plate with sucrose drop show more developed embryos compared to the control treatment

Figure 14 shows the clear difference in development of a low responding accession YS-033 (pvBR020159) between the plate treated with a sucrose drop from NLN13 to NLN10 (right) and the control NLN13 treatment (left). This difference remained present at the time of harvest. The picture was taken at 17th of august, 2 weeks after the microspores were isolated.

3.5 Monitoring of microspore cultures

From the first microspore cultures it was clearly seen that after 2 weeks a difference in the number of visible events was seen in the sucrose drop treatment compared to the other two treatments. In order to see what happens with the microspores during the first days of culturing two brocoletto accessions were monitored together with *B. napus* as control. One accession was chosen that (BRO-103) regenerated many embryos in a previous isolation and the second accession (BRO-25) had a poor response in earlier isolations.

A sample of 100 µl was pipetted out of the plates which should be representive for the present microspores in that plate culture without damaging or infecting the microspores in the petri dish. The third day after isolation no samples were taken.

The results of the monitoring are shown in the tables 6 to 8 and figures 15 to 18. Table 6 lists the observed development for while tables 7 and 8 do the same for the accessions BRO-103 and BRO-025. In these tables a description is given that summarizes the observed cultures. No actual counting took place but an estimation in percentages was given when possible. Pictures that represent the observed development are shown in appendix 4 and a selection is present in figure 15.

Table 6: Observations during the first 8 days of microspore culture of cultured in NLN17 for the different and the mean number of embryos observed after 14 and 24 days for each treatment.

Day	Control (NLN17)	Refresh (NLN17>NLN17)	Drop (NLN17>NLN10)
0	Mid/late uni-nucleate 20%/80%		
1	Mid/Late/Bi nucleate 5%/90%/5% 1 symmetrical division	Late/bi nucleate 60%/40% Few symmetrical divisions	Late/bi nucleate 40%/60% some symmetrical divisions
2	30% symmetrical division 5 % 2nd division(4 cells)	30% symmetrical division 5 % 2nd division(4 cells)	40% symmetrical division 5 % 2nd division(4 cells)
3	No data collected	No data collected	No data collected
4	To low no. of cells A few uni-nucleate	To low no. of cells Few 2nd divisions	1 3rd division (8 nucleus) Few 2nd division
5	Symmetrical divisions Single 2nd and 3rd division	Many symmetrical divisions Some second divisions	To low no. of cells
6	One multi cellular structure	Few multi cellular structures	Several multicellular structures
7	Some 2nd divisions	Symmetrical divisions Some 2nd divisions	Many multi cellular structures Some 2nd and 3rd division
8	Tri nucleates 3rd divisions	Some 3rd divisions Few multi cellular structures	Many multi cellular structures Some suspensor formation
14	1embryo	2 embryos	~100 embryos
28	~100 embryos	~150 embryos	~350embryos

From table 6 it becomes clear that the *B. napus* microspores present at the start of the monitoring (day 0) were close to the optimal stage, late uni-nucleate with up to 20% bi-nucleate. Starting from day 2 on a difference can be seen in the development. At the first day minor variation in symmetrical divisions were observed. From day 2 the percentage of symmetrical divisions was higher when treated with a sucrose drop compared to the other two treatments but the percentage of second divisions was equal over the treatments. At day four and five not all collected samples could be observed well due to low number of microspores present in the sample. Samples collected at day 6 showed a clear increase in the number of multicellular structures in the sucrose reduction treatment (several) compared to the control (one) and refreshment treatment (a few). For the last days this difference in development remained present.

In figure 15 an overview is shown of different observed structures after 8 days for the sucrose reduction treatment of *B. napus*. This figure clearly illustrates than not all microspores present in a culture are embryogenic. Furthermore it shows that there is a large variation in development among the microspores present in the culture. Late uni nucleate (red arrow) and bi nucleate (white arrow) microspores are present as well as symmetrical divisions (white arrowhead), 2nd divisions (red arrowhead), 3rd divisions (green arrowhead) and multi cellular structures (blue arrowhead) are being observed in this sample.

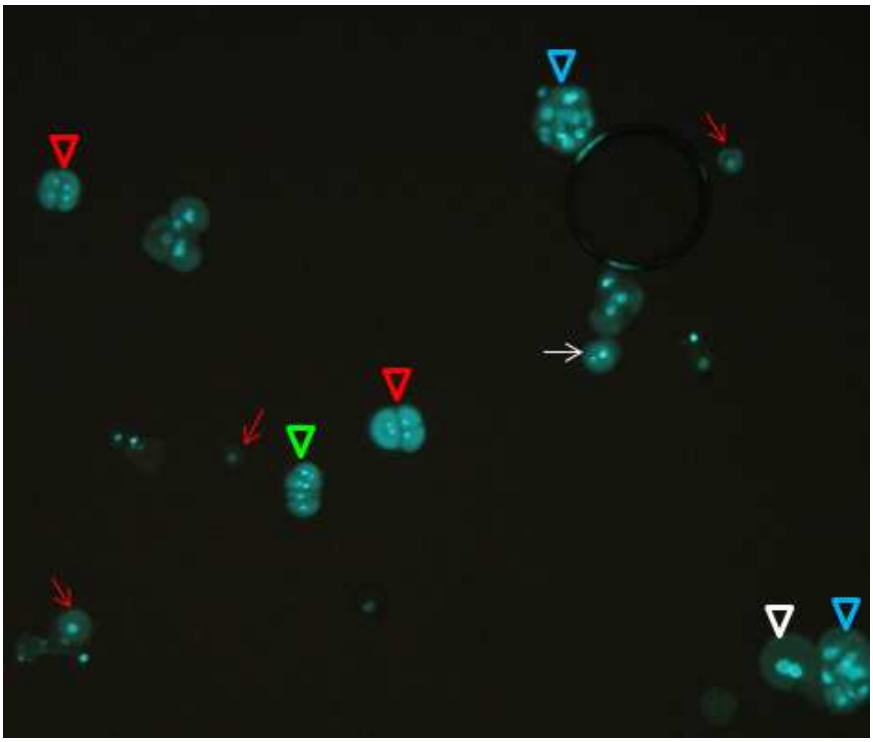


Figure 15: Overview of different stages of microspore development after 8 days of culturing treated with a sucrose reduction from NLN17. Present are:
Late uni nucleate (red arrow);bi-nucleate (white arrow) symmetrical divisions (white arrowhead),2nd divisions (red arrowhead), 3rd divisions (green arrowhead) and multicellular structures (blue arrowhead)

Table 7 illustrates that the microspores of accession BRO-103 present at the start of the monitoring (day 0) were at the optimal stage, late uni-nucleate with up to 20% bi-nucleate. Also for this accession a difference can be seen in the development from day 2; were a second divisions and some symmetrical divisions were observed in the sucrose treatment while in the refreshment treatment only a few symmetrical divisions were observed. Also many tri nucleate microspores were observed after the first two days of culturing in all treatments. Starting from day four "starch cells" were observed in the cultures of all treatments. Samples collected the last days contained hardly any visible cells and no multicellular structures were observed after 8 days as for *B. napus*.

The observations for accession BRO-025 are listed in table 8. The microspores were older than the optimal stage at the start of the monitoring (day 0). At day 1 a few symmetrical divisions were observed when treated with the sucrose drop which were not observed in the other two treatments at that day. These were observed at day 2 for the control treatment and at day 4 also for the refreshment treatment. From day four "starch cells" were observed in samples of the different treatments, similar to accession BRO-103. However these "starch cells" were not observed in samples of *B. napus*. At day 5 both the control treatment and the sucrose drop treatment show 2nd divisions. In samples from the last days no further embryogenic development was observed.

Table 7: Observations during the first 8 days of microspore culture of BRO-103 cultured in NLN17 for the different treatments and the mean number of embryos observed after 14 and 24 days for each treatment.

	pvBR020061 (BRO-103)		
Day	Control (NLN17)	Refresh (NLN17>NLN17)	Drop (NLN17>NLN10)
0	Late + bi nucleate 80%/20%		
1	Late/Bi/ tri nucleate 50%/45%/5%	Late/Bi/ tri nucleate 50%/45%/5%	Late/Bi/ tri nucleate 50%/45%/5% Few symmetrical divisions
2	Low no. of cells mainly tri nucleate	Mainly tri nucleate a few symmetrical divisions	Some symmetrical divisions 1 second division Starch cells
3	No data collected	No data collected	No data collected
4	No cells visible	Few symmetrical divisions Starch cells	1 second division Starch cells
5	"Starch cells"	No cells visible	Single 2nd division, "starch cells"
6	Single 2nd division	Some 3rd division (mcs)	No cells visible
7	Some old uni nucleate Many "Starch cells"	Few 3rd divisions Many tri nucleae and "Starch cells"	Some symmetrical and 2nd divisions
8	No cells visible	No cells visible	No cells visible
14	0 embryos	0 embryos 0	0 embryos
28	0 embryos	0 embryos	0 embryos

Table 8: Observations during the first 8 days of microspore culture of BRO-25 cultured in NLN17 for the different treatments and the mean number of embryos observed after 14 and 24 days for each treatment.

	pvBR020117 (BRO-025)		
Day	Control (NLN17)	Refresh (NLN17>NLN17)	Drop (NLN17>NLN10)
0	Late + bi nucleus 40%/60%		
1	Bi/ tri nucleate 99%/1%	Late/Bi/ tri nucleate 5%/90%/5%	Bi/ tri nucleate 80%/20% Few symmetrical divisions
2	Many tri nucleate Few symmetrical divisions	No cells visible	No cells visible
3	No data collected	No data collected	No data collected
4	Many tri nucleate Some "Starch cells"	Many tri nucleate Few symmetrical divisions	Many tri nucleate Few symmetrical divisions
5	Single 2nd division	No cells visible	Single 2nd division, "Starch cells"
6	Some "Starch cells"	Some "Starch cells"	Some "Starch cells"
7	Few symmetrical divisions	Few symmetrical divisions "Starch cells"	No cells visible
8	No cells visible	No cells visible	No cells visible
14	0 embryos	0 embryos	0 embryos
28	0 embryos	0 embryos	1 embryo

Two weeks after the start of the microspore cultures pictures were taken from all 3 accessions from all treatments. These are showed in figures 16 to 18 for respectively *B. napus*, BRO-103 and BRO-025. For *B. napus* (figure 16) you can see that after 14 days of culturing a mean number of embryos per plate of one and two were observed for the control and refreshment treatment (embryos indicated by arrow). This was significantly lower compared to the sucrose reduction treatment which yielded approximately 100 embryos per plate after 14 days of culturing. The other accessions BRO-103 and BRO-025 showed no response after 2 weeks of culturing (figure 17 and 18).

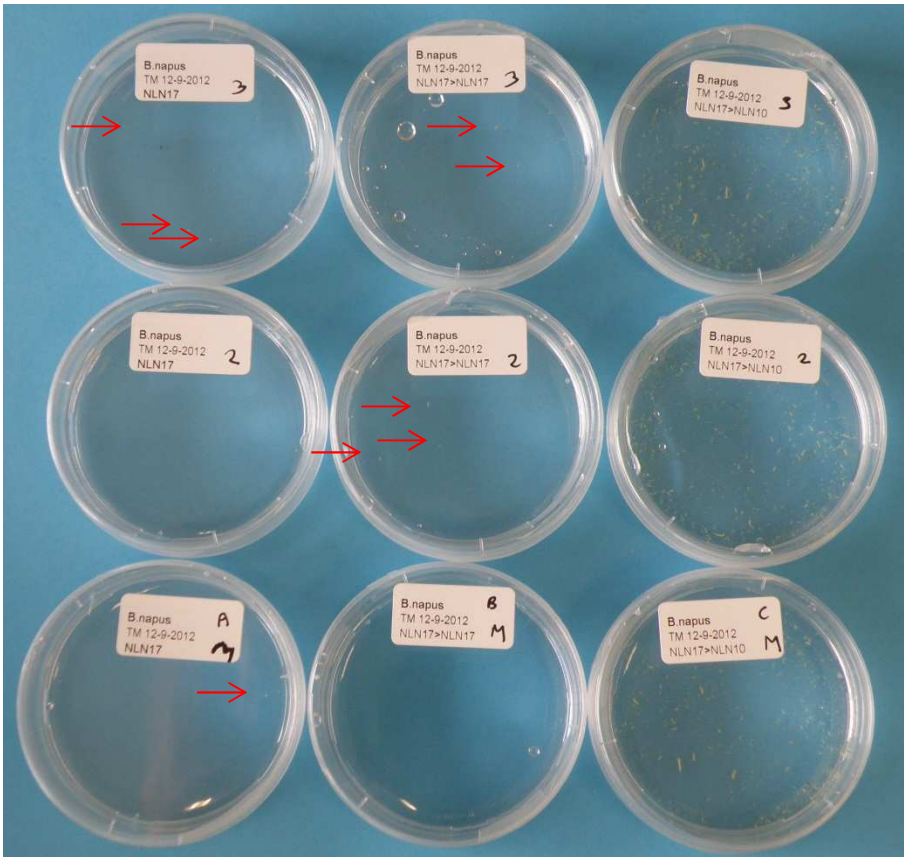


Figure 16: Observed *B. napus* microspore cultures after 2 weeks with different treatments; control (left), refreshment (middle) and sucrose drop (right). The arrow indicate the present embryos in the refreshment treatment and the control treatments. For the sucrose drop treatment many embryos are visible.

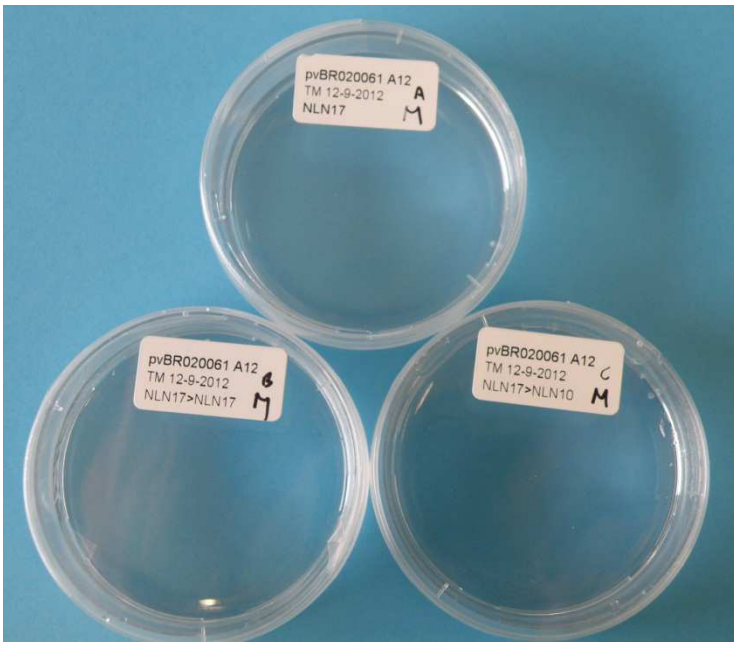


Figure 17: Observed BRO-103 microspore cultures after 2 weeks with different treatments; control (top), refreshment (left) and sucrose drop (right). No embryos were observed in any of the cultures.

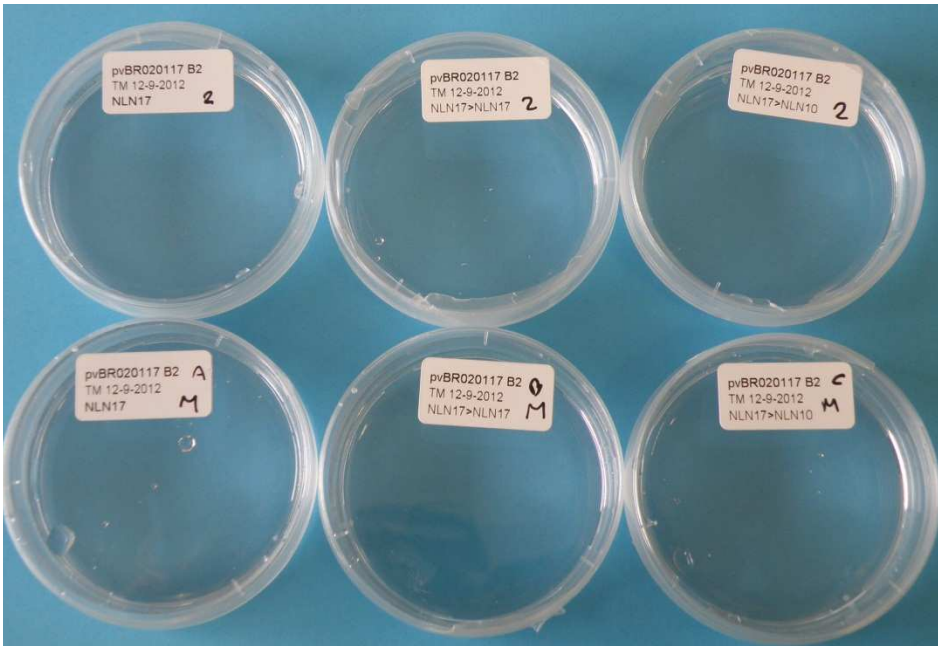


Figure 18: Observed BRO-025 microspore cultures after 2 weeks with different treatments; control (left), refreshment (middle) and sucrose drop (right). No embryos were observed in any of the cultures.

At the time of embryo harvest, after 28 days one embryo was observed for accession BRO-025 when treated with the sucrose drop treatment (see table 8). The other petri dishes of the broccoletto accessions remained empty. So is seems that the observed divisions in the control and refreshment treatment for accession BRO-025 and the divisions for accession BRO-103 did not result in embryos. It might be that the presence of "starch cells" is related to the development of the microspores and the final embryo production.
The amount of embryos produced by *B. napus* varied for the first time for the different treatments where the sucrose treatment yielded approximately twice as much embryos compared to the refreshment and control treatment (see table 3).

4 Discussion

In this chapter the observed results will be discussed in the same order as the results were presented. At the end relevant topics that were addressed before will be discussed.

4.1 Literature review

For the literature review papers were selected with the primary focus on *B. rapa* Doubled Haploid production. Often papers were found with cross references to other paper with protocols that were designed for other *Brassica* genera. These were also included in the overview table since *B. rapa* gave response to that protocol. Many papers show a minimal amount of information on the procedure to produce DH and many minor variations in for example plating density or heat-shock temperature were observed. This made it difficult to compare all protocols and convert the data into one ideal procedure for *B. rapa* microspore isolation. Therefore the key criteria were selected which resulted in the production or improvement of DH production and based on these parameters I designed my experiments and defined the standard treatment.

This standard treatment included a stress treatment at 32ºC and addition of 0.015% active charcoal. Both parameters were adjusted at the start of the experiments. The available incubator for the experiments was set at 33ºC instead of the proposed 32ºC. Temperature can be a critical factor for the switch to the sporophytic pathway (Custers, et al. 1994) and might result in less embryos. However the goal of this thesis was to come with a general protocol to produce DH for the brassica group and since this is the only available incubator, the protocol should work with this temperature.

Also active charcoal was left out of the protocol due to practical reasons even though experiments in the past and several publications (Gu, Zhou and Hagberg 2003), (Na, et al. 2009) and (Zhang, Wang, et al. 2012) show the improved embryo generation of addition of active charcoal. Addition of active charcoal could lead to improved embryogenesis embryo formation, growth and development, due to interference with secondary metabolites and other toxic compounds in the present in the media (Dias 2001) (Kott, et al. 1988).

4.2 Tested accessions

The major goal of this thesis was to optimize the existing microspore culture protocol in order to obtain embryos from all *B. rapa* accessions representing all the different morphotypes including recalcitrant lines. Therefor a selection was made based on available accessions, DH response of previous studies and data from earlier sowings about time to flower. The preferred DH response was low to moderate in order to see an increase or decrease in embryo production as an result of the applied test treatment.

Some of these accessions were not represented in this screen because seeds did not germinate (BRO-026 and OR216). For both accessions it was not possible to replace these accessions with another. BRO-026 was not replaced because there were no other broccoletto accessions available and OR-216 was not replaced because of the estimated time to flowering of the other accessions was too long. The other reason that some accessions are not represented was caused by the fact that these did not flower in time in order to isolate microspores (FT-097, NG215V, PC-Vr.930V and T1283V). All these four accession were selected because they gave a DH response in previous studies. Turnip lines (FT-097 and T1283V) flower very late, which can be more than 200 days which clearly explains the fact that they did not flower in time.

The other two accessions (NG215V, PC-Vr.930V) had an estimated flowering time between 40 and 120 days and therefor these lines were expected to flower. It might be that these accessions or even morphotypes flowering is more temperature/ daylight dependant like in cauliflower.

The fact that not all morphotypes are represented in this screen means that the results from this thesis cannot be implemented for all *B. rapa* subspecies. Never the less, from the 18 tested accessions representing 11 morphotypes, 17 respond in various levels to the used microspore culture protocol. This indicates that the microspores from most accessions are capable to be converted from gametophytic to a sporophytic pathway. The average response per accession is defined as mean measured over all isolations with different parameters like media and treatments. The effect of these parameters can be different for individual accessions and will be discussed later.

Another observation seen in embryo response was the variation of plant effect within an accession e.g. microspores were isolated from accession BRO-127 four times, three times plant A1 and one time from plant B1. The isolated microspores were at the same stage and exposed to the same treatments. However, only plant B1 gave a response. This so called plant effect can be explained by the origin of the used seed.
The seeds used to grow the donor-plants are derived from the original accessions which are heterogeneous and heterozygous. Therefore these plants will cause more genotype and plant effects compared to plants from DH1 seeds which are fixed and therefore with uniform progeny. Since DH1 seeds have already successfully undergone microspore culture selection there is the possibility that these plants respond better to the microspore culture protocol than unfixed accessions (only the responsive allelic combinations gave rise to DH plants).
When using a single plant or a few plants from the original accession, not only plant effect can influence the results of the experiments but also the natural variation present within the different seeds of the original accession will be reduced drastically. This can lead to the loss of certain interesting traits. For both reasons it would be better to use multiple plants of an accession to subject to microspore culture.
When doing so plants that give no response can be left out for analyses of specific treatment which could lead to a better indication of responsiveness of different morphotypes to microspore culture and the effect of applied treatments.
Furthermore, the Doubled Haploid plants that are produced will show more variation and therefore a better representative set of plant for that specific accession can be selected, which might lead to more variation when used in association mapping populations.

4.3 Stage of microspores

The stage of the cultured microspores is important for the success of the microspore culture (Touraev, Vicente and Heberle-Bors 1997) and the most optimal stage for *B. rapa* microspores is late uni-nucleate with up to 20 % bi-nucleate microspores (Keller, Rajhathy and Lacapra 1975) (Custers, et al. 1994) (Babbar, et al. 2004) (Shariatpanahi, et al. 2006). This optimal stage is covered in the stage groups 9-11. As figure 5 and table 4 show more than half of the number of plates contain microspores older than the optimal stage. This could be a cause why often low number of embryos are obtained as shown in figure 6. Kott et al (1988) mention that a relative high number of old microspores increases the level of produced negative growth factors which are inhibiting continuous embryo growth.

Despite that, there are embryos formed in the older stage groups. This can be partly explained by the fact that till stage group 19 some microspores at the late uni-nucleate stage are present. Custers et al (1994) also divided the bi-nucleate microspores in three classes, early, mid and late, of which the first is also responsive to microspore culture. This discrimination was not made in my observation. Both the presence of uni-nucleate microspores and young bi-nucleate microspores can explain the formation of embryos in the older microspore stage groups.

Furthermore, Binarova et al (1997) mention that microspores that are to old still can be converted in to the sporophytic pathway when the brassica pollen, which already contain starch grains, are treated by a precisely timed strong heat shock.

The reason of this large spread of microspore developmental stages might be explained by the selection procedure. Buds are selected at approximately 2.9 mm which should cover the optimal developmental stage of microspores for all tested accessions, based on previous stage determination experiments. Therefore the selection of the buds is indirect and based on assumptions, since the actual stage is determined after the isolation. Factors like plant developmental stage, age and stress are circumstances that can influence the stage of the microspores in the buds of the selected size. Therefore microspores can be older or the range of microspores stage can show more variation than expected. In order to prevent the large variation in microspore stage present in the culture a direct method could be applied. For this the stage of the microspores is determined before the actual bud harvest. Buds from specific sizes need to be selected at random from each plant or even more specific from each inflorescence stalk depending on the available amount of buds. The selected buds are squashed in dapi and stage can be determined under an UV microscope. Than buds corresponding to the correct stage(s) can be harvested.

4.4 Culture media

The effect of the different sucrose levels in the culture media NLN, and more specific the reduction of the sucrose level, was the major parameter of this thesis project. The sucrose level was reduced from a high level (17%) to a low level of sucrose (10 %) However, in the current protocol NLN13 was used as culture media. In order to exclude a negative effect of the increase of sucrose in the culture media accessions were continuously cultured with both NLN13 and NLN17. The results showed that there was no differences in these treatments with the exception of two accessions, BRO-029 and WO-080. The large variation between the two media can be explained by the fact that different donor-plants were used for the isolations with the different media.
In order to exclude donor plant effects between treatments the number of harvested buds could be split into batches or the pellet could be split before the last washing step. However the number of available buds was often limiting which resulted in a low number of plates per parameter and therefore one type of culture media was used at a time.

The effect of the three different treatments was tested with both culture media because the results were not available at the time the experiments should be conducted. Therefore the data of both media were combined in order to see the effect of the treatments per accession. The variation observed in figure 9, where the number of petri-dishes per treatment per accession is shown can be explained by the fact that some accessions were not cultured under all three different conditions. Mostly this was due to a low amount of microspores resulting in only a single petri-dish, which was than treated as control. For other isolations often it was difficult to divide the petri-dishes equally over the number of treatments. In those cases the refreshment treatment was often left out.

The observation from figure 10 that accessions with a moderate to high number of embryos produced in the control treatment show less increase in embryos in the other treatments can be explained by the fact that these accessions are already responsive to microspore culture and that the sucrose drop and /or refreshment treatments have less effect on the total embryo production. Embryos were counted 4 to 6 weeks after microspore isolation. Monitoring of the cultures after 14 days of initiation shown (figures 12 and 13) that there is a clear difference between the sucrose drop and the other treatments but that this difference disappear in time for well responding lines. These results are comparable to the observations in studies of Baillie et al (1992), Ferrie and Keller (1995) and Gu et al (2003).

The other interesting observation was the strong increase in embryo response for some accession which have a low embryo response in the control treatment. From the experimental data it was not clear that this increase was related to the sucrose drop, or due to the refreshment of the culture. Only for accession BRO-103 the data clearly showed that this was due to the sucrose drop treatment. For the other accessions it could be the case that the refreshment of the treatment which occurs simultaneously with the sucrose drop was sufficient to get rid of toxic elements released from the older bi-nucleate microspores, resulting in normal embryo initiation and development as observed by Kott et al.(1988).
However, the picture in figure 14 shows more developed embryos for the sucrose drop treatment compared to the control treatment which in in line with the observations of Dunwell and Thurling (1985) where they discovered that a high level of sucrose in beneficial for the initial microspore survival and divisions but a lower level is required for the continuation of this division.

As figure 11 shown a large difference was present between both the sucrose drop and refreshment treatment performed with NLN13 as starting culture media compared to the same treatments performed with NLN17 as starting culture media. This outcome was in contrast to the results of the control treatments which showed a comparable response in embryo production between both media.
It has to be noticed that the data were combined over all isolations. Therefore variation due to plant effects can have a large influence on these results since not all plants were isolated with both media for the comparable treatments. Due to this reason it is very likely that NLN17 could have resulted in a similar response as the NLN13 media. Especially when taking in consideration that a medium containing 17% sucrose generates an osmotic potential similar to that of the anther homogenate as discovered by Dunwell and Thurling (1985).
The fact that there is no difference between the sucrose drop and the refreshment treatment seen when cultured in NLN13 can indicate that the increase in the total numbers of embryos compared to the control treatment are an effect of the refreshment of the microspore cultures rather than the sucrose drop. However, the development and the time of flight of the embryos when treated with a sucrose drop show that sucrose reduction does affect embryogenesis.

4.5 Monitoring

From the refreshment and sucrose reduction treatments it was seen that both could lead to an increased number of embryos per petri dish when compared to the control treatment. Also it was seen that after two weeks of culturing a sucrose reduction showed more visible embryos compared to the refreshment treatment. However, during both treatments the media is refreshed and therefor it is not proven that the observed results in the sucrose reduction experiment were a result of the sucrose reduction alone. To monitor development of the microspores during the first days of culturing two broccoletto accessions and *B. napus* were monitored.

The results showed that after 1 day of culturing, which is still during the stress treatment, the first symmetrical divisions were already visible. These observations were in line with a publication of Aionesei et al (2005) who describes that about 8h of in vitro culture at 32°C is sufficient to induce sporophytic development.

Surprisingly, some variation in observed microspore development is already present between the three different treatments of all three tested accessions day 1. At this point the microspores were divided over the different plates for the varying treatment but the actual treatment did not take place at time of sampling. So the differences were not all related to treatments but presented the general variation among plates.
Sampling occurred though gentle mixture by pipetting without disturbing too much in order to get an representative sample. Rough mixture creates the cultures strong forces to the microspores which kills the microspores. Apparently the samples were not always representative for the culture resulting in observed variations in development. To gently mixing also explains the fact that sometimes hardly or no embryos were observed at certain days since they tend to stick at the surface of the plates.

Even though variation in sampling was present clear variations were observed between the different treatments, especially for *B. napus*. The sucrose reduction resulted in earlier development microspores at higher numbers compared to the other treatments for the tested accessions. The observed development is in line with the observed studies of Baillie et al (1992), Ferrie and Keller (1995) and Gu et al (2003). However all applied the sucrose reduction 48 hours after isolation of the microspores while the results reported here were observed from treatments 24 hours after microspore isolation.
It might be that applying the treatments 48 hours after isolation could have and enhanced effect on the embryogenesis of the tested accessions.

Although some symmetrical and even 2nd divisions were observed for the 2 broccoletto accession only one embryo was obtained. A clear explanation cannot be found for this aborted development. Aionesei et al (2005) report that in all systems studied the majority of the dividing microspores do not develop into embryos but stop dividing and abort. It might be that the presence of "starch cells" are an indication of or stimulate abortion of the development. In tobacco microspore culture microspores are able to continue the sporophytic pathway after symmetrical divisions were observed resulting in starch formation inside the pollen (Touraev, et al. 1995). For this reason the cell were scored as "starch cells". However, starch accumulation in older microspores was never observed for Brassica microspore culture.
An more plausible explanation is that these "starch cells" are a kind of syncytium. Aionesei et al (2005) describe that microspores can undergo different pathways and that in one (B pathway), the microspore nucleus divides into two vegetative-type nuclei without subsequent cytokinesis. This may result in a syncytium of up to 30 nuclei, which may or may not cellularize at various numbers of nuclei. Non-cellularizing syncytia die.
The formation of these syncytia correspond to the observations for the monitored broccoletto accessions which showed some symmetrical divisions but no embryo formation.

4.6 General remarks

Besides the factors discussed above another critical factor for the success of microspore cultures is the viability of the microspores. Viability can have a large effect on the embryo response of the culture and a high amount of death cells will produce additional toxic compounds in the culture (Custers, et al. 1994). During the development of the plant and the process of isolation several factors can influence the viability of the microspores. In example plants were sprayed against aphids in the climate cell which will have an effect on the quality and the amount of available microspores. Also the grinding of the buds and the washing steps in the protocol contain several handlings that can damage the microspores. Samples were not tested for their viability but it might be that some cultures did not respond due to the lack of viable microspores.

Although the focus of this thesis was on protocol development to induce embryos, continuation of the development of the embryos was also noticed. The majority of the produced embryos remained brownish and ceased development when transferred to solid media. Part of this observation can be explained by the fact that embryos were transferred relatively late (after 4 to 6 weeks) to solid media due to time. However also fine looking embryos (yellow/green) turned brown after transferring to solid media. Therefor it is critical to improve the process of embryo harvest and acclimation to come to an optimal protocol for Doubled Haploid production.

5 Conclusion and Recommendations

In this last chapter the conclusions of this thesis research are given and some recommendations for the improvements of the Doubled Haploid production protocol for *B. rapa*.

5.1 Conclusions

Based on the results of this thesis research it can be concluded that:

A reduction of sucrose in the culture media from 17% or 13% to 10% leads to an increase in the number of embryos produced and reduces the time before the embryos become visible in the microspore culture for the tested accessions. Divisions occur earlier and more frequent when a reduction of sucrose, from NLN17 to NLN10, in the culture media takes places.

Most morphotypes are responsive to microspore culture, 17 out of 18 accessions representing 11 out of 15 morphotypes resulted in one or more embryos, although variation in response between different accessions can be large.

The stage of the isolated microspores are likely having an effect on the embryo response of the culture and should contain no more that 30% bi-nucleates.

5.2 Recommendations.

In order to produce Doubled Haploid plants I would recommend to use or investigate the following steps:

Use at least 10 different plants from each original accession to observe plant effects and benefit more from the variation present within an accession.

Determine the stage of the microspores before the actual isolation in order to narrow the variation of isolated microspores in the cultures.

A NLN culture medium with 17% or 13 % sucrose. The optimal for each accession should be explored further.

Add active charcoal (0.015%) to the cultures to remove toxic compounds produced by older and no vital microspores.

Apply an sucrose reduction after 24 hours by refreshing the culture media with NLN10. The timing of this sucrose reduction can be postponed with another 24 hours.

Harvest of the embryos should be earlier, preferably 3 weeks after isolation. Ideally, microspores are placed on a shaker under dim light conditions after they become visible.

Further investigation on embryo harvest and acclimation of the microspores.

References.

Agarwal, Pradeep K., Parinita Agarwal, Jan B. M. Custers, Chun-ming Liu, and S. S. Bhojwani. "PCIB an antiauxin enhances microspore embryogenesis in microspore culture of Brassica juncea." *Plant Cell Tiss Organ Cult* 86 (2006): 201-210.

Aionesei, Tatiana, Alisher Touraev, and Erwin Heberle-Bors. *Pathways to Microspore Embryogenesis.* Vol. 56, in *Haploids in Crop Improvement II*, by Palmer, W.A. Keller and K.J. Kasha, 11-34. Berlin: Springer-Verlag, 2005.

Ajisaka, Hidetoshi, Yasuhisa Kuginuki, Motokazu Shiratori, Keizo Ishiguro, Sueo Enomoto, and Masashi Hitai. "Mapping of loci affecting the cultural efficiency of microspore culture of Brassica rapa L. syn campestrus L. using DNA polumorphism." *Breeding Science* 49 (1999): 187-192.

Babbar, Shasjo B., Pradeep K. Agarwal, Shalini Sahay, and Sant S. Bhojwani. "Isolated microspore culture of Brassica: An experimental tool for developmental studies and crop improvement." *Indian Journal of Biotechnology* 3 (2004): 185-202.

Baillie, A.M.R., D.J. Epp, D. Hutcheson, and W. A. Keller. "In vitro culture of isolated microspores and regeneration of plants in Brassica campestris." *Plant Cell Reports* 11 (1992): 234-237.

Bedinger, Patricia. "The Remarkable Biology of Pollen." *The Plant Cell* 4 (1992): 879-887.

Binarova, Pavla, Gerd Hause, Cenklová Vera, H.G. Cordewener Jan, Lookeren Campagne Michiel M. van, and Pavla Binavora. "A short severe heat shock is required to induce embryogenesis in late bicellular pollen of Brassica napus L." *Sex Plant Reprod* 10 (1997): 200-208.

Burnett, Laurie, Stephen Yarrow, and Bin Huang. "Embryogenesis and plant regeneration from isolated microspores of Brassica rapa L. ssp. Oleifera." *Plant Cell Reports* 11 (1992): 215-218.

Cao, Ming Qing, Yan Li, Fan Liu, and Claire Doré. "Embryogenesis and plant regeneration of pakchoi (Brassica rapa L. ssp. chinensis) via in vitro isolated microspore culture." *Plant Cell Reports* 13 (1994): 447-450.

Custers, Jan B.M., Jan H. G. Cordewener, Yvonne Niillen, Hans J.M. Dons, and Michiel M. Van Lookeren Campagne. "Temperature controls both gametophytic and sporophytic development in microspore cultures of Brassica napus." *Plant Cell Reports* 13 (1994): 267-271.

Dias, João Carlos da Silva. "Effect of incubation temperature regimes and culture medium on broccoli microspore culture embryogenesis." *Euphytica*, no. 109 (2001): 389-394.

FAO, Online Statistics. *faostat.fao.org.* 2012. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor (accessed march 8, 2012).

Ferrie, A. M. R., D.J. Epp, and W.A. Keller. "Evaluation of Brassica rapa L. genotypes for microspore culture response and identification of a highly embryogenic line." *Plant Cell Reports* 14 (1995): 580-584.

Ferrie, A. M. R., J. Dirpaul, P. Krishna, J. Krochko, and W. A. Keller. "Effects of Brassinosteroids on Microspore Embryogenesis in Brassica Species." *In Vitro Cellular & Developmental Biology. Plant,* 41, no. 6 (2005): 742-745.

Ferrie, Alison M. R., and Christian Möllers. "Haploids and doubled haploids in Brassica spp. for genetic and genomic research." *Plant Cell Tiss Organ Cult* 104 (2011): 375-386.

Friedt, Wolfgang, and M. Karim Zarhloul. *Haploids in the Improvement of Crucifers.* Vol. 56, in *Haploids in Crop Improvement II*, by C.E. Palmer, W.A. Keller and and K.J. Kasha, 191-213. Berlin: Springer-Verslag, 2005.

Germana, Maria Antonietta. "Gametic embryogenesis and haploid technology as valuable support to plant breeding." *Plant Cell Rep*, 2011: 839-857.

Gil-Humanes, J., and F. Barro. "Production of Doubled Haploids in Brassica." In *Advances in Haploid Production in Higher Plants*, by A. Touraev et al., 65-73. Springer Science + Business Media B.V., 2009.

Gu, H.H., W.J. Zhou, and P. Hagberg. "High frequency spontaneous production of doubled haploid plants in microspore cultures of Brassica rapa ssp. chinensis." *Euphytica* 134 (2003): 239-245.

Guo, Yang-Dong, and Seppo Pulli. "High-frequency embryogenesis in Brassica campestris microspore culture." *Plant Cell, Tissue and Organ Culture* 46 (1996): 219-225.

Keller, W.A., T. Rajhathy, and J. Lacapra. "IN VITRO PRODUCTION OF PLANTS FROM POLLEN IN BRASSICA CAMPESTRIS." *Can. J. Genet. Cytol* 17 (1975): 655-666.

Kott, L.S., L. Polsoni, B. Ellis, and W. D. Beversdorf. "Autotoxicity in isolated microspore cultures of Brassica napus." *Can. J. Bot.* 66 (1988): 1665-1670.

Lionneton, E., W. Beuret, C. Delaitre, S. Ochatt, and M. Rancillac. "Improved microspore culture and doubled-haploid plant regeneration in the brown condiment mustard (Brassica juncea)." *Plant Cell Reports* 20 (2001): 126-130.

Lou, Ping, et al. "Quantitative Trait Loci for Glucosinolate Accumulation in Brassica Rapa Leaves." *New Phytologist* 179, no. 4 (2008): 1017-1032.

Na, Haeyoung, Suhyoung Park, Guiyoung Hwanh, Moo-Kyoung Yoon, and Changhoo Chun. "Medium, AgNO3, activated Carcoal and NAA effects on microspore culture in Brassica rapa." *Kor. J. Hort. Sci. Technol* 24, no. 4 (2009): 657-661.

Nishioka, Miki, et al. "Mapping of QTLs for bolting time in Brassica rapa (syn. campestris) under different enviromental conditions." *Breeding Science* 55 (2005): 127-133.

Parihar, Dwarkesh S., and Sanjit Aradhye. "Microspore Culture for Induction of Doubled Haploidy in B.juncea and B.rapa." *10th International Rapeseed Congress.* Canberra, 1999.

Rakow, G. *Species Origin and Economic Importance of Brassica.* Vol. 54, in *Biotechnology in agriculture and Forestry*, by E.C. Pua and C.J. Douglas. Berlin: Springer-Verslag, 2004.

Rodríguez-Serrano, María, Ivett Bárány, Deepak Prem, María-José Coronado, and María C. Risuenõ and Pilar S. Testillano. "NO, ROS, and cell death associated with caspase-like activity increase in stress-induced microspore embryogenesis of barley." *Journal of Experimental Botany*, 2011: 1-18.

Sato, Seike, Norio Katoh, Sumio Iwai, and Manabu Hagimori. "Effect of low temperature pretreatment of buds or inflorescence on isolated microspore culture in Brassica rapa (syn. B. campestris)." *Breeding Science* 52 (2002): 23-26.

Sato, Takanori, Takeshi Nishio, and Masashi Hirai. "Plant regeneration from isolated microspore cultures of Chinese cabbage (Brassica campestris spp. pekinensis)." *Plant Cell Reports* 8 (1989): 486-488.

Shariatpanahi, Mehran E., Ugur Bala, Erwin Heberle-Bors, and Alisher Touraev. "Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis." *Physiologia Plantarum* 127 (2006): 519–534.

Simmonds, Daina H., and Wilfred A. Keller. "Significance of preprophase bands of microtubules in the induction of microspore embryogenesis of Brassica napus." *Planta* 208 (1999): 383-391.

Takuno, Shohei, Taihachi Kawahara, and Ohmi Ohnishi. "Phylogenetic relationships among cultivated types of Brassica rapa L. em. Metzg. as revealed by AFLP analysis." *Genetic Resources and Crop Evolution* 54 (2007): 279-285.

Touraev, A., A. Indrianto, O. Vicente, and E. Heberle-Bors. "Efficient microspore embryogenesis in wheat (Triticum aestivum L.) induced by starvation at high temperature." *Sex Plant Reprod* 9 (1996): 209-215.

Touraev, Alisher, Oscar Vicente, and Erwin Heberle-Bors. "Initiation of microspore embryogenesis by stress." *Trends in plant science* 2, no. 8 (1997): 297-302.

Touraev, Lezin, Heberle-Bors, and Vicente. "Maintenance of gametophytic development after symmetrical division in tobacco microspore culture." *Sex Plant Reprod* 8 (1995): 70-76.

Twell, David, Soon Ki Park, and Eric Lalanne. "Asymmetric division and cell-fate determination in developing pollen." *Trends in plant science* 3, no. 8 (1998): 305-310.

U N. "Genome analysis in Brassica with special reference to the experimental formation of B.napus and peculiar mode of fertilization." *Japanes Journal of Botany* 7 (1935): 389-452.

Wang, Taotao, HanXia Li, Junhong Zhang, Bo Ouyang, Yongen Lu, and Zhibiao Ye. "Initiation and development of microspore embryogenesis in recalcitrant purple flowering stalk (Brassica campestris ssp. chinensis var. purpurea Hort.) genotypes." *Scientia Horticulturae* 121 (2009): 419–424.

Wong, R. S. C., S.Y. Zee, and E.B. Swanson. "Isolated microspore culture of Chinese flowering cabbage (Brassica campestris ssp. parachinensis)." *Plant Cell Reports* 15 (1996): 396-400.

Zaki, M.A.M., and H.G. Dickinson. "Microspore-derived embryos in Brassica: the significance of division symmetry in pollen mitosis I to embryogenic development." *Sex Plant Reprod* 4 (1991): 48-55.

Zhang, F.L., and Y. Takahata. "Inheritance of microspore embryogenic ability in Brassica crops." *Theor Appl Genet* 103 (2001): 254-258.

Zhang, Yun, AiJie Wang, Yang Liu, YuShu Wang, and Hui Feng. "Effects of the antiauxin PCIB on microspore embryogenesis and plant regeneration in Brassica rapa." *Scientia Horticulturae* 130 (2011): 32-37.

Zhang, Yun, AiJie Wang, Yang Liu, Yushu Wang, and Hui Feng. "Improved production of doubled haploids in Brassica rapa through microspore culture." *Plant Breeding* 131 (2012): 164-169.

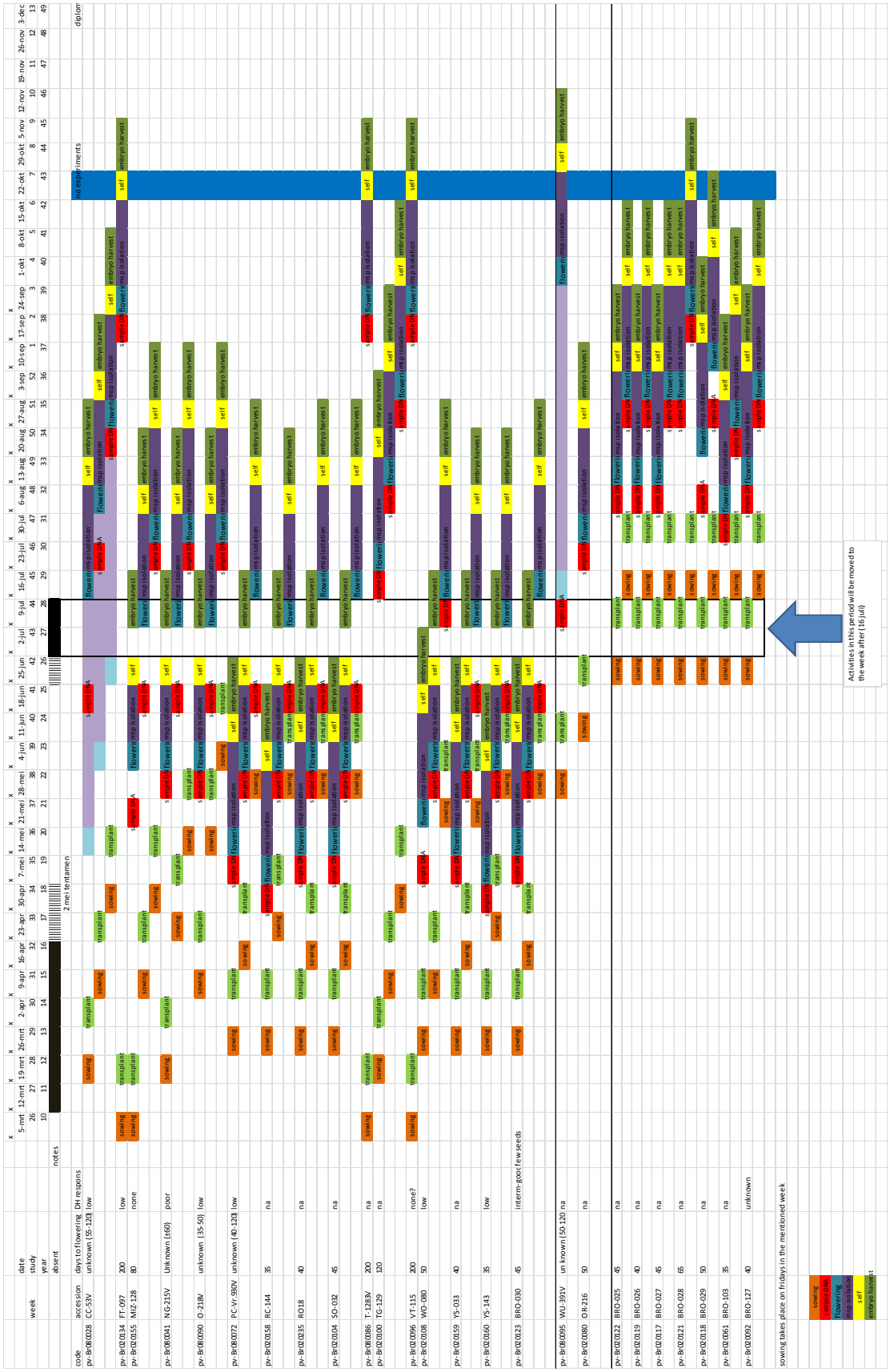
Zhao, Jianjun, et al. "Design of a Brassica rapa core collection for association mapping studies." *Genome* 53 (2010): 884-898.

Zhao, Jianjun, et al. "Genetic relationships within Brassica rapa as inferred from AFLP fingerprints." *Theor Appl Genet*, 2005: 1301–1314.

Zhao, Jianjun, Vani Kulkarni, Nini Liu, Dunia Pino Del Carpio, Johan Bucher, and Guusje Bonnema. "BrFLC2 (FLOWERING LOCUS C) as a candidate gene for a vernalization response QTL in Brassica rapa." *Journal of Experimental Botany* 61, no. 6 (2010): 1817-1825.

Appendix

Appendix 1: Overview of work scheme



Appendix 2: Overview of used media

Ingredients of the NLN medium

Component	Ingredient	Stock preparation				Final medium conc. (mg/l)
		Stock No. (strength)	Amount (mg or g)	Total volume (ml)	Stored aliquots (ml) [x]	
Macro-elements	KNO_3	1 (20x)	2.5 g	1,000	50	125
	$Ca(NO_3)_2.4H_2O$		10 g			500
	$MgSO_4.\ 7H_2O$		2.5 g			125
	KH_2PO_4		2.5 g			125
Micro-elements	$MnSO_4.\ 4H_2O$	2 (200x)	1250 mg	250	5	25
	$ZnSO_4.\ 7H_2O$		500 mg			10
	H_3BO_3		500 mg			10
	$Na_2MoO_4.$		12.5 mg			0.25
	$CuSO_4.\ 5H_2O$		1.25 mg[xx]			0.025
	$CoCl_2.\ 6H_2O$		1.25 mg[xx]			0.025
Iron source	NaFe(III)EDTA	3 (200x)	2 g	250	5	40
Organic components 1	Nicotinic acid	4 (1000x)	500 mg	100	1	5
	Thiamine HCl		50 mg			0.5
	Pyridoxine HCl		50 mg			0.5
	Folic acid		50 mg			0.5
	Biotin		5 mg			0.05
	Glycin		200 mg			2
Organic components 2	L-Serine	5 (50x)	2.5 g	500	20	100
	L-Glutamine		20 g			800
	Glutathion		0.75 g			30
	Myo-inositiol		2.5 g			100
Carbon	Sucrose	NLN13			130 g	130,000
		NLN17			170 g	170,000
		NLN10			100 g	100,000

[x] Aliquots to be combined to achieve the correct medium composition, for 1 litre. Set the pH at 5.8 and sterilize with a filter.

[x x] For convenience, first dissolve both these micro-elements at 10x higher strength

Appendix 3: Data collection table

At the next pages the table is shown will all recorded parameters for microspore isolations.

Accession nr

Plant used for isolation

Number of plates after isolation

Isolation date

Plating density in msp/ml (3 ml per plate)

Stage group

Observed stage

Media used

Treatment applied to that plate

Number of embryos observed

Marking table for the exact media in each plate

First count /harvest date

Number of embryos transferred to solid media

Notes

Second count /harvest date

Number of embryos transferred to vessels solid media

Transfer date

Notes

accession nr	plant	plate no	isolation date	plating density (msp/ml)	stage group	stage	Media	treatment	# embryos	NLN17	NLN17> NLN17	NLN17 > NLN10	NLN13	NLN13> NLNL13	NLN13 > NLN10	NLN10	count/ harvest date 1	transferred embryos	notes	count/ harvest date 2	trans to vessel #	transfer date	note
B. napus			22-mei	17000	9	L 100	13	Control	>100				x				19-jul	x		24-aug			
B. napus		1	30-mei	11250	19	B 100	13	Control	>100				x				19-jul		till globular /hart shape stage	24-aug			
B. napus		2	30-mei	10000	19	B 100	13	Control	>100				x				19-jul		till globular /hart shape stage	24-aug			
B. napus		1	6-jun	9583	16	L/B 30/70	13	Control	>100				x				19-jul	x		24-aug			
B. napus		1	6-jun	6666	16	L/B 30/70	17	Control	>100	x							19-jul	x		24-aug			
B. napus		1	7-jun	20833	19	B 100	13	Control					x				19-jul		contaminated	24-aug			
B. napus		1	7-jun	9583	19	B 100	17	Control	5	x							19-jul			24-aug			
B. napus		1	13-jun	15000	5	M/L 40/60	17	Control	>100	x							19-jul	10		24-aug	5		
B. napus		1	20-jun	19166	6	M/L 30/70	17	Control	19	x							19-jul			24-aug			
B. napus		1	20-jun	19166	6	M/L 30/70	17	Drop	64			x					19-jul		appears larger than non refreshed	24-aug			
B. napus		1	25-jun	15208	4	M/L 50/50	13	Drop	>100						x		19-jul	10		24-aug	5		
B. napus		1	25-jun	15208	4	M/L 50/50	13	Refresh	>100					x			19-jul			24-aug			
B. napus		1	25-jul	19583	6	M/L 30/70	17	Control	>100	x							24-aug						
B. napus		1	26-jul	17291	7	M/L 20/80	13	Control	>100				x				24-aug						
B. napus		2	26-jul	17291	7	M/L 20/80	13	Control	>100				x				24-aug						
B. napus		1	26-jul	17291	7	M/L 20/80	13	Drop	>100						x		24-aug						
B. napus		2	26-jul	17291	7	M/L 20/80	13	Drop	>100						x		24-aug						
B. napus		1	26-jul	17291	7	M/L 20/80	13	Refresh	>100					x			24-aug						
B. napus		2	26-jul	17291	7	M/L 20/80	13	Refresh	>100					x			24-aug						
B. napus		1	1-aug	21041	6	M/L 30/70	13	Control	>100				x				24-aug						
B. napus		2	1-aug	21041	6	M/L 30/70	13	Drop	>100						x		24-aug						
B. napus		1	2-aug	15694	7	M/L 20/80	17	Control	>100	x							24-aug						
B. napus		1	2-aug	15694	7	M/L 20/80	17	Drop	>100			x					24-aug		more then NLN17> NLN17				
B. napus		1	2-aug	15694	7	M/L 20/80	17	Refresh	>100		x						24-aug		more then NLN17				
B. napus		1	6-aug	21994	5	M/L 40/60	17	Control	4	x							24-aug						
B. napus		1	6-aug	21994	5	M/L 40/60	17	Drop	>200			x					24-aug						
B. napus		1	6-aug	21994	5	M/L 40/60	17	Refresh	~70		x						24-aug						
B. napus		1	15-aug	10833	18	L/B/T 10/80/10	13	Control	100				x				10-okt						
B. napus		1	16-aug	15833	14	L/B 50/50	17	Control	100	x							10-okt					7-sep	
B. napus		1	22-aug	20000	5	M/L 40/60	10		0							x	10-okt						
B. napus		2	22-aug	20000	5	M/L 40/60	10		0							x	10-okt						
B. napus		3	22-aug	20000	5	M/L 40/60	10		0							x	10-okt						
B. napus		1	29-aug	20500	5	M/L 40/60	17	Control	63	x							10-okt						
B. napus		1	29-aug	20500	5	M/L 40/60	17	Drop	46			x					10-okt		larger				
B. napus		2	29-aug	20500	5	M/L 40/60	17	Drop	35			x					10-okt		larger				
B. napus		1	29-aug	20500	5	M/L 40/60	17	Refresh	100		x						10-okt						
B. napus		2	29-aug	20500	5	M/L 40/60	17	Refresh	100		x						10-okt						
B. napus		1	5-sep	19167	5	M/L	17	Control	100	x							10-okt						
B. napus		1	5-sep	19167	5	M/L	17	Drop	100			x					10-okt						
B. napus		2	12-sep	20231	7	M/L 20/80	17	Control	0	x							10-okt		vapored				
B. napus		3	12-sep	20231	7	M/L 20/80	17	Control	200	x							10-okt						
B. napus		M	12-sep	20231	7	M/L 20/80	17	Control	0	x							10-okt						
B. napus		2	12-sep	20231	7	M/L 20/80	17	Drop	400			x					10-okt						

B. napus		3	12-sep	20231	7	M/L 20/80	17	Drop	400			x					10-okt						
B. napus		M	12-sep	20231	7	M/L 20/80	17	Drop	300			x					10-okt						
B. napus		2	12-sep	20231	7	M/L 20/80	17	Refresh	150		x						10-okt						
B. napus		3	12-sep	20231	7	M/L 20/80	17	Refresh	150		x						10-okt						
B. napus		M	12-sep	20231	7	M/L 20/80	17	Refresh	150		x						10-okt						
B. napus		1	13-sep	20833	9	L 100	13	Control	50				x				10-okt						
B. napus		2	13-sep	20833	9	L 100	13	Control	50				x				10-okt					7-sep	
B. napus		1	13-sep	20833	9	L 100	13	Drop	10						x		10-okt		contaminated				
B. napus		2	13-sep	20833	9	L 100	13	Drop	70						x		10-okt		bigger				
B. napus		1	26-sep	22292	5	M/L 40/60	17	Control	45	x							17-okt						
B. napus		2	26-sep	22292	5	M/L 40/60	17	Control	60	x							17-okt						
B. napus		1	26-sep	22292	5	M/L 40/60	17	Drop	80			x					17-okt						
B. napus		2	26-sep	22292	5	M/L 40/60	17	Drop	80			x					17-okt						
pvBR020061	A1	1	6-aug	21458	10	L/B 95/5	17	Control	1	x							24-aug	1		17-okt			
pvBR020061	A1	2	6-aug	21458	10	L/B 95/5	17	Control	0	x							24-aug						
pvBR020061	A2	1	16-aug	16944	18	L/B 10/90	17	Control	0	x							10-okt						
pvBR020061	A1	1	16-aug	20417	6	M/L 30/70	17	Control	2	x							10-okt	2		17-okt			
pvBR020061	A1	2	16-aug	20417	6	M/L 30/70	17	Control	2	x							10-okt	2		17-okt			
pvBR020061	A2	1	16-aug	16944	18	L/B 10/90	17	Drop	0			x					10-okt						
pvBR020061	A1	1	16-aug	20417	6	M/L 30/70	17	Drop	13			x					10-okt	5		17-okt			
pvBR020061	A1	2	16-aug	20417	6	M/L 30/70	17	Drop	12			x					10-okt						
pvBR020061	A1	3	16-aug	20417	6	M/L 30/70	17	Drop	14			x					10-okt	5		17-okt			
pvBR020061	A2	1	16-aug	16944	18	L/B 10/90	17	Refresh	0		x						10-okt						
pvBR020061	A1+2	1	29-aug	19881	9	L 100	17	Control	1	x							10-okt	1		17-okt			
pvBR020061	A1+2	2	29-aug	19881	9	L 100	17	Control	1	x							10-okt	1		17-okt			
pvBR020061	A1+2	1	29-aug	19881	9	L 100	17	Drop	5			x					10-okt	5		17-okt			
pvBR020061	A1+2	2	29-aug	19881	9	L 100	17	Drop	7			x					10-okt	7		17-okt			
pvBR020061	A1+2	3	29-aug	19881	9	L 100	17	Drop	7			x					10-okt						
pvBR020061	A1+2	1	29-aug	19881	9	L 100	17	Refresh	1		x						10-okt	1		17-okt			
pvBR020061	A1+2	2	29-aug	19881	9	L 100	17	Refresh	3		x						10-okt	3		17-okt			
pvBR020061	A1+2	1	5-sep	19271	9	L 100	17	Control	0	x							10-okt						
pvBR020061	A1+2	2	5-sep	19271	9	L 100	17	Control	0	x							10-okt						
pvBR020061	A1+2	1	5-sep	19271	9	L 100	17	Drop	1			x					10-okt	1		17-okt			
pvBR020061	A1+2	2	5-sep	19271	9	L 100	17	Drop	1			x					10-okt	1		17-okt			
pvBR020061	A1+2	M	12-sep	16389	11	L/B 80/20	17	Control	0	x							10-okt						
pvBR020061	A1+2	M	12-sep	16389	11	L/B 80/20	17	Drop	0			x					10-okt						
pvBR020061	A1+2	M	12-sep	16389	11	L/B 80/20	17	Refresh	0		x						10-okt						
pvBR020061	A2	1	26-sep	20833	10	L/B 90/10	17	Control	0	x							17-okt						
pvBR020061	A2	1	26-sep	20833	10	L/B 90/10	17	Drop	0			x					17-okt						
pvBR020092	A1	1	29-aug	20093	12	L/B 70/30	17	Control	0	x							10-okt		contaminated				
pvBR020092	A1	2	29-aug	20093	12	L/B 70/30	17	Control	0	x							10-okt		contaminated				
pvBR020092	A1	3	29-aug	20093	12	L/B 70/30	17	Control	0	x							10-okt		contaminated				
pvBR020092	A1	1	29-aug	20093	12	L/B 70/30	17	Drop	0			x					10-okt		contaminated				
pvBR020092	A1	2	29-aug	20093	12	L/B 70/30	17	Drop	0			x					10-okt		contaminated				
pvBR020092	A1	3	29-aug	20093	12	L/B 70/30	17	Drop	0			x					10-okt		contaminated				
pvBR020092	A1	1	29-aug	20093	12	L/B 70/30	17	Refresh	0		x						10-okt		contaminated				
pvBR020092	A1	2	29-aug	20093	12	L/B 70/30	17	Refresh	0		x						10-okt		contaminated				
pvBR020092	A1	3	29-aug	20093	12	L/B 70/30	17	Refresh	0		x						10-okt		contaminated				
pvBR020092	A1	1	5-sep	20347	14	L/B 50/50	17	Control	0	x							10-okt						
pvBR020092	A1	2	5-sep	20347	14	L/B 50/50	17	Control	0	x							10-okt						

pvBR020092	A1	1	5-sep	20347	14	L/B 50/50	17	Drop	0			x					10-okt		contaminated				
pvBR020092	A1	2	5-sep	20347	14	L/B 50/50	17	Drop	0			x					10-okt		contaminated				
pvBR020092	A1	1	5-sep	20347	14	L/B 50/50	17	Refresh	0		x						10-okt		contaminated				
pvBR020092	A1	2	5-sep	20347	14	L/B 50/50	17	Refresh	0		x						10-okt		contaminated				
pvBR020092	B1	1	13-sep	21806	14	L/B 50/50	13	Control	0				x				10-okt						
pvBR020092	B1	2	13-sep	21806	14	L/B 50/50	13	Control	1				x				10-okt	1		17-okt			
pvBR020092	B1	1	13-sep	21806	14	L/B 50/50	13	Drop	1						x		10-okt		?				
pvBR020092	B1	2	13-sep	21806	14	L/B 50/50	13	Drop	9						x		10-okt	8		17-okt			
pvBR020092	B1	1	13-sep	21806	14	L/B 50/50	13	Refresh	1					x			10-okt		?				
pvBR020092	B1	2	13-sep	21806	14	L/B 50/50	13	Refresh	0					x			10-okt						
pvBR020092	A1	1	26-sep	20000	12	L/B 70/30	17	Control	0	x							17-okt						
pvBR020092	A1	2	26-sep	20000	12	L/B 70/30	17	Control	0	x							17-okt						
pvBR020092	A1	1	26-sep	20000	12	L/B 70/30	17	Drop	0			x					17-okt						
pvBR020092	A1	2	26-sep	20000	12	L/B 70/30	17	Drop	0			x					17-okt						
pvBR020092	A1	1	26-sep	20000	12	L/B 70/30	17	Refresh	0		x						17-okt						
pvBR020092	A1	2	26-sep	20000	12	L/B 70/30	17	Refresh	0		x						17-okt						
pvBR020096	A1	1	13-jun	19166	18	L/B 10/90	17	Control	1	x							19-jul	1	callus	24-aug	0		
pvBR020096	A1	2	13-jun	19166	18	L/B 10/90	17	Control	1	x							19-jul	1	callus	24-aug	0		
pvBR020096	A1	3	13-jun	19166	18	L/B 10/90	17	Control	1	x							19-jul	1	callus	24-aug	0		
pvBR020096	A1	1	25-jun	25416	3	M/L 60/40	13	Control	0				x				19-jul			24-aug			
pvBR020096	A2	1	25-jul	19895	14	L/B 50/50	17	Control	2	x							7-sep	2					
pvBR020096	A2	2	25-jul	19895	14	L/B 50/50	17	Control	5	x							24-aug	3		17-okt			
pvBR020096	A2	1	25-jul	19895	14	L/B 50/50	17	Drop	4			x					24-aug	2	larger than NLN17>NLN17	17-okt			
pvBR020096	A2	2	25-jul	19895	14	L/B 50/50	17	Drop	6			x					7-sep	8	and NLN17	17-okt			
pvBR020096	A2	3	25-jul	19895	14	L/B 50/50	17	Drop	5			x					24-aug	2		17-okt			
pvBR020096	A2	1	25-jul	19895	14	L/B 50/50	17	Refresh	2		x						7-sep	2					
pvBR020096	A2	2	25-jul	19895	14	L/B 50/50	17	Refresh	1		x						24-aug						
pvBR020096	A2	3	25-jul	19895	14	L/B 50/50	17	Refresh	0		x						24-aug						
pvBR020096	A1	1	26-jul	19675	11	M/L/B 15/80/5	13	Control	1				x				7-sep	1					
pvBR020096	A1	2	26-jul	19675	11	M/L/B 15/80/5	13	Control	1				x				7-sep	1					
pvBR020096	A1	3	26-jul	19675	11	M/L/B 15/80/5	13	Control	0				x				24-aug	4	callus	17-okt			
pvBR020096	A1	1	26-jul	19675	11	M/L/B 15/80/5	13	Drop	1						x		24-aug						
pvBR020096	A1	2	26-jul	19675	11	M/L/B 15/80/5	13	Drop	1						x		7-sep	1					
pvBR020096	A1	3	26-jul	19675	11	M/L/B 15/80/5	13	Drop	3						x		24-aug	5	callus	17-okt			
pvBR020096	A1	1	26-jul	19675	11	M/L/B 15/80/5	13	Refresh	0					x			24-aug	2	callus	17-okt			
pvBR020096	A1	2	26-jul	19675	11	M/L/B 15/80/5	13	Refresh	1					x			24-aug	2	callus	17-okt			
pvBR020096	A1	3	26-jul	19675	11	M/L/B 15/80/5	13	Refresh	0					x			24-aug	2		17-okt			
pvBR020096	A2	1	1-aug	19166	8	M/L 10/90	13	Control	6				x				7-sep	6					
pvBR020096	A2	1	1-aug	19166	8	M/L 10/90	13	Drop	3						x		7-sep	3					
pvBR020096	A2	1	1-aug	19166	8	M/L 10/90	13	Refresh	4					x			7-sep	4					
pvBR020096	A2	1	6-aug	20583	12	L/B 70/30	17	Control	0	x							24-aug						
pvBR020096	A2	1	6-aug	20583	12	L/B 70/30	17	Drop	1			x					24-aug						
pvBR020096	A2	2	6-aug	20583	12	L/B 70/30	17	Drop	0			x					24-aug						
pvBR020096	A2	1	6-aug	20583	12	L/B 70/30	17	Refresh	0		x						24-aug						
pvBR020096	A2	2	6-aug	20583	12	L/B 70/30	17	Refresh			x						24-aug		lost when taping				
pvBR020100	B1	1	2-aug	19958	7	M/L 20/80	17	Control	6	x							7-sep	6					
pvBR020100	B1	2	2-aug	19958	7	M/L 20/80	17	Control	16	x							24-aug	7		17-okt			
pvBR020100	B1	3	2-aug	19958	7	M/L 20/80	17	Control	5	x							24-aug						
pvBR020100	B1	4	2-aug	19958	7	M/L 20/80	17	Control	8	x							24-aug	8		17-okt			
pvBR020100	B1	1	2-aug	19958	7	M/L 20/80	17	Drop	14			x					7-sep	10					

pvBR020100	B1	2	2-aug	19958	7	M/L 20/80	17	Drop	6			x					24-aug						
pvBR020100	B1	3	2-aug	19958	7	M/L 20/80	17	Drop	13			x					24-aug						
pvBR020100	B1	1	2-aug	19958	7	M/L 20/80	17	Refresh	24		x						7-sep	10					
pvBR020100	B1	2	2-aug	19958	7	M/L 20/80	17	Refresh	13		x						24-aug						
pvBR020100	B1	3	2-aug	19958	7	M/L 20/80	17	Refresh	22		x						24-aug						
pvBR020100	B1	1	6-aug	18750	11	L/B 80/20	17	Control	1	x							24-aug						
pvBR020100	B1	2	6-aug	18750	11	L/B 80/20	17	Control	2	x							24-aug						
pvBR020100	B1	1	6-aug	18750	11	L/B 80/20	17	Refresh	14		x						24-aug						
pvBR020100	B1	2	6-aug	18750	11	L/B 80/20	17	Refresh	4		x						24-aug						
pvBR020100	B1	1	15-aug	20278	5	M/L 40/60	13	Control	10				x				10-okt						
pvBR020100	B1	2	15-aug	20278	5	M/L 40/60	13	Control	13				x				10-okt						
pvBR020100	B1	3	15-aug	20278	5	M/L 40/60	13	Control	3				x				10-okt						
pvBR020100	B1	1	15-aug	20278	5	M/L 40/60	13	Drop	16						x		10-okt		larger				
pvBR020100	B1	2	15-aug	20278	5	M/L 40/60	13	Drop	10						x		10-okt		larger				
pvBR020100	B1	3	15-aug	20278	5	M/L 40/60	13	Drop	20						x		10-okt		larger				
pvBR020100	B1	1	15-aug	20278	5	M/L 40/60	13	Refresh	8					x			10-okt						
pvBR020100	B1	2	15-aug	20278	5	M/L 40/60	13	Refresh	15					x			10-okt						
pvBR020100	B1	3	15-aug	20278	5	M/L 40/60	13	Refresh	16					x			10-okt						
pvBR020104	A2	1	30-mei	19083	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020104	A2	2	30-mei	19083	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020104	A2	3	30-mei	19083	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020104	A2	4	30-mei	19083	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020104	A2	5	30-mei	19083	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020104	A2	1	30-mei	18416	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020104	A2	2	30-mei	18416	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020104	A2	3	30-mei	18416	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020104	A2	4	30-mei	18416	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020104	A2	5	30-mei	18416	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020104	A1	1	6-jun	18472	14	L/B 50/50	13	Control	1				x				19-jul	1		24-aug	2		
pvBR020104	A1	2	6-jun	18472	14	L/B 50/50	13	Control	6				x				19-jul	6		24-aug			
pvBR020104	A1	3	6-jun	18472	14	L/B 50/50	13	Control	5				x				19-jul	3		24-aug			
pvBR020104	A1	1	6-jun	20416	14	L/B 50/50	17	Control	7	x							19-jul	3	4 globular not transferred	24-aug	0		
pvBR020104	A1	2	6-jun	20416	14	L/B 50/50	17	Control	2	x							19-jul	2		24-aug	0		
pvBR020104	A1	3	6-jun	20416	14	L/B 50/50	17	Control	3	x							19-jul	3		24-aug	0		
pvBR020104	A2	1	13-jun	19940	12	L/B 70/30	17	Control	29	x							19-jul	10		24-aug	7		
pvBR020104	A2	2	13-jun	19940	12	L/B 70/30	17	Control	16	x							19-jul			24-aug			
pvBR020104	A2	3	13-jun	19940	12	L/B 70/30	17	Control	25	x							19-jul			24-aug			
pvBR020104	A2	4	13-jun	19940	12	L/B 70/30	17	Control	18	x							19-jul	10		24-aug			
pvBR020104	A2	5	13-jun	19940	12	L/B 70/30	17	Control	16	x							19-jul			24-aug			
pvBR020104	A2	6	13-jun	19940	12	L/B 70/30	17	Control	19	x							19-jul			24-aug			
pvBR020104	A2	7	13-jun	19940	12	L/B 70/30	17	Control	14	x							19-jul			24-aug			
pvBR020104	A1	1	14-jun	21250	20	B/Tri 80/20	13	Control	0				x				19-jul		contaminated	24-aug		7-sep	poor
pvBR020104	A1	2	14-jun	21250	20	B/Tri 80/20	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020104	A1	3	14-jun	21250	20	B/Tri 80/20	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020104	B2	1	20-jun	19861	15	L/B 40/60	17	Control	1	x							19-jul	1	callus	24-aug	0		
pvBR020104	B2	2	20-jun	19861	15	L/B 40/60	17	Control	0	x							19-jul			24-aug			
pvBR020104	B2	3	20-jun	19861	15	L/B 40/60	17	Control	0	x							19-jul			24-aug			
pvBR020104	B2	4	20-jun	19861	15	L/B 40/60	17	Control	0	x							19-jul			24-aug			
pvBR020104	B2	1	20-jun	19861	15	L/B 40/60	17	Drop	1			x					19-jul	1		24-aug	1		
pvBR020104	B2	2	20-jun	19861	15	L/B 40/60	17	Drop	0			x					19-jul			24-aug			

pvBR020104	B2	3	20-jun	19861	15	L/B 40/60	17	Drop	0			x					19-jul			24-aug		7-sep	5 g 2 p
pvBR020104	B2	4	20-jun	19861	15	L/B 40/60	17	Drop	0			x					19-jul			24-aug			
pvBR020104	B2	1	20-jun	19861	15	L/B 40/60	17	Refresh	1		x						19-jul	1		24-aug	0		
pvBR020104	B2	2	20-jun	19861	15	L/B 40/60	17	Refresh	1		x						19-jul	1		24-aug	0		
pvBR020104	B2	3	20-jun	19861	15	L/B 40/60	17	Refresh	0		x						19-jul			24-aug			
pvBR020104	B2	4	20-jun	19861	15	L/B 40/60	17	Refresh	0		x						19-jul			24-aug			
pvBR020104	B1	1	25-jun	22916	4	M/L 50/50	13	Control	0				x				19-jul			24-aug			
pvBR020104	B1	2	25-jun	22916	4	M/L 50/50	13	Control	0				x				19-jul			24-aug			
pvBR020104	B1	3	25-jun	22916	4	M/L 50/50	13	Control	0				x				19-jul			24-aug			
pvBR020104	B1	1	1-aug	19583	9	L 100	13	Control	0				x				24-aug						
pvBR020104	B1	2	1-aug	19583	9	L 100	13	Control	0				x				24-aug						
pvBR020104	C1	1	15-aug	20938	9	L 100	13	Drop	20						x		10-okt	5		17-okt			
pvBR020104	C1	2	15-aug	20938	9	L 100	13	Drop	16						x		10-okt	5		17-okt			
pvBR020104	C1	1	15-aug	20938	9	L 100	13	Refresh	12					x			10-okt	6		17-okt			
pvBR020104	C1	2	15-aug	20938	9	L 100	13	Refresh	4					x			10-okt	4		17-okt			
pvBR020108	A1	1	20-jun	18166	14	L/B 50/50	17	Control	16	x							19-jul	4		24-aug	7		
pvBR020108	A1	2	20-jun	18166	14	L/B 50/50	17	Control	24	x							19-jul	6		24-aug			
pvBR020108	A1	1	20-jun	18166	14	L/B 50/50	17	Drop	16			x					19-jul	5		24-aug	4		
pvBR020108	A1	2	20-jun	18166	14	L/B 50/50	17	Drop	19			x					19-jul	5		24-aug			
pvBR020108	A1	3	20-jun	18166	14	L/B 50/50	17	Drop	16			x					19-jul	0		24-aug			
pvBR020108	B1+2	1	25-jul	20666	18	L/B/T 10/50/40	17	Control	0	x							24-aug						
pvBR020108	B1+2	2	25-jul	20666	18	L/B/T 10/50/40	17	Control	0	x							24-aug						
pvBR020108	B1+2	1	25-jul	20666	18	L/B/T 10/50/40	17	Drop	1			x					7-sep	1					
pvBR020108	B1+2	2	25-jul	20666	18	L/B/T 10/50/40	17	Drop	2			x					7-sep	2					
pvBR020108	B1+2	3	25-jul	20666	18	L/B/T 10/50/40	17	Drop	0			x					24-aug						
pvBR020108	B1+2	1	25-jul	20666	18	L/B/T 10/50/40	17	Refresh	0		x						24-aug						
pvBR020108	B1+2	2	25-jul	20666	18	L/B/T 10/50/40	17	Refresh	0		x						24-aug						
pvBR020108	B1+2	3	25-jul	20666	18	L/B/T 10/50/40	17	Refresh	0		x						24-aug						
pvBR020108	A1	1	26-jul	22916	18	L/B 10/90	13	Control	1				x				7-sep	1					
pvBR020108	A1	3	26-jul	22916	18	L/B 10/90	13	Drop	1						x		7-sep	1					
pvBR020108	A1	2	26-jul	22916	18	L/B 10/90	13	Refresh	2					x			7-sep	2					
pvBR020108	B1+2	1	6-aug	19129	19	L/B 1/99	17	Control	0	x							24-aug					7-sep	
pvBR020108	B1+2	2	6-aug	19129	19	L/B 1/99	17	Control	0	x							24-aug						
pvBR020108	B1+2	3	6-aug	19129	19	L/B 1/99	17	Control	0	x							24-aug					7-sep	
pvBR020108	B1+2	1	6-aug	19129	19	L/B 1/99	17	Drop	0			x					24-aug						
pvBR020108	B1+2	2	6-aug	19129	19	L/B 1/99	17	Drop	0			x					24-aug						
pvBR020108	B1+2	3	6-aug	19129	19	L/B 1/99	17	Drop	0			x					24-aug						
pvBR020108	B1+2	4	6-aug	19129	19	L/B 1/99	17	Drop	0			x					24-aug						
pvBR020108	B1+2	1	6-aug	19129	19	L/B 1/99	17	Refresh	0		x						24-aug						
pvBR020108	B1+2	2	6-aug	19129	19	L/B 1/99	17	Refresh	0		x						24-aug						
pvBR020108	B1+2	3	6-aug	19129	19	L/B 1/99	17	Refresh	0		x						24-aug						
pvBR020108	B1+2	4	6-aug	19129	19	L/B 1/99	17	Refresh	0		x						24-aug						
pvBR020108	B1+2	1	15-aug	18750	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020108	B1+2	2	15-aug	18750	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020108	B1+2	3	15-aug	18750	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020108	B1+2	1	15-aug	18750	11	L/B 80/20	13	Drop	0						x		10-okt						
pvBR020108	B1+2	2	15-aug	18750	11	L/B 80/20	13	Drop	0						x		10-okt						
pvBR020108	B1+2	3	15-aug	18750	11	L/B 80/20	13	Drop	0						x		10-okt						
pvBR020108	B1+2	1	15-aug	18750	11	L/B 80/20	13	Refresh	0					x			10-okt						
pvBR020108	B1+2	2	15-aug	18750	11	L/B 80/20	13	Refresh	0					x			10-okt						

pvBR020108	B1+2	3	15-aug	18750	11	L/B 80/20	13	Refresh	0					x			10-okt						
pvBR020117	A1	1	16-aug	17604	10	L/B 90/10	17	Control	0	x							10-okt						
pvBR020117	A1	2	16-aug	17604	10	L/B 90/10	17	Control	0	x							10-okt						
pvBR020117	A1	1	16-aug	17604	10	L/B 90/10	17	Drop	0			x					10-okt						
pvBR020117	A1	2	16-aug	17604	10	L/B 90/10	17	Drop	1			x					10-okt	1	callus	17-okt			
pvBR020117	A2	1	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	2	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	3	22-aug	20566	17	L/B 20/80	13	Control	1				x				10-okt	1	callus	17-okt			
pvBR020117	A2	4	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	5	22-aug	20566	17	L/B 20/80	13	Control	1				x				10-okt	1	callus	17-okt			
pvBR020117	A2	6	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	7	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	8	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	9	22-aug	20566	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020117	A2	1	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	2	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	3	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	4	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	5	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	6	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	7	22-aug	18988	17	L/B 20/80	10		0							x	10-okt						
pvBR020117	A2	1	29-aug	21389	8	M/L 10/90	17	Control	0	x							10-okt						
pvBR020117	A2	2	29-aug	21389	8	M/L 10/90	17	Control	0	x							10-okt						
pvBR020117	A2	1	29-aug	21389	8	M/L 10/90	17	Drop	0			x					10-okt						
pvBR020117	A2	2	29-aug	21389	8	M/L 10/90	17	Drop	1			x					10-okt	1		17-okt			
pvBR020117	A2	1	29-aug	21389	8	M/L 10/90	17	Refresh	0		x						10-okt						
pvBR020117	A2	2	29-aug	21389	8	M/L 10/90	17	Refresh	1		x						10-okt	1		17-okt			
pvBR020117	B1	1	5-sep	18854	18	L/B 10/90	17	Control	0	x							10-okt						
pvBR020117	B1	2	5-sep	18854	18	L/B 10/90	17	Control	0	x							10-okt						
pvBR020117	B1	1	5-sep	18854	18	L/B 10/90	17	Drop	0			x					10-okt						
pvBR020117	B1	2	5-sep	18854	18	L/B 10/90	17	Drop	0			x					10-okt						
pvBR020117	B1	3	5-sep	18854	18	L/B 10/90	17	Drop	0			x					10-okt						
pvBR020117	B1	1	5-sep	18854	18	L/B 10/90	17	Refresh	0		x						10-okt						
pvBR020117	B1	2	5-sep	18854	18	L/B 10/90	17	Refresh	0		x						10-okt						
pvBR020117	B1	3	5-sep	18854	18	L/B 10/90	17	Refresh	0		x						10-okt						
pvBR020117	B2	2	12-sep	18750	15	L/B 40/60	17	Control	0	x							10-okt						
pvBR020117	B2	M	12-sep	18750	15	L/B 40/60	17	Control	0	x							10-okt						
pvBR020117	B2	2	12-sep	18750	15	L/B 40/60	17	Drop	1			x					10-okt		callus				
pvBR020117	B2	M	12-sep	18750	15	L/B 40/60	17	Drop	0			x					10-okt						
pvBR020117	B2	2	12-sep	18750	15	L/B 40/60	17	Refresh	0		x						10-okt						
pvBR020117	B2	M	12-sep	18750	15	L/B 40/60	17	Refresh	0		x						10-okt						
pvBR020117	A2	1	26-sep	18750	14	L/B 50/50	17	Control	0	x							17-okt						
pvBR020117	A2	1	26-sep	18750	14	L/B 50/50	17	Drop	0			x					17-okt						
pvBR020117	A2	1	26-sep	18750	14	L/B 50/50	17	Refresh	0		x						17-okt						
pvBR020118	A1	1	29-aug	19444	10	L/B 95/5	17	Control	0	x							10-okt						
pvBR020118	A1	2	29-aug	19444	10	L/B 95/5	17	Control	0	x							10-okt						
pvBR020118	A1	3	29-aug	19444	10	L/B 95/5	17	Control	0	x							10-okt						
pvBR020118	A1	1	29-aug	19444	10	L/B 95/5	17	Drop	0			x					10-okt						
pvBR020118	A1	2	29-aug	19444	10	L/B 95/5	17	Drop	0			x					10-okt						
pvBR020118	A1	3	29-aug	19444	10	L/B 95/5	17	Drop	0			x					10-okt						

pvBR020118	A1	1	29-aug	19444	10	L/B 95/5	17	Refresh	0		x						10-okt						
pvBR020118	A1	2	29-aug	19444	10	L/B 95/5	17	Refresh	0		x						10-okt						
pvBR020118	A1	3	29-aug	19444	10	L/B 95/5	17	Refresh	0		x						10-okt						
pvBR020118	A1	1	5-sep	20341	11	L/B 80/20	17	Control	0	x							10-okt						
pvBR020118	A1	2	5-sep	20341	11	L/B 80/20	17	Control	0	x							10-okt						
pvBR020118	A1	3	5-sep	20341	11	L/B 80/20	17	Control	0	x							10-okt						
pvBR020118	A1	1	5-sep	20341	11	L/B 80/20	17	Drop	0			x					10-okt						
pvBR020118	A1	2	5-sep	20341	11	L/B 80/20	17	Drop	0			x					10-okt						
pvBR020118	A1	3	5-sep	20341	11	L/B 80/20	17	Drop	0			x					10-okt						
pvBR020118	A1	4	5-sep	20341	11	L/B 80/20	17	Drop	0			x					10-okt						
pvBR020118	A1	1	5-sep	20341	11	L/B 80/20	17	Refresh	1		x						10-okt		callus				
pvBR020118	A1	2	5-sep	20341	11	L/B 80/20	17	Refresh	0		x						10-okt						
pvBR020118	A1	3	5-sep	20341	11	L/B 80/20	17	Refresh	0		x						10-okt						
pvBR020118	A1	4	5-sep	20341	11	L/B 80/20	17	Refresh	0		x						10-okt						
pvBR020118	A2	1	13-sep	20278	9	L 100	13	Control	5				x				10-okt						
pvBR020118	A2	2	13-sep	20278	9	L 100	13	Control	21				x				10-okt	10		17-okt			
pvBR020118	A2	1	13-sep	20278	9	L 100	13	Drop	25						x		10-okt						
pvBR020118	A2	2	13-sep	20278	9	L 100	13	Drop	0						x		10-okt						
pvBR020118	A2	1	13-sep	20278	9	L 100	13	Refresh	13					x			10-okt						
pvBR020118	A2	2	13-sep	20278	9	L 100	13	Refresh	21					x			10-okt	10		17-okt			
pvBR020118	B1	1	26-sep	24757	14	L/B 50/50	17	Control	0	x							17-okt						
pvBR020118	B1	2	26-sep	24757	14	L/B 50/50	17	Control	0	x							17-okt						
pvBR020118	B1	3	26-sep	24757	14	L/B 50/50	17	Control	0	x							17-okt						
pvBR020118	B1	4	26-sep	24757	14	L/B 50/50	17	Control	2	x							17-okt	2					
pvBR020118	B1	1	26-sep	24757	14	L/B 50/50	17	Drop	1			x					17-okt	1					
pvBR020118	B1	2	26-sep	24757	14	L/B 50/50	17	Drop	0			x					17-okt						
pvBR020118	B1	3	26-sep	24757	14	L/B 50/50	17	Drop	0			x					17-okt						
pvBR020118	B1	4	26-sep	24757	14	L/B 50/50	17	Drop	0			x					17-okt						
pvBR020118	B1	1	26-sep	24757	14	L/B 50/50	17	Refresh	0		x						17-okt						
pvBR020118	B1	2	26-sep	24757	14	L/B 50/50	17	Refresh	0		x						17-okt						
pvBR020118	B1	3	26-sep	24757	14	L/B 50/50	17	Refresh	0		x						17-okt						
pvBR020118	B1	4	26-sep	24757	14	L/B 50/50	17	Refresh	0		x						17-okt						
pvBR020121	A1	1	13-sep	19583	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020121	A1	2	13-sep	19583	17	L/B 20/80	13	Control	0				x				10-okt						
pvBR020121	A1	1	13-sep	19583	17	L/B 20/80	13	Drop	1						x		10-okt	1	callus	17-okt			
pvBR020121	A1	2	13-sep	19583	17	L/B 20/80	13	Drop	0						x		10-okt						
pvBR020121	A1	1	13-sep	19583	17	L/B 20/80	13	Refresh	0					x			10-okt						
pvBR020121	A1	2	13-sep	19583	17	L/B 20/80	13	Refresh	1					x			10-okt	1	callus	17-okt			
pvBR020121	B1	1	26-sep	22639	16	L/B 30/70	17	Control	0	x							17-okt						
pvBR020121	B1	2	26-sep	22639	16	L/B 30/70	17	Control	0	x							17-okt						
pvBR020121	B1	1	26-sep	22639	16	L/B 30/70	17	Drop	0			x					17-okt						
pvBR020121	B1	2	26-sep	22639	16	L/B 30/70	17	Drop	0			x					17-okt						
pvBR020121	B1	1	26-sep	22639	16	L/B 30/70	17	Refresh	0		x						17-okt						
pvBR020121	B1	2	26-sep	22639	16	L/B 30/70	17	Refresh	0		x						17-okt						
pvBR020122	A1	1	16-aug	18958	10	L/B 90/10	17	Control	0	x							10-okt						
pvBR020122	A1	2	16-aug	18958	10	L/B 90/10	17	Control	0	x							10-okt						
pvBR020122	A1	1	16-aug	18958	10	L/B 90/10	17	Drop	0			x					10-okt						
pvBR020122	A1	2	16-aug	18958	10	L/B 90/10	17	Drop	0			x					10-okt						
pvBR020122	A1	3	16-aug	18958	10	L/B 90/10	17	Drop	1			x					10-okt		poor				
pvBR020122	A1	1	16-aug	18958	10	L/B 90/10	17	Refresh	0		x						10-okt						

pvBR020122	A1	2	16-aug	18958	10	L/B 90/10	17	Refresh	0		x						10-okt						
pvBR020122	A1	3	16-aug	18958	10	L/B 90/10	17	Refresh	0		x						10-okt						
pvBR020122	A1	1	22-aug	21333	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020122	A1	2	22-aug	21333	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020122	A1	3	22-aug	21333	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020122	A1	4	22-aug	21333	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020122	A1	5	22-aug	21333	11	L/B 80/20	13	Control	0				x				10-okt						
pvBR020122	A1	1	22-aug	20729	11	L/B 80/20	10		0							x	10-okt						
pvBR020122	A1	2	22-aug	20729	11	L/B 80/20	10		0							x	10-okt						
pvBR020122	A1	3	22-aug	20729	11	L/B 80/20	10		0							x	10-okt						
pvBR020122	A1	4	22-aug	20729	11	L/B 80/20	10		0							x	10-okt						
pvBR020122	A2	1	29-aug	21042	10	L/B 90/10	17	Control	0	x							10-okt						
pvBR020122	A2	2	29-aug	21042	10	L/B 90/10	17	Control	0	x							10-okt						
pvBR020122	A2	1	29-aug	21042	10	L/B 90/10	17	Drop	0			x					10-okt						
pvBR020122	A2	2	29-aug	21042	10	L/B 90/10	17	Drop	0			x					10-okt						
pvBR020122	B1	1	5-sep	22500	9	L 100	17	Control	0	x							10-okt						
pvBR020122	B1	1	5-sep	22500	9	L 100	17	Drop	0			x					10-okt						
pvBR020122	B1	1	5-sep	22500	9	L 100	17	Refresh	0		x						10-okt						
pvBR020122	B1	1	13-sep	18287	15	L/B 40/60	13	Control	0				x				10-okt						
pvBR020122	B1	2	13-sep	18287	15	L/B 40/60	13	Control	0				x				10-okt						
pvBR020122	B1	3	13-sep	18287	15	L/B 40/60	13	Control	0				x				10-okt						
pvBR020122	B1	1	13-sep	18287	15	L/B 40/60	13	Drop	0						x		10-okt						
pvBR020122	B1	2	13-sep	18287	15	L/B 40/60	13	Drop	0						x		10-okt						
pvBR020122	B1	3	13-sep	18287	15	L/B 40/60	13	Drop	0						x		10-okt						
pvBR020122	B1	1	13-sep	18287	15	L/B 40/60	13	Refresh	0					x			10-okt						
pvBR020122	B1	2	13-sep	18287	15	L/B 40/60	13	Refresh	0					x			10-okt						
pvBR020122	B1	3	13-sep	18287	15	L/B 40/60	13	Refresh	0					x			10-okt						
pvBR020122	B2	1	26-sep	20139	18	L/B 10/90	17	Control	0	x							17-okt						
pvBR020122	B2	1	26-sep	20139	18	L/B 10/90	17	Drop	0			x					17-okt						
pvBR020122	B2	1	26-sep	20139	18	L/B 10/90	17	Refresh	0		x						17-okt						
pvBR020123	A1	1	30-mei	19444	18	L/B 10/90	13	Control	2				x				19-jul	2	callus	24-aug	0		
pvBR020123	A1	2	30-mei	19444	18	L/B 10/90	13	Control	1				x				19-jul	1	callus	24-aug	0		
pvBR020123	A1	3	30-mei	19444	18	L/B 10/90	13	Control	1				x				19-jul	1	callus	24-aug	0		
pvBR020123	A1	1	30-mei	19166	18	L/B 10/90	17	Control	2	x							19-jul	2	callus	24-aug	0		
pvBR020123	A1	1	6-jun	10833	14	L/B 50/50	13	Control	1				x				19-jul	1	callus	24-aug	0		
pvBR020123	A1	1	6-jun	5833	14	L/B 50/50	17	Control	0	x							19-jul			24-aug			
pvBR020123	A2	1	7-jun	18333	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020123	A2	2	7-jun	18333	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR020123	A2	1	7-jun	5833	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020123	B2	1	20-jun	18819	2	M/L 70/30	17	Control	0	x							19-jul			24-aug			
pvBR020123	B2	2	20-jun	18819	2	M/L 70/30	17	Control	0	x							19-jul			24-aug			
pvBR020123	B2	1	20-jun	18819	2	M/L 70/30	17	Drop	0			x					19-jul			24-aug			
pvBR020123	B2	2	20-jun	18819	2	M/L 70/30	17	Drop	0			x					19-jul			24-aug			
pvBR020123	B2	1	20-jun	18819	2	M/L 70/30	17	Refresh	0		x						19-jul			24-aug			
pvBR020123	B2	2	20-jun	18819	2	M/L 70/30	17	Refresh	0		x						19-jul			24-aug			
pvBR020123	B2	1	25-jun	20737	8	M/L 10/90	13	Control	0				x				19-jul			24-aug			
pvBR020123	B2	2	25-jun	20737	8	M/L 10/90	13	Control	0				x				19-jul			24-aug			
pvBR020123	B2	3	25-jun	20737	8	M/L 10/90	13	Control	0				x				19-jul			24-aug			
pvBR020123	B2	4	25-jun	20737	8	M/L 10/90	13	Control	0				x				19-jul			24-aug			
pvBR020123	B2	5	25-jun	20737	8	M/L 10/90	13	Control	0				x				19-jul			24-aug			

pvBR020123	B2	1	25-jun	20737	8	M/L 10/90	13	Drop	0						x		19-jul			24-aug			
pvBR020123	B2	2	25-jun	20737	8	M/L 10/90	13	Drop	0						x		19-jul			24-aug			
pvBR020123	B2	3	25-jun	20737	8	M/L 10/90	13	Drop	0						x		19-jul			24-aug			
pvBR020123	B2	4	25-jun	20737	8	M/L 10/90	13	Drop	0						x		19-jul			24-aug			
pvBR020123	B2	1	25-jun	20737	8	M/L 10/90	13	Refresh	0					x			19-jul			24-aug			
pvBR020123	B2	2	25-jun	20737	8	M/L 10/90	13	Refresh	0					x			19-jul			24-aug			
pvBR020123	B2	3	25-jun	20737	8	M/L 10/90	13	Refresh	0					x			19-jul			24-aug			
pvBR020123	B2	4	25-jun	20737	8	M/L 10/90	13	Refresh	0					x			19-jul			24-aug			
pvBR020123	B2	1	1-aug	21145	15	L/B 40/60	13	Control	0				x				24-aug						
pvBR020123	B2	2	1-aug	21145	15	L/B 40/60	13	Control	0				x				24-aug						
pvBR020123	B2	1	1-aug	21145	15	L/B 40/60	13	Drop	0						x		24-aug						
pvBR020123	B2	2	1-aug	21145	15	L/B 40/60	13	Drop	0						x		24-aug						
pvBR020123	B2	1	2-aug	19861	9	L 100	17	Control	0	x							24-aug						
pvBR020123	B2	2	2-aug	19861	9	L 100	17	Control	0	x							24-aug						
pvBR020123	B2	1	2-aug	19861	9	L 100	17	Drop	0			x					24-aug						
pvBR020123	B2	2	2-aug	19861	9	L 100	17	Drop	0			x					24-aug						
pvBR020123	B2	1	2-aug	19861	9	L 100	17	Refresh	0		x						24-aug						
pvBR020123	B2	2	2-aug	19861	9	L 100	17	Refresh	0		x						24-aug						
pvBR020123	B1	1	6-aug	21019	9	L 100	17	Control	1	x							24-aug	1	callus	17-okt			
pvBR020123	B1	2	6-aug	21019	9	L 100	17	Control	1	x							24-aug	1	callus	17-okt			
pvBR020123	B1	3	6-aug	21019	9	L 100	17	Control	0	x							24-aug						
pvBR020123	B1	1	6-aug	21019	9	L 100	17	Drop	1			x					24-aug	1	callus	17-okt			
pvBR020123	B1	2	6-aug	21019	9	L 100	17	Drop	0			x					24-aug						
pvBR020123	B1	3	6-aug	21019	9	L 100	17	Drop	3			x					24-aug	3	callus	17-okt			
pvBR020123	B1	1	6-aug	21019	9	L 100	17	Refresh	0		x						24-aug						
pvBR020123	B1	2	6-aug	21019	9	L 100	17	Refresh	0		x						24-aug						
pvBR020123	B1	3	6-aug	21019	9	L 100	17	Refresh	1		x						24-aug	1	large	17-okt			
pvBR020150		1	6-aug	19583	15	L/B 40/60	17	Control	0	x							24-aug						
pvBR020150		2	6-aug	19583	15	L/B 40/60	17	Control	0	x							24-aug						
pvBR020150		3	6-aug	19583	15	L/B 40/60	17	Control	0	x							24-aug						
pvBR020150		4	6-aug	19583	15	L/B 40/60	17	Control	0	x							24-aug						
pvBR020150		1	6-aug	19583	15	L/B 40/60	17	Drop	0			x					24-aug						
pvBR020150		2	6-aug	19583	15	L/B 40/60	17	Drop	0			x					24-aug						
pvBR020150		3	6-aug	19583	15	L/B 40/60	17	Drop	0			x					24-aug						
pvBR020150		1	6-aug	19583	15	L/B 40/60	17	Refresh	0		x						24-aug						
pvBR020150		2	6-aug	19583	15	L/B 40/60	17	Refresh	0		x						24-aug						
pvBR020150		3	6-aug	19583	15	L/B 40/60	17	Refresh	0		x						24-aug						
pvBR020153		1	2-aug	19167	5	M/L 40/60	17	Control	0	x							24-aug						
pvBR020153		2	2-aug	19167	5	M/L 40/60	17	Control	0	x							24-aug						
pvBR020153		3	2-aug	19167	5	M/L 40/60	17	Control	0	x							24-aug						
pvBR020153		1	2-aug	19167	5	M/L 40/60	17	Drop	1			x					7-sep	1					
pvBR020153		2	2-aug	19167	5	M/L 40/60	17	Drop	0			x					24-aug						
pvBR020153		3	2-aug	19167	5	M/L 40/60	17	Drop	0			x					24-aug						
pvBR020153		1	2-aug	19167	5	M/L 40/60	17	Refresh	0		x						24-aug						
pvBR020153		2	2-aug	19167	5	M/L 40/60	17	Refresh	0		x						24-aug						
pvBR020153		3	2-aug	19167	5	M/L 40/60	17	Refresh	0		x						24-aug						
pvBR020155	A2	1	25-jul	13750	10	L/B 90/10	17	Control	0	x							24-aug						
pvBR020155	A1	1	2-aug	17917	7	M/L 20/80	17	Control	0	x							24-aug		contaminated				
pvBR020155	A1	1	15-aug	24167	5	M/L 40/60	13	Control	0				x				10-okt						
pvBR020155	A1	2	15-aug	24167	5	M/L 40/60	13	Control	0				x				10-okt						

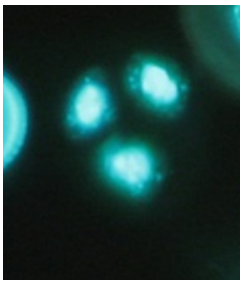
pvBR020155	B1	1	22-aug	29583	5	tetra/m/l 35/60/5	13	Control	0				x				10-okt		contaminated				
pvBR020155	B1	1	29-aug	20208	5	M/L 40/60	17	Control	0	x							10-okt						
pvBR020155	B1	2	29-aug	20208	5	M/L 40/60	17	Control	0	x							10-okt						
pvBR020155	B1	1	29-aug	20208	5	M/L 40/60	17	Drop	0			x					10-okt						
pvBR020155	B1	2	29-aug	20208	5	M/L 40/60	17	Drop	0			x					10-okt						
pvBR020158	A1	1	22-mei	4000		NDT	13	Control	0				x				19-jul			24-aug			
pvBR020158	B1	1	6-jun	2083	20	tri	13	Control	0				x				19-jul			24-aug			
pvBR020158	B1	1	6-jun	2916	20	tri	17	Control	0	x							19-jul			24-aug			
pvBR020158	B2	1	13-jun	17708	20	tri	17	Control	0	x							19-jul			24-aug			
pvBR020158	B2	2	13-jun	17708	20	tri	17	Control	0	x							19-jul			24-aug			
pvBR020159	A1	1	22-mei	2000		NDT	13	Control	1				x				19-jul	1		24-aug	1		
pvBR020159	A2	1	30-mei	10000	18	L/B 10/90	13	Control	1				x				19-jul	1	callus	24-aug	0		
pvBR020159	A2	1	30-mei	24583	18	L/B 10/90	17	Control	1	x							19-jul	1		24-aug	0		
pvBR020159	A1	1	6-jun	19166	18	L/B/T 10/60/30	13	Control	0				x				19-jul			24-aug		7-sep	poor
pvBR020159	A1	2	6-jun	19166	18	L/B/T 10/60/30	13	Control	0				x				19-jul			24-aug			
pvBR020159	A1	1	6-jun	18333	18	L/B/T 10/60/30	17	Control	0	x							19-jul			24-aug			
pvBR020159	A2	1	14-jun	12916		?	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020159	B1	1	20-jun	22638	12	L/B 70/30	17	Control	1	x							19-jul		callus	24-aug			
pvBR020159	B1	2	20-jun	22638	12	L/B 70/30	17	Control	3	x							7-sep	3	callus	24-aug			
pvBR020159	B1	3	20-jun	22638	12	L/B 70/30	17	Control	0	x							19-jul			24-aug			
pvBR020159	C1	1	1-aug	22777	14	L/B 50/50	13	Control	2				x				24-aug		contaminated				
pvBR020159	C1	1	1-aug	22777	14	L/B 50/50	13	Drop	8						x		7-sep	8					
pvBR020159	C1	1	1-aug	22777	14	L/B 50/50	13	Refresh	0					x			24-aug		contaminated				
pvBR020160	A1	1	30-mei	17083	19	L/B 1/99	13	Control	1				x				19-jul	1		24-aug	0		
pvBR020160	A1	1	30-mei	17916	19	L/B 1/99	17	Control	0	x							19-jul			24-aug			
pvBR020160	A2	1	7-jun	20416		NDT	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020160	A2	2	7-jun	20416		NDT	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020160	A2	1	7-jun	27500		NDT	17	Control	0	x							19-jul		contaminated	24-aug			
pvBR020160	A1	1	14-jun	2291	11	L/B 80/20	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020160	A1	2	14-jun	2291	11	L/B 80/20	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020160	B1	1	20-jun	18437	2	M/L 70/30	17	Control	1	x							19-jul	1		24-aug	1		
pvBR020160	B1	2	20-jun	18437	2	M/L 70/30	17	Control	1	x							19-jul	1	callus	24-aug	0	7-sep	poor
pvBR020160	B2	1	25-jun	18125	1	M/L 80/20	13	Control	4				x				19-jul	4		24-aug	2		
pvBR020160	B2	2	25-jun	18125	1	M/L 80/20	13	Control	2				x				19-jul	2		24-aug		7-sep	1g 1p a albino?
pvBR020160	B2	1	25-jun	18125	1	M/L 80/20	13	Drop	22						x		19-jul	10		24-aug	5		
pvBR020160	B2	2	25-jun	18125	1	M/L 80/20	13	Drop	12						x		19-jul			24-aug		7-sep	3g 2p
pvBR020160	C1+2	1	26-jul	20833	14	L/B 50/50	13	Control	0				x				24-aug						
pvBR020160	C1+2	2	26-jul	20833	14	L/B 50/50	13	Control	0				x				24-aug						
pvBR020235	A1	1	22-mei	18000	20	MU->tri	13	Control	1				x				19-jul	1	callus	24-aug	0		
pvBR020235	A2	1	7-jun	17291	20	B/Tri 60/40	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020235	A2	2	7-jun	17291	20	B/Tri 60/40	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR020235	A2	1	7-jun	14583	20	B/Tri 60/40	17	Control	0	x							19-jul			24-aug			
pvBR020235	A1	1	13-jun	18437	9	LU ?	17	Control	0	x							19-jul			24-aug			
pvBR020235	A1	2	13-jun	18437	9	LU ?	17	Control	0	x							19-jul			24-aug			
pvBR020235	A1	3	13-jun	18437	9	LU ?	17	Control	0	x							19-jul			24-aug			
pvBR020235	A1	4	13-jun	18437	9	LU ?	17	Control	0	x							19-jul			24-aug			
pvBR020235	B1	1	20-jun	22500	9	LU?	17	Control	0	x							19-jul			24-aug			
pvBR020235	B2	1	25-jun	21666	9	L 100	13	Control	0				x				19-jul			24-aug			
pvBR020235	B2	2	25-jun	21666	9	L 100	13	Control	0				x				19-jul			24-aug			
pvBR020235	C1	1	15-aug	25000	13	L/B 60/40	13	Control	1				x				10-okt	1		17-okt			

pvBr080028	B2	1	25-jul	18854	16	L/B 30/70	17	Control	0	x							24-aug						
pvBr080028	B2	2	25-jul	18854	16	L/B 30/70	17	Control	0	x							24-aug						
pvBr080028	B2	1	25-jul	18854	16	L/B 30/70	17	Drop	0			x					24-aug						
pvBr080028	B2	2	25-jul	18854	16	L/B 30/70	17	Drop	0			x					24-aug						
pvBr080028	B2	3	25-jul	18854	16	L/B 30/70	17	Drop	0			x					24-aug						
pvBr080028	B2	1	25-jul	18854	16	L/B 30/70	17	Refresh	0		x						24-aug						
pvBr080028	B2	2	25-jul	18854	16	L/B 30/70	17	Refresh	0		x						24-aug						
pvBr080028	B2	3	25-jul	18854	16	L/B 30/70	17	Refresh	0		x						24-aug						
pvBr080028	A2	1	26-jul	20000	5	M/L 40/60	13	Control	43				x				7-sep	10+10		17-okt			
pvBr080028	A2	2	26-jul	20000	5	M/L 40/60	13	Control	41				x				24-aug						
pvBr080028	A2	1	26-jul	20000	5	M/L 40/60	13	Drop	54						x		7-sep	10					
pvBr080028	A2	2	26-jul	20000	5	M/L 40/60	13	Drop	47						x		24-aug						
pvBr080028	A2	3	26-jul	20000	5	M/L 40/60	13	Drop	46						x		24-aug						
pvBr080028	A2	1	26-jul	20000	5	M/L 40/60	13	Refresh	44					x			7-sep	10					
pvBr080028	A2	2	26-jul	20000	5	M/L 40/60	13	Refresh	51					x			24-aug						
pvBr080028	A2	3	26-jul	20000	5	M/L 40/60	13	Refresh	52					x			24-aug						
pvBr080028	B1+2	1	1-aug	18009	4	M/L 50/50	13	Control	25				x				7-sep	10					
pvBr080028	B1+2	2	1-aug	18009	4	M/L 50/50	13	Control	35				x				24-aug						
pvBr080028	B1+2	3	1-aug	18009	4	M/L 50/50	13	Control	25				x				24-aug						
pvBr080028	B1+2	1	1-aug	18009	4	M/L 50/50	13	Drop	31						x		7-sep	10					
pvBr080028	B1+2	2	1-aug	18009	4	M/L 50/50	13	Drop	42						x		24-aug						
pvBr080028	B1+2	3	1-aug	18009	4	M/L 50/50	13	Drop	46						x		24-aug	10		17-okt			
pvBr080028	B1+2	1	1-aug	18009	4	M/L 50/50	13	Refresh	52					x			7-sep	10					
pvBr080028	B1+2	2	1-aug	18009	4	M/L 50/50	13	Refresh	54					x			24-aug						
pvBr080028	B1+2	3	1-aug	18009	4	M/L 50/50	13	Refresh	48					x			24-aug						
pvBr080028	A1	1	2-aug	21563	13	L/B 60/40	17	Control	70	x							7-sep	10					
pvBr080028	A1	2	2-aug	21563	13	L/B 60/40	17	Control	70	x							24-aug						
pvBr080028	A1	1	2-aug	21563	13	L/B 60/40	17	Drop	66			x					7-sep	12		17-okt			
pvBr080028	A1	2	2-aug	21563	13	L/B 60/40	17	Drop	85			x					24-aug						
pvBr080028	A1	1	6-aug	17083	9	L/B 99/1	17	Control	25	x							24-aug	8		17-okt			
pvBr080028	A1	1	6-aug	17083	9	L/B 99/1	17	Drop	45			x					24-aug	8		17-okt			
pvBr080028	A1	1	6-aug	17083	9	L/B 99/1	17	Refresh	31		x						24-aug	8		17-okt			
pvBR080090	A2	1	30-mei	17500	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR080090	A2	1	30-mei	14583	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR080090	A2	2	30-mei	17500	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR080090	A2	3	30-mei	17500	19	L/B 1/99	13	Control	0				x				19-jul			24-aug			
pvBR080090	A1	1	7-jun	23750	16	L/B 30/70	13	Control	0				x				19-jul			24-aug			
pvBR080090	A1	2	7-jun	23750	16	L/B 30/70	13	Control	0				x				19-jul			24-aug			
pvBR080090	A1	1	7-jun	19166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A1	2	7-jun	19166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	1	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	2	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	3	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	4	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	5	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	6	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	7	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	8	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	9	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			
pvBR080090	A2	10	13-jun	27166	16	L/B 30/70	17	Control	0	x							19-jul			24-aug			

pvBR080090	A2	1	14-jun	21527	20	B/T	13	Control	0				x				19-jul			24-aug			
pvBR080090	A2	2	14-jun	21527	20	B/T	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR080090	A2	3	14-jun	21527	20	B/T	13	Control	0				x				19-jul		contaminated	24-aug			
pvBR080090	C2	1	25-jul	20729	16	L/B/T 33/33/33	17	Control	0	x							24-aug						
pvBR080090	C2	2	25-jul	20729	16	L/B/T 33/33/33	17	Control	0	x							24-aug						
pvBR080090	C2	1	25-jul	20729	16	L/B/T 33/33/33	17	Drop	0			x					24-aug		contaminated ?				
pvBR080090	C2	2	25-jul	20729	16	L/B/T 33/33/33	17	Drop	0			x					24-aug						
pvBR080090	C2	3	25-jul	20729	16	L/B/T 33/33/33	17	Drop	0			x					24-aug						
pvBR080090	C2	1	25-jul	20729	16	L/B/T 33/33/33	17	Refresh	0		x						24-aug						
pvBR080090	C2	2	25-jul	20729	16	L/B/T 33/33/33	17	Refresh	0		x						24-aug						
pvBR080090	C2	3	25-jul	20729	16	L/B/T 33/33/33	17	Refresh	0		x						24-aug						
pvBR080090	C1	1	26-jul	17430	19	L/B 2/98	13	Control	0				x				24-aug						
pvBR080090	C1	2	26-jul	17430	19	L/B 2/98	13	Control	0				x				24-aug						
pvBR080090	C1	1	26-jul	17430	19	L/B 2/98	13	Drop	0						x		24-aug						
pvBR080090	C1	2	26-jul	17430	19	L/B 2/98	13	Drop	1						x		7-sep	1					
pvBR080090	C1	1	26-jul	17430	19	L/B 2/98	13	Refresh	0					x			24-aug						
pvBR080090	C1	2	26-jul	17430	19	L/B 2/98	13	Refresh	0					x			24-aug						
pvBR080095	A1	1	16-aug	17500	19	B 100	17	Control	1	x							10-okt						
pvBR080095	A1	2	16-aug	17500	19	B 100	17	Control	1	x							10-okt	1		17-okt			
pvBR080095	A1	1	16-aug	17500	19	B 100	17	Drop	0			x					10-okt		contaminated				
pvBR080095	A1	2	16-aug	17500	19	B 100	17	Drop	0			x					10-okt						
pvBR080095	A1	1	29-aug	19333	18	L/B 10/90	17	Control	0	x							10-okt						
pvBR080095	A1	1	29-aug	19333	18	L/B 10/90	17	Drop	1			x					10-okt	1		17-okt			
pvBR080095	A1	2	29-aug	19333	18	L/B 10/90	17	Drop	1			x					10-okt						
pvBR080095	A1	1	29-aug	19333	18	L/B 10/90	17	Refresh	1		x						10-okt						
pvBR080095	A1	2	29-aug	19333	18	L/B 10/90	17	Refresh	1		x						10-okt		callus				
pvBR080095	A2	1	5-sep	18917	14	L/B 50/50	17	Control	1	x							10-okt	1	callus	17-okt			
pvBR080095	A2	1	5-sep	18917	14	L/B 50/50	17	Drop	3			x					10-okt	3		17-okt			
pvBR080095	A2	2	5-sep	18917	14	L/B 50/50	17	Drop	2			x					10-okt	2		17-okt			
pvBR080095	A2	1	5-sep	18917	14	L/B 50/50	17	Refresh	3		x						10-okt	3		17-okt			
pvBR080095	A2	2	5-sep	18917	14	L/B 50/50	17	Refresh	0		x						10-okt						
pvBR080095	B1	1	13-sep	19167	19	L/B 5/95	13	Control	0				x				10-okt						
pvBR080095	B1	2	13-sep	19167	19	L/B 5/95	13	Control	0				x				10-okt						
pvBR080095	B1	1	13-sep	19167	19	L/B 5/95	13	Drop	0						x		10-okt						
pvBR080095	B1	2	13-sep	19167	19	L/B 5/95	13	Drop	1						x		10-okt	1		17-okt			

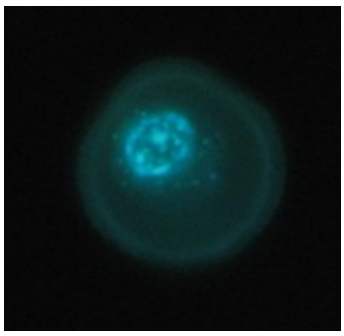
Appendix 4: Different stages during microspore development into embryo

Tetrad

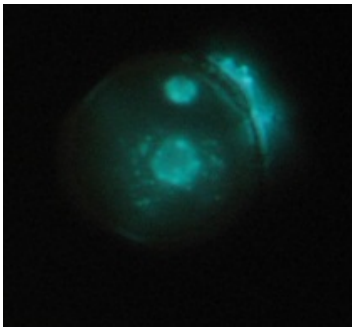


Mid uni-nucleate
Nucleus in centre of msp,
strong visible exine

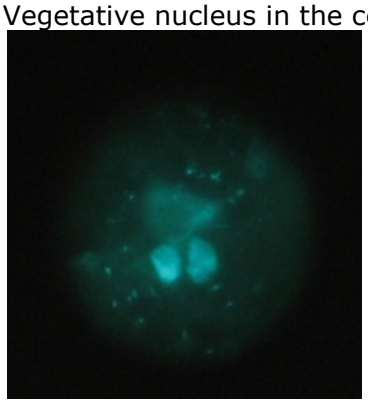
Late uni-nucleate
nucleus more towards the exine
exine less visible



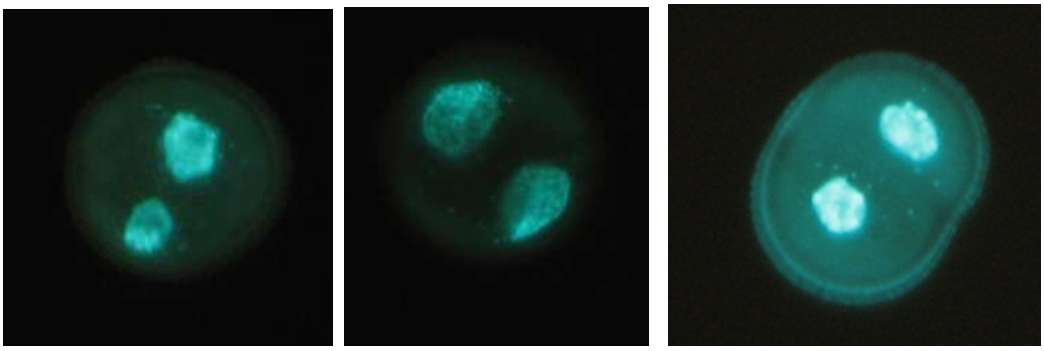
Bi nucleate
Large vegetative nucleus and small generative nucleus which is positioned more to the exine.



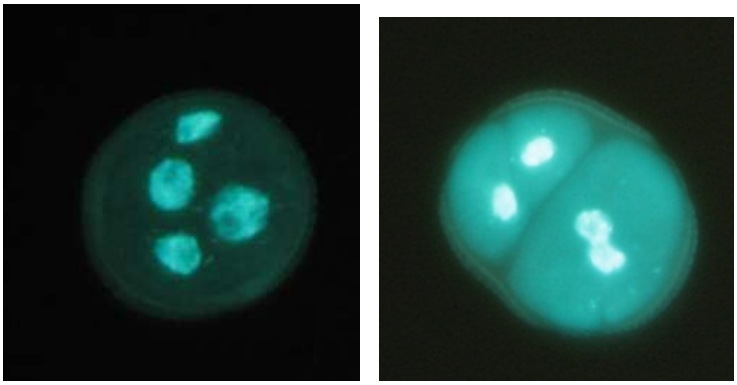
Tri nucleate
Vegetative nucleus in the centre of the cell and two smaller generative nucleus



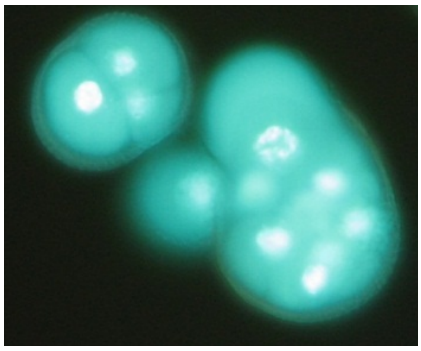
Symmetrical division
Symmetrical division of the vegetative nucleus after stress treatment.
On the right picture the formation of a new cell wall is visible (resulting in cell division)



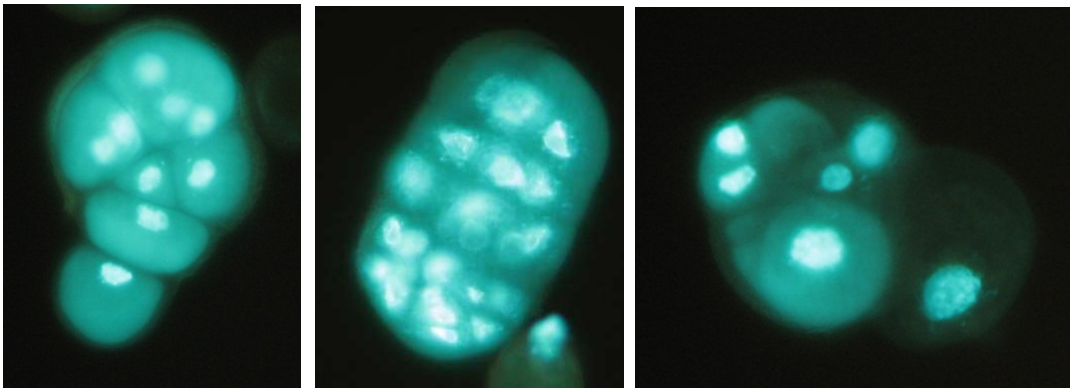
Second division
Division of the vegetative nucleus for the second time (2x2).
On the right picture the formation of a new cell walls is visible (resulting in 4 cells)



3rd division



Multi cellular structure



Starch