



**Barley Anther Culture: Determining the optimal pre-treatment
for green plant regeneration.**

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A thesis submitted in partial fulfilment of the requirements for the degree

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BARLEY ANTHOR CULTURE: DETERMINING THE OPTIMAL PRETREATMENT
FOR GREEN PLANT REGENERATION.

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KEYWORDS

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Pre-treatment

Anther culture

Doubled haploid

Plant regeneration

Albinism



ABSTRACT

BARLEY ANTHHER CULTURE: DETERMINING THE OPTIMAL PRETREATMENT FOR GREEN PLANT REGENERATION.

C. MEYER

M.Sc thesis, Department of Biotechnology, University of the Western Cape.

Doubled Haploid (DH) Technology is an important tool for plant breeding and biotechnological applications as it accelerates the breeding cycle of plants by shortening the time required to attain homozygosity. Anther culture has become one of the most frequent and well-established methods for the induction of haploid embryogenesis and regeneration in barley. Anther culture is easily reproduced and workable for a wide range of genotypes. The aim of this study was to determine the optimal pre-treatment for barley anther culture. Three pre-treatments, 0.3 M Mannitol, 0.7 M Mannitol and a cold treatment with a moist cloth (CMC), were studied. The results suggest that CMC is the optimal pre-treatment to use for green plant regeneration. Anthers treated with CMC showed a higher response percentage than that of 0.7 M Mannitol and 0.3 M Mannitol. CMC also induced a significantly higher callus formation and green plant regeneration frequencies than 0.7 M Mannitol and 0.3 M Mannitol. Further research has to be conducted to further optimize green plant yields per treatment as well as reduce the number of albinos regenerated through barley anther culture.

DECLARATION

I declare that, *Barley Anther Culture: Determining the optimal pre-treatment for green plant regeneration* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Crezelda Meyer

Nov 2013

Signed: _____



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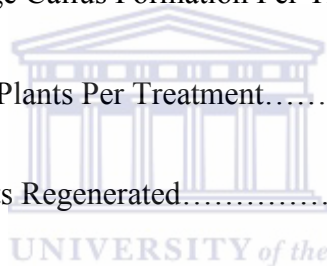
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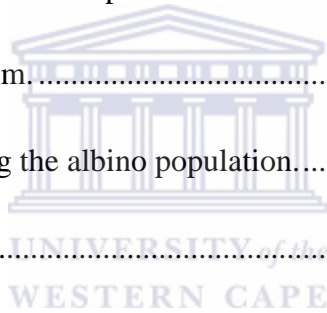
AC	Anther Culture
Alb	Albino
BAP	6-Benzylaminopurine solution
CMC	Cold treatment with Moist Cloth
DH	Doubled Haploid
GP	Green Plants
HSP	Heat Shock Protein
Man	Mannitol
MC	Microspore Culture
MS medium	Murashige and Skoog medium
PI	Pedigree Inbreeding
R&D	Research and Development
SABBI	South African Barley Breeding Institute
SSD	Single Seed Descent



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CHAPTER 1

INTRODUCTION

The use of doubled haploid plants in a breeding program serves as a useful tool for plant breeders to accelerate the production of homozygous lines of their crops (Jaquard *et al.*, 2009). Barley (*Hordeum vulgare* L.) is a member of the cereal family (Gramineae) and is mainly used for animal feed, human consumption and in the malting industry.

The research was performed at the South African Barley Breeding Institute (SABBI), situated in Caledon, Western Cape. The institute specialises in the development of new barley cultivars for the malting industry in South Africa. It is important for the barley breeders to provide superior malting barley varieties to the malting industry as they are always looking to deliver better malt quality to their ultimate clients, the brewers. The barley breeders are thus under immense pressure to deliver barley with good quality through their breeding program.

The institute found it necessary to incorporate new breeding techniques to reduce the time it takes to release a cultivar commercially. This was done through researching and implementing doubled haploid techniques in the Research and Development (R&D) program. However, it was found that there is a need to improve on the doubled haploid production technique to better suit the barley varieties found in the Southern Cape as well as the laboratory capacity.

Traditionally, it takes the institute 16 years for a new barley cultivar to be released experimentally, and approximately 18 years to be produced commercially (SABBI, 2013). Using doubled haploid barley plants reduces the time it takes to release a new barley variety

commercially by 4 years. This is because barley breeders can obtain a genetically pure line in one year or one crossing cycle through the use of doubled haploids. By using doubled haploids in a breeding program, the breeders will save valuable time and money in terms of labour, machinery and fuel compared to conventional breeding practices.

There are various protocols available to produce doubled haploids in crop plants. The protocols differ for each crop but the principle remains the same. To produce doubled haploids successfully certain steps has to be followed. Firstly the donor plant has to be grown in an optimal environment. Secondly the spikes from the donor plant have to be harvested and placed under stress to trigger the change of the microspore development pathway from gametophytic to sporophytic. Thirdly, depending on the type of tissue culture used, the anthers or microspores have to be placed on an induction media to induce embryogenesis. Thereafter, the embryos that have developed on the induction media are transferred to a regeneration media where roots and shoots develop. These plantlets are then transferred to a rooting media where it promotes vertical growth of the plantlet as well as root development. The final step in the process of doubled haploid production in barley is the maturing phase in a greenhouse. This is where the plant is grown in an optimally controlled environment within a greenhouse (Maluszynski *et al.*, 2003).

In barley, various scientists have recorded a spontaneous chromosome doubling capacity of 70% (Wan and Widholm, 1994). This means that 70% of all surviving green plantlets grown in the greenhouse will be doubled haploid plants. However, in the past, the institute have struggled to obtain that same success rate, the highest obtained was approximately 45% (Meyer, unpublished data). This success rate for a doubled haploid program that supports a commercial breeding program is not sufficient. Hence it became important to improve the technique to suit the needs of the institution.

The institute have experimented with various parts of the barley anther culture protocol. The institute have determined the optimal induction media, regeneration media as well as rooting media for the barley donor material. Through optimising the various medium protocols the institute have observed a remarkable increase in the barley doubled haploid plant numbers year on year. However, the institute believe that when the optimal pre-treatment for barley anther culture is determined that the regenerated barley doubled haploid plants would increase to an optimal percentage that would support the breeding program sufficiently.

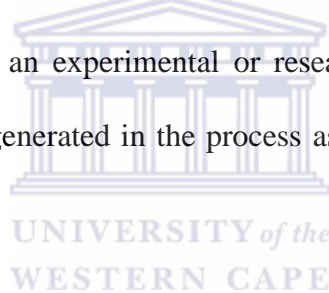
The objective of the study is to determine the most effective pre-treatment option for the production of double haploid barley through the use of anther culture for a breeding program. There are multiple parties that will benefit from obtaining homozygous barley lines in a shorter period of time. The breeding program will benefit as it will be able to produce the required amount of doubled haploid lines annually as to make the program sustainable. Also, the barley program would be able to produce a commercial variety in a shorter time-frame compared to conventional breeding methods. The commercial barley industry would benefit through producing superior malting quality local barley to the malting industry. Thus obtaining competitive market prices for their produce and making their farms lucrative. The malting industry would benefit through producing superior quality malt to the local breweries. The malting industry would be able to sell their malt at a competitive price to the breweries when using local barley for their malt instead of imported barley, thus rendering their business profitable. The breweries would in turn be able to produce superior quality beer at lower prices due to using locally produced malt.

To determine the optimal pre-treatment we have researched the various stressors used by previous researchers (Maluszynski *et al.*, 2003; Huang and Sunderland, 1982). The most common pre-treatment used is cold treatment, either used on its own or in combination with mannitol. We use a cold treatment (cold with humidity) as a standard, and will be testing the

use of mannitol in two forms (liquid 0.3 M Mannitol and solid 0.7 M Mannitol). The results of these would be recorded and analysed to determine the best stressor for barley anther culture in South Africa.

To measure the objectives, the number of calli induced per 100 anthers cultured, the number of green plants, sterile plants, albino plantlets as well as doubled haploids generated per 100 anthers cultured will be measured. As well as the number of green plants, albinos, doubled haploids and sterile plants generated per 100 calli cultured. The spontaneous chromosome doubling rate will be determined by the number of doubled haploids and sterile plants regenerated per 100 green plants.

It is important for us at SABBI to understand what factors affects albinism. We function as a production laboratory instead of an experimental or research laboratory, and thus need to decrease the number of albinos generated in the process as it negatively influence the green plant regeneration frequency.



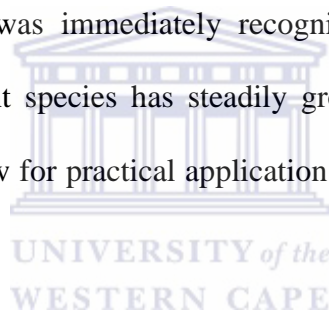
CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

2.1.1. The history of doubled haploid production

In 1921, A.D. Bergner observed the first natural sporophytic haploid whilst working with Jimson weed (*Datura Stramonium* L). This was reported in Science by Blakeslee *et al.*, in 1922 (Maluszynski *et al.*, 2003 and Germaná, 2011). The importance of haploids in plant breeding and genetic research was immediately recognized. Since then, the number of spontaneous haploids in different species has steadily grown. However the frequency of spontaneous haploids was too low for practical application in plant breeding (Maluszynski *et al.*, 2003).



In the early 1960's Guha and Maheshwari (1964) discovered that it was possible through *in vitro* culture of immature anthers to change the gametophytic development stage of microspores into a sporophytic one. This enabled them to produce embryos and plants with a haploid chromosome number (Germaná, 2011). This discovery made further and extensive research possible for anther culture that was predominantly successful in the *Solanaceae*, *Brassicaceae* and *Graminaceae* species. However, not all of the angiosperm crops of interest responded efficiently to embryogenesis induction. Barley (*Hordeum vulgare* L.), rapeseed (*Brassica napus* L.), tobacco (*Nicotiana spp.*) and wheat (*Triticum aestivum* L.) are considered to be the model species for use in microspore embryogenesis research due to their high regeneration efficiency (Forster *et al.*, 2007). There is however other scientifically or economically interesting species that still remain resistant to this type of *in vitro*

morphogenesis, such as *Arabidopsis* (Rock cress). Rock cress is a small flowering plant that is related to the cabbage and mustard family, many woody plants or members of the legumes family (Sangwa-Norreel *et al.*, 1986; Bajaj, 1990; Raghavan, 1990; Wenzel *et al.*, 1995; Germaná, 2006, 2009, 2011). In 1972, Thompson reported that the first doubled haploid crop plant, cultivar ‘Maris Haplona’ of rapeseed (*Brassica napus*) was released, followed by cultivar ‘Mingo’ in barley (*Hordeum vulgare*) in 1980 (Ho and Jones, 1980).

The interest in haploids was apparent in the organization of the 1st International Symposium “Haploids in higher plants”, which was held at Guelph, Canada, in 1974. Riley, one of the presenters, provided an interesting introduction to haploidy and covered some terminology as well as a historical perspective for those working in that area (Kasha, 1974 b; Kasha and Maluszynski, 2003 a).

Since then, extensive research has been done to establish efficient techniques for haploid and doubled haploid production with the purpose to increase varieties of different plant species. Approximately 300 new varieties of different plant species have been produced and Maluszynski *et al.*, (2003) provide a list of these in their book “Doubled Haploid Production in Crop plants: A Manual”. A variety of methods were used to obtain these such as chromosome elimination subsequent to wide hybridization, the ‘Bulbosum method’ by Kasha and Koa (1970), pollination with irradiated pollen selection of twin seedlings, *in vivo* or *in vitro* pollination with pollen from triploid plant gynogenesis, and pollen embryogenesis through *in vitro* anther or isolated microspore culture (Forster and Thomas, 2005; Germaná, 2011).

Microspore culture provides the most efficient and uniform route to mass production of doubled haploids. However it is technically demanding and requires an optimally controlled environment for donor plant growth. It is also more expensive to set up a laboratory for

microspore culture than for anther culture, as more specialised equipment is needed. Also, the risk of contamination is fairly higher for microspore culture, and the protocol calls for working under strict aseptic conditions (Maluszynski *et al.*, 2003).

Alternatively, *in vitro* anther culture is labour intensive and the cost involved is relatively high. However, it is technically less demanding and the risk for culture contamination is fairly lower. Anther culture in barley remains of high interest to the R&D program of SABBI, as it suits the facilities, small team and production needs.

2.1.2. Androgenesis

Androgenesis (or microspore embryogenesis) is defined as the condition of an embryo that contains only paternal chromosomes. Thus, androgenic haploids are produced from the nucleus of the male gametophyte only (Pandey, 1973 and Segui-Simarro, 2010). Microspore embryogenesis is the most commonly used method to produce doubled haploids. Microspore embryogenesis is based on the ability of a single haploid cell, the microspore, to de-differentiate and regenerate into a whole plant after it was exposed to some form of stress (Shariatpanahi *et al.*, 2006).

In 1973, Pandey stated that there may be two types of androgenesis. The first type of androgenesis is (1) Ovule Androgenesis. Ovule Androgenesis is where a male gamete is developed in the female cytoplasm, within the embryo-sac of an ovule, resulting in a paternal haploid. The second is (2) Anther Androgenesis. In Anther Androgenesis the female cytoplasm has no role in development of the paternal haploid. Furthermore Pandey suggests that (2) Anther Androgenesis can be classified into two forms, (2a) spore androgenesis and (2b) callus androgenesis. In spore androgenesis the sporophytes are initiated into embryos either inside or outside the anther. In callus androgenesis the sporophytes are initiated as

plantlets, or shoots and roots, from the callus. These are usually grown outside the anther. This type of androgenesis is commonly seen in barley.

It is known that the process of androgenesis can be separated into three phases or steps. The first phase of androgenesis is the pre-treatment or induction phase. During this phase the gametophytic pathway is switched to the sporophytic embryonic pathway. The second phase of androgenesis is the culture phase. During this phase the microspore is developed into an embryo. The final phase of the androgenesis is the regeneration phase. During this phase the embryos are developed into haploid plants (Jacquard *et al.*, 2003). These phases may not always be named the same by all the researchers, but the principle around the process of androgenesis remains the same.

In a review by Islam and Tuteja (2012), the various uses of abiotic stresses and pre-treatments and their impact on androgenesis was discussed. It highlights the relevance of research in optimising the protocols for developing doubled haploid plants for certain crop species. Optimizing the protocols for each crop would ensure obtaining the optimal results in all crops, instead of using a generic broad based protocol for all crops that may deliver results but not in the volumes desired. In simple terms, the anther culture protocol for wheat may be successfully used for producing doubled haploid barley plants. However, the average number of doubled haploid plants for regenerated for barley may be lower than that of wheat due to using the same protocol for both crops. Using the optimal protocol for a crop is particularly important for breeding institutes where the mass production of doubled haploids is required.

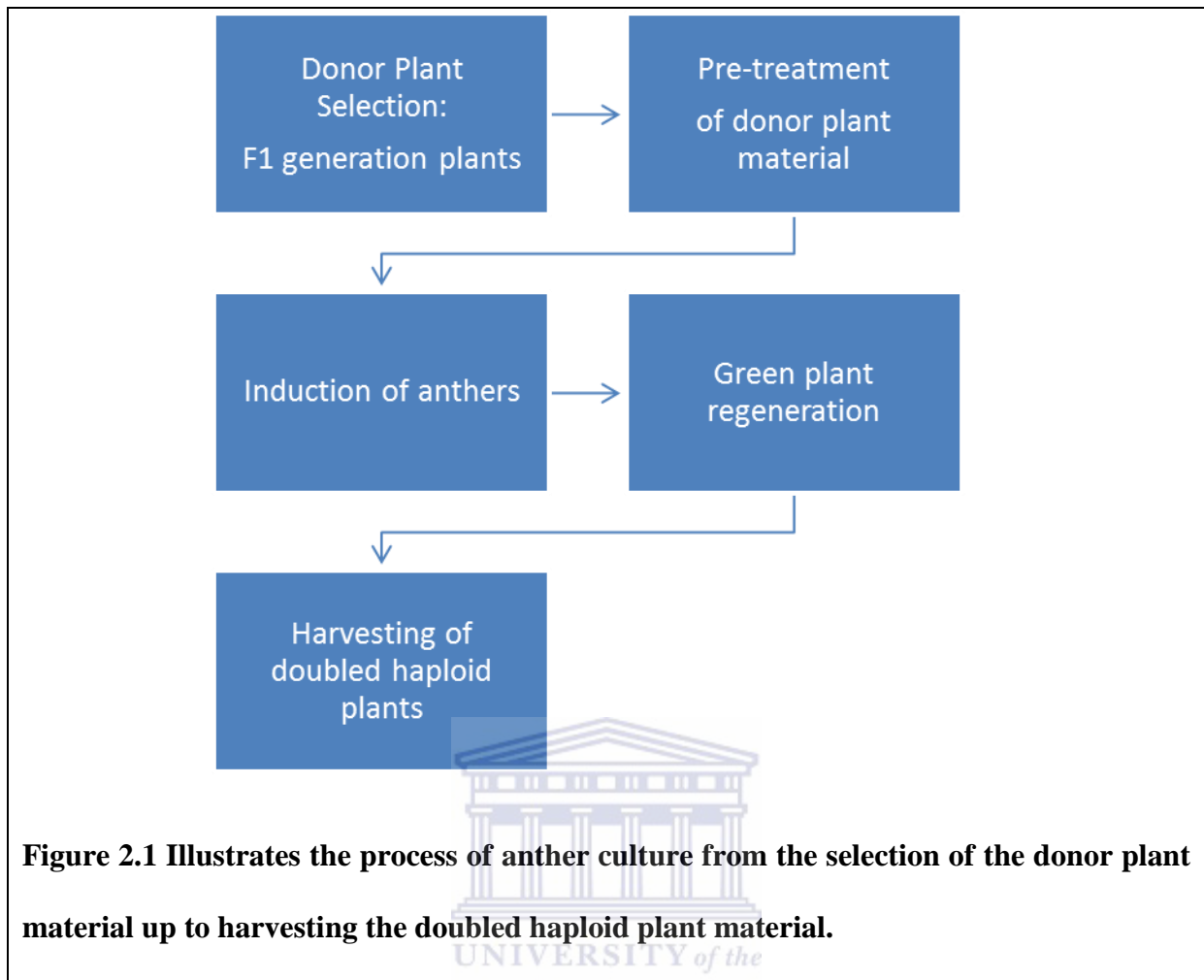
Breeding institutes may not always have the resources to research and experiment with all the available tissue culture techniques for their specialised crop, due to mass production requirements. Research institutes however, do not have the dilemma of producing mass volumes of doubled haploids to be used for production. Thus, research institutes have the

freedom to experiment with tissue culture techniques and optimising doubled haploid protocols.

2.1.3. Anther Culture

Anther culture is a plant culture technique in which haploid plants are obtained from isolated immature microspores of cultured anthers. The anthers are placed on a culture medium from which embryos will grow from the microspores. The embryos will differentiate into roots and shoots. The plantlets that are regenerated from this technique will have one set of chromosomes. Doubled haploid plants will be the result of spontaneous chromosome doubling. The technique was first reported by Guha and Maheswari in 1964.

The process for anther culture is depicted in Figure 2.1 and Figure 2.2. Figure 2.1 illustrates the various phases of anther culture whereas Figure 2.2 shows the effect of anther culture on the chromosomal number throughout the various phases. In Figure 2.1, F1 generation plants are used as the donor plants for producing doubled haploids. The spikes harvested or collected from the donor plants will be placed on some form of pre-treatment to initiate the process of androgenesis. The pre-treatment can be a cold treatment, starvation of anthers, heat shock or a combination of these. After pre-treatment the anthers are placed on an induction medium. This will induce the generation of calli or embryos which in turn will regenerate plantlets. These can be albino plantlets, doubled haploid plantlets or haploid plantlets. However, the albino plantlets will be discarded and the green plants will mature and be harvested when the seed sets are ripe. In Figure 2.2, the genetic process of Anther culture is shown through highlighting the chromosomal count throughout the process.



In Figure 2.2, the genetic process of anther culture is shown through highlighting the chromosomal count throughout the process. The donor plant has 14 chromosomes; however the anther only has 7 chromosomes. The anthers undergo some form of stress during the pre-treatment period. Where it will spontaneously double its chromosome number and give rise to calli. The calli will have 14 chromosomes too, and will consequently generate doubled haploid plantlets.

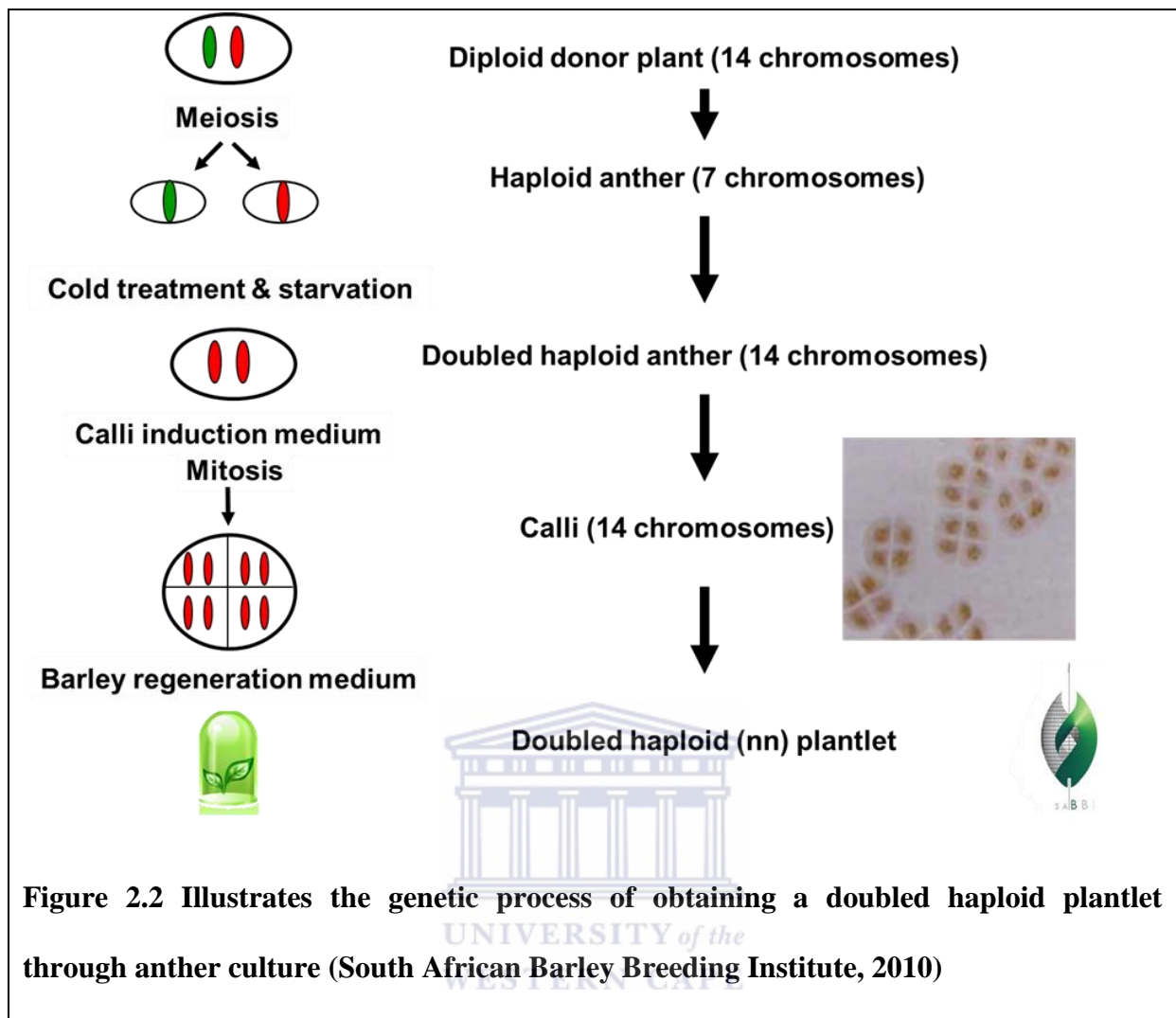


Figure 2.2 Illustrates the genetic process of obtaining a doubled haploid plantlet through anther culture (South African Barley Breeding Institute, 2010)

Most researchers (Jacquard *et al.*, 2003 and Kasha *et al.*, 2003 b) who work on doubled haploids agree that the state of the donor plants that will be used is critical to the success of anther culture and microspore culture. Any form of stress during their growth should be avoided. Stresses include dehydration, broad fluctuations in temperature and daylight, pesticide treatment and disease. However, Jacquard *et al.*, (2003) suggests using a preventative pesticide treatment at least once a week. They believed that this can be administered without having any significant changes in the yield.

Another important factor to consider with regards to the donor material is the tillers that the material is harvested from. It is recommended that primary tillers be used for anther culture

and microspore culture. Even though secondary tillers can be used, it does not deliver the same results as that for primary tillers. It has been reported that the use of secondary tillers in anther culture will result in a lower green plant regeneration frequency. However, a higher albino regeneration frequency (Jacquard *et al.*, 2006 and Kumari *et al.*, 2009) will be observed through the use of secondary tillers in anther culture. Over the past few years it has been observed in the laboratory of the South African Barley Breeding Institute that the tiller position does play a role in the quality of the embryos produced during anther culture. It has been observed in the laboratory that the age of the donor plant has a direct effect on the regeneration capacity. Also, higher contamination percentages and albino regeneration frequency was observed (Meyer, unpublished data). These observations, with regards to the effect of secondary tillers on green plant regeneration, by SABBI support the findings of Jacquard *et al.*, (2006) and Kumari *et al.*, (2009).

An interaction between organellar and nuclear genes controls the formation of microspores that are able to undergo embryogenesis (Heberle-Bors, 1985). Studies on potato (*Solanum tuberosum*) showed that the ability of microspores to undergo embryogenesis is a heritable recessive trait that is controlled by more than one gene (Chupeau *et al.*, 1998; Rudolf *et al.*, 1999, Smykal, 2000). Asakaviciute (2008) also stated that the morphogenetic potential of callus and embryoids is genetically predetermined. In 1982 Foroughi-Wehr *et al.*, identified four independent and differently inherited traits, namely callus induction, callus stabilization, plantlet regeneration and albino versus green plantlet formation. Petolino and Thompson demonstrated in 1987 that it is possible to improve responsiveness in maize breeding.

In 2006, Shim *et al.*, stated that efficient chromosome doubling is important in the production of haploids plants. The ideal time for chromosome doubling for the production of doubled haploid plants is during the first couple of cell divisions in the gametes (Kasha, 2004). The results obtained by Asakaviciute (2008) confirmed that the induction response in anther

culture, embryo formation, regeneration potential and the ratio of green regenerates to albino are controlled genetically. Their research showed that the *shd1* gene, which is found on the second chromosome of barley, affected the formation of green plants from embryos by 65%. Thus the formation of green plants in anther culture is dependent on the genetic predetermination of the donor plant.

The pollen development stage within the anther is a rather complicated factor and it greatly affects the success of anther culture. The developmental window of embryonic competence is species specific, but generally the period of sensitivity to inductive treatments is around the first pollen mitosis. For barley the best results is obtained when the microspores are in the mid- to late uninucleate stage (just before, during or after the first mitotic division). After the pollen grains begin to accumulate their storage reserves, they usually lose their embryonic capacity and follow the gametophytic developmental pathway (Pandey, 1973; Herberle-Bors, 1989; Raghaven, 1990 and Telmer *et al.*, 1992). This sensitivity may be due to the low metabolic content of microspores during the first mitotic division, as there is increased nuclear activity during this time. Pandey (1973) stated that it has been observed that embryos were unlikely to be produced by microspores in which starch accumulation has occurred after mitosis. Dunwell (2010) stated that the percentage of anthers producing microspore embryos and the number of regenerates produced per anther appear to be determined independently.

Taking into account all factors mentioned above, namely the tiller position and the pollen stage, it is imperative to acknowledge the role of genotype resistance on the efficacy of anther culture. Not all genotypes will show the same response to anther culture. Over the past few years it has been observed at SABBI that certain combinations would perform better than another using the same protocol (Meyer, unpublished data). Factors such as growth conditions and pre-treatment options was eliminated as variables to poor regeneration of doubled haploids because the donor plant material was sourced at the same time, and pre-

treatment used was identical for all. The only reasonable explanation for poor performance was thus attributed to genotype resistance.

2.2. The significance of doubled haploids in the malting barley industry

The malting industry in South Africa greatly depends on locally grown barley in the Southern Cape area. Figure 2.3 illustrates the various commercial production areas for barley in South Africa. The Southern Cape area is the biggest production area for barley in South Africa. The Northern Cape region produces a small amount of barley compared to that of the Southern Cape region. Between the two, it produces the majority of barley needed for the malting industry in South Africa. The beer beverage industry is driven by brand popularity and the maltsters always strive for a superior blend of malt to ensure that brand superiority. It is vital for the brewers, maltsters and barley breeders to work together in determining what the end result needs to be. To have the best beer it requires using superior malt. Superior malt begins with superior barley. Barley breeders thus have the responsibility to constantly produce new varieties that contains the properties the brewers require. Unfortunately, the conventional method of breeding may take an average 16 years to produce a new variety. This time delay is very costly and prevents breeders from responding rapidly to their clients' needs. Using doubled haploids in commercial breeding can overcome this because homozygosity can be achieved within one generation, thus reducing the time it takes to potentially release a new variety commercially.

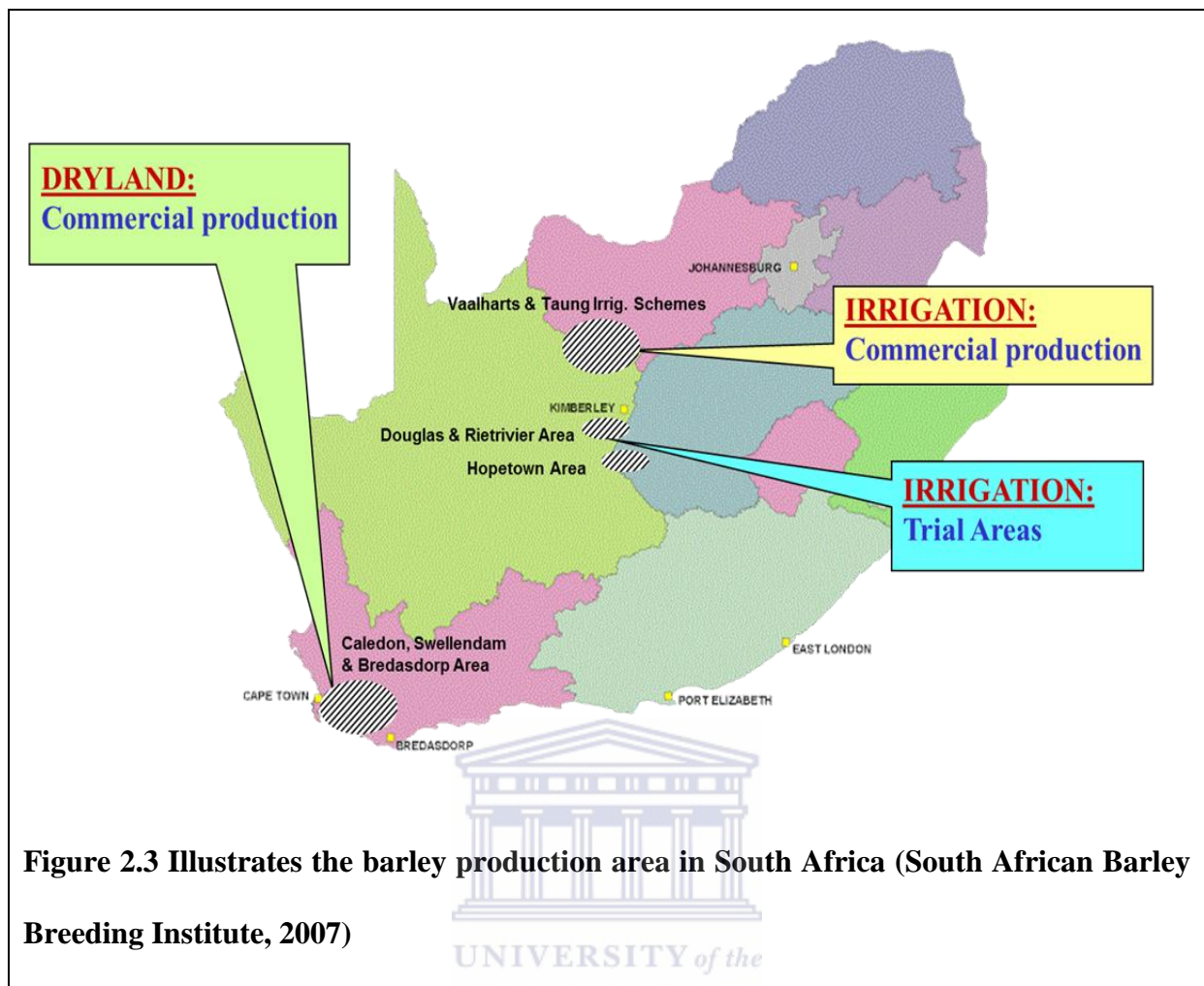


Figure 2.3 Illustrates the barley production area in South Africa (South African Barley Breeding Institute, 2007)

2.2.1. Conventional Breeding

There are many drawbacks in conventional breeding, besides the extensive time it takes to release a new cultivar. There is also the issue where in the early stages all the individuals are unique. There is a biasing effect of dominance on the phenotype during the early generations. Another drawback is that the non-competitive individuals are rejected by breeders and early selection is based on individuals grown in non-crop conditions without replication (Thomas *et al.*, 2003). In conventional breeding, selection of lines cannot be made until the lines approach homozygosity and enough seed is available for field trials. To help with the lagging of homozygous development, breeders can use another method, such as Single Seed Decent (SSD), to help speed up the development of homozygous lines. Single seed decent is a

method to rapidly fix genes in breeding lines. This involves planting one seed per plant (from the harvested first generation plants) for each generation seed. The advantage of using SSD is that a genetically stable generation can be obtained in a shorter time period than through conventional breeding practices

However, SSD also suffers from time delays and competitive interaction between plants (Thomas *et al.*, 2003). SSD also requires constant monitoring as the plants have to undergo certain stress periods and other environmental conditions.

Another disadvantage is that the labour cost of maintaining the SSD plants in a greenhouse will be higher than that of maintaining the doubled haploid progeny in the glasshouse. This is because the SSD plants will have to be planted multiple times before a genetically pure generation can be obtained. However, with doubled haploid plants it can be obtained within a single planting cycle and the resulting seed would be planted directly into the field for trial evaluation as Elite 1 trials. This has been observed numerous times at the South African Barley Breeding Institute.

As mentioned before, conventional breeding practices takes approximately 18 years before a new barley cultivar can be produced for commercial purposes. The South African Barley Breeding Institute (2013) shared Figure 2.4 to illustrate the timeline for the production of barley using the conventional barley breeding method. During the 1st year and 2nd year, crosses of the selected plants are made and the resultant F1 plants are grown in the greenhouse. During the 3rd to 6th year the F2 – F5 generation plants are planted in the field. During this period single plants are selected from the different generations. Year 7 through to the 12th year is the trial period, where the selected barley varieties are evaluated. The commercial evaluation period spreads over year 13 until the 16th year. During the final

release of the new barley variety, the seeds will be multiplied during the 17th year and will be available the following season for commercial production.

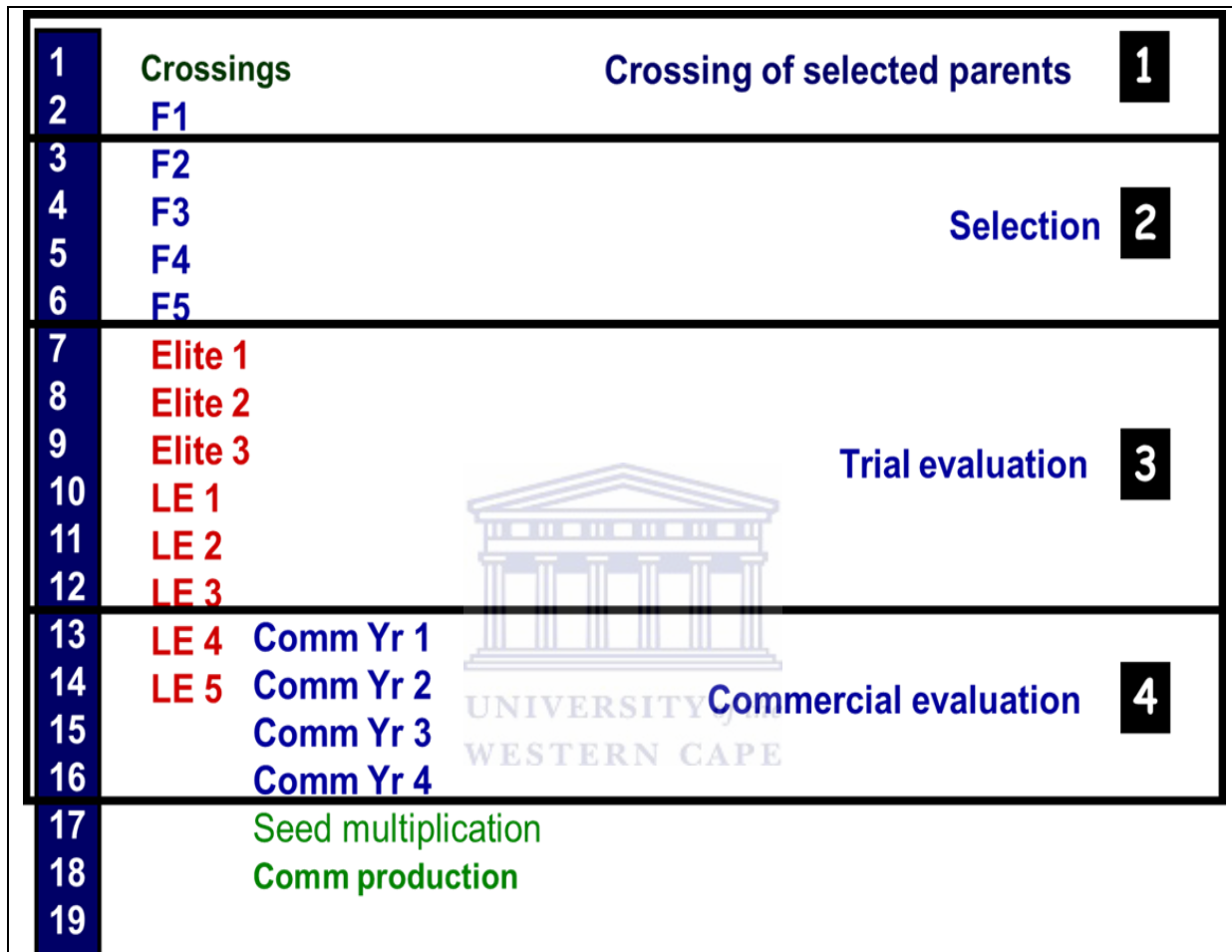


Figure 2.4 Illustrates the time-line for a conventional barley breeding program at SABBI (South African Barley Breeding Institute, 2013)

Using doubled haploids (DH's) in a breeding program can overcome many of the above mentioned problems. Homozygosity can be achieved in a single generation and it can be performed at any generation in a breeding program. Doubled haploid production saves the breeder valuable time as it is possible to cross parental lines and conduct field trials of the derived doubled haploid progeny within a two year period. Selection of superior doubled haploid progeny lines through Marker Assisted Selection prior to placing the lines in the field trails. Also, it is possible to conduct selections for disease resistance in the greenhouse thus spending resources only on the most promising lines. Since doubled haploid lines are pure, selection on these lines are more reliable as there are no dominance related effects (Thomas *et al.*, 2003).



2.2.2. Doubled haploid production vs. other methods

In 1976 Snape compared the theoretical properties of progenies produced through doubled haploidy and single seed decent and concluded that there were no difference between the two in the absence of linkage. However, when linkage is present, SSD would have a higher expectancy of recombination frequency. Snape suggested that the choice of method used would be dependent on whether the breeder wishes to preserve the linkage blocks, which would be achieved through doubled haploidy, or whether to break them, which can be achieved through SSD and Pedigree Inbreeding (PI). Numerous comparisons of populations produced by the different breeding methods from a wide range of species, mostly cereals, have been made (Snape, 1976). All agree that doubled haploidy does not lead to any bias populations. It has even been found that random doubled haploids can be compared to selected lines produced by PI (Friedt *et al.*, 1986). Thus there is no reason not to use doubled haploids in a breeding programme.

Another important consideration, when choosing a breeding method, is the number of lines per combination involved in the breeding programme. The reason for this is because the potential number of homozygous lines in a DH laboratory may be far too large for a breeding programme to handle effectively. In the case where segregating characters have high heritability, conventional breeding can decrease the number of potential lines in early generations (Thomas *et al.*, 2003) through single plant selection.

Many breeders rely on a mixture of these methods in their breeding programs. This is most evident in the commercial plant breeding sector. The South African Breeding Institute makes use of both conventional breeding as well as doubled haploid technology, although there is sufficient evidence to prove that DH is reliable. There are many programs or institutions that have switched exclusively to the use of DH's. However, the main reason for not using DH in a breeding program is due to the lack of resources. When setting up a DH laboratory, the program leader has to realise that in order for it to work, the skills of at least two technicians would be required whose sole purpose would be to produce doubled haploid plantlets. The selected genotypes require at least 100 lines per combination or alternatively 2000 lines per year to be beneficial to the breeding program. Thus, it is essential to follow the optimal protocol to produce the number of lines mentioned above. Besides the skilled labour required, one has to be cognitive of the financial expenditure of a doubled haploid laboratory. The financial expenditure would include specialised laboratory chemicals and reagents, laboratory equipment, laboratory consumables as well as the cost related to glasshouse maintenance. The doubled haploid technique requires the technician to work in aseptic conditions, which means that the environment, equipment and consumables have to be sterile.

It is important to realise that the number of progeny produced by doubled haploids would not be the same as for the F₂ population in a conventional programme. However, it has been

reported numerous times that the F2 single plant selection is at best random and that haploidy is competitive (Thomas *et al.*, 2003).

2.3. Barley Anther Culture

2.3.1. The importance of pre-treatment in Anther Culture

The purpose of applying pre-treatment is to create the stress necessary to change the development pathway of microspores from gametophytic to sporophytic. The most common pre-treatments used are cold treatment, where the donor material is kept at a low temperature. An alternative method is starvation through the use of mannitol. Some researchers have found that a combination of the two methods mentioned above increases the frequency of green plant regeneration for certain plant species (Shariatpanahi *et al.*, 2006).

Li and Devaux (2005) stated that the success of microspore embryogenesis is affected by the appropriate pollen stage. However, they also state that the pollen stage is associated with various pre-treatments, suggesting that each type of pre-treatment would have an appropriate pollen developmental stage associated to it that would enhance microspore embryogenesis. The authors stated that in mannitol pre-treatment it would be best to use microspores at the early-late uninucleate or late uninucleate stage. Furthermore, if using a cold pre-treatment it would be advised to use microspores at the early to early-mid uninucleate stage.

In 2006, Shariatpanahi *et al.*, published a review article based on the various stresses applied to donor material for the re-programming of microspores toward embryogenesis. They found that there were multiple approaches in anther culture as well as microspore culture. Some of these pre-treatments included chilling, heat application, high humidity, water stress, anaerobic treatment, centrifugation, sucrose starvation, nitrogen starvation, microtubule

disruptive agents, electro-stimulation and high medium pH. They concluded that researchers have yet to establish exactly how stress affects pollen differentiation. However, Shariatpanahi *et al.*, (2006) believed that stress seems to alter the polarity of the division at the first haploid mitosis. This involves the re-organization of the cytoskeleton.

Jacquard *et al.*, (2009) stated that stress treatment is vital to switch the developmental pathways. Jacquard *et al* stated as well that the quality of the stress treatment directly affect the success of doubled haploid production. The authors believed that there were several abiotic stressors, which could be used alone or in combination, to trigger microspore embryogenesis. These stressors could include cold shock, heat-shock, osmotic shock, pH variation but in barley microspore embryogenesis is mainly triggered by using a combination of cold- and osmotic shock. Their study showed that barley anthers are able to perceive abiotic stress conditions early during treatment, and respond by triggering various aspects of stress-related physiology. These included inducing an oxidative burst in anthers and stimulation of defence and stress-related gene expression.

2.3.1.1. Cold Pre-treatment

Temperature shock has been reported to be one of the most effective treatments. However the optimum temperature and pre-treatment vary with the genotype. Nitsch and Norreel first reported the use of cold pre-treatment on *Datura* anthers in 1973.

In 1982 Huang and Sunderland tested different pre-treatment methods for optimal callus and green plant production on the barley cultivar 'Sabarlis'. Huang and Sunderland found that there were greater callus yields at 4°C than 25°C, and that maximum yields were best produced at 4°C incubated over 3-5 weeks and 3-5 days for 25°C.

Huang and Sunderland (1982) also compared the effectiveness of using spikes and tillers. They found that spikes required a shorter pre-treatment than tillers and that spikes were more effective at 4°C than 20°C, and that individual spikelets were as effective as whole spikes.

Huang and Sunderland (1982) determined in their study that green plant regeneration was more effective from spikes than from tillers. Higher yields of green plants from 4°C than 25°C from both spikes and tillers were observed. However, green plant regeneration treatment time exceeds those for callus production. Approximately 60% of the calli formed gave rise to plantlets at 4°C. Amongst the plantlets regenerated every 3 plantlets were albinos and every 2 were green plants. This was equivalent to 5 plants per 100 anthers or 2 spikes. The ratio of green plants to albinos was lower for all.

In a review on cereal microspore culture by Jähne and Lörz (1995), they state that cold shock has a dual effect by providing interruption of microspore mitosis in order to produce embryos, as well as providing ample time for microspores to be nurtured by the anther. They identify that the most common cold shock protocol is 28 days at 4°C at high relative humidity in the dark. They emphasized that optimal pre-treatment is genotype-dependent.

Hou *et al.*, (1993) claimed that fresh anthers treated for 3 days in a mannitol solution was less effective for green plant production than a 28 day cold pre-treatment.

In 2001, Ritala *et al.*, published their work on barley isolated microspore culture in Finland, Europe. They tested 3 different cold pre-treatments, and the donor material was incubated at 4°C for up to 4 weeks in the dark. The only difference between the pre-treatments was the method of incubation. The methods were as follows; (a) collected spikes, covered by the flag leaf sheath, were wrapped in aluminium foil and kept in water; (b) harvested spikes were kept in petri dishes with a water insert (authors failed to mention the volume of the water); (c) harvested spikes kept on water-moistened filter paper in petri dishes. The researchers

observed a seasonal difference in the regeneration frequency. There was an increase in regeneration frequency during the period of March until October, with an average of 300 plants regenerated. During the period of November and February the regeneration frequency decreased to an average of 100 plants. They also observed that the best results were obtained for an incubation period of 4 weeks and that results decreased dramatically for incubation periods above 4 weeks. They also observed that there was no difference seen in the regeneration capacity with the addition of copper to the culture media. This study provided guidelines on choosing the optimal cold pre-treatment method for this study.

Szarejko (2003) was able to produce green plants from donor plants that were grown in a greenhouse as well as from the field. The spikes were placed in a 2 compartment petri dish with a few drops of sterile water in one of the compartments. The plates were incubated in the dark at 4-5 °C for 3-4 weeks. Szarejko obtained an average of 12.2 green plants per 100 anthers. The recalcitrant varieties only produced, on average, 2-3 green plants per 100 anthers where the more responsive varieties produced 50-60 green plants on average. However, Szarejko obtained a 70-80% spontaneous chromosome doubling for the regenerated plants. This particular study provides evidence on the impact of genotype dependence on green plant regeneration.

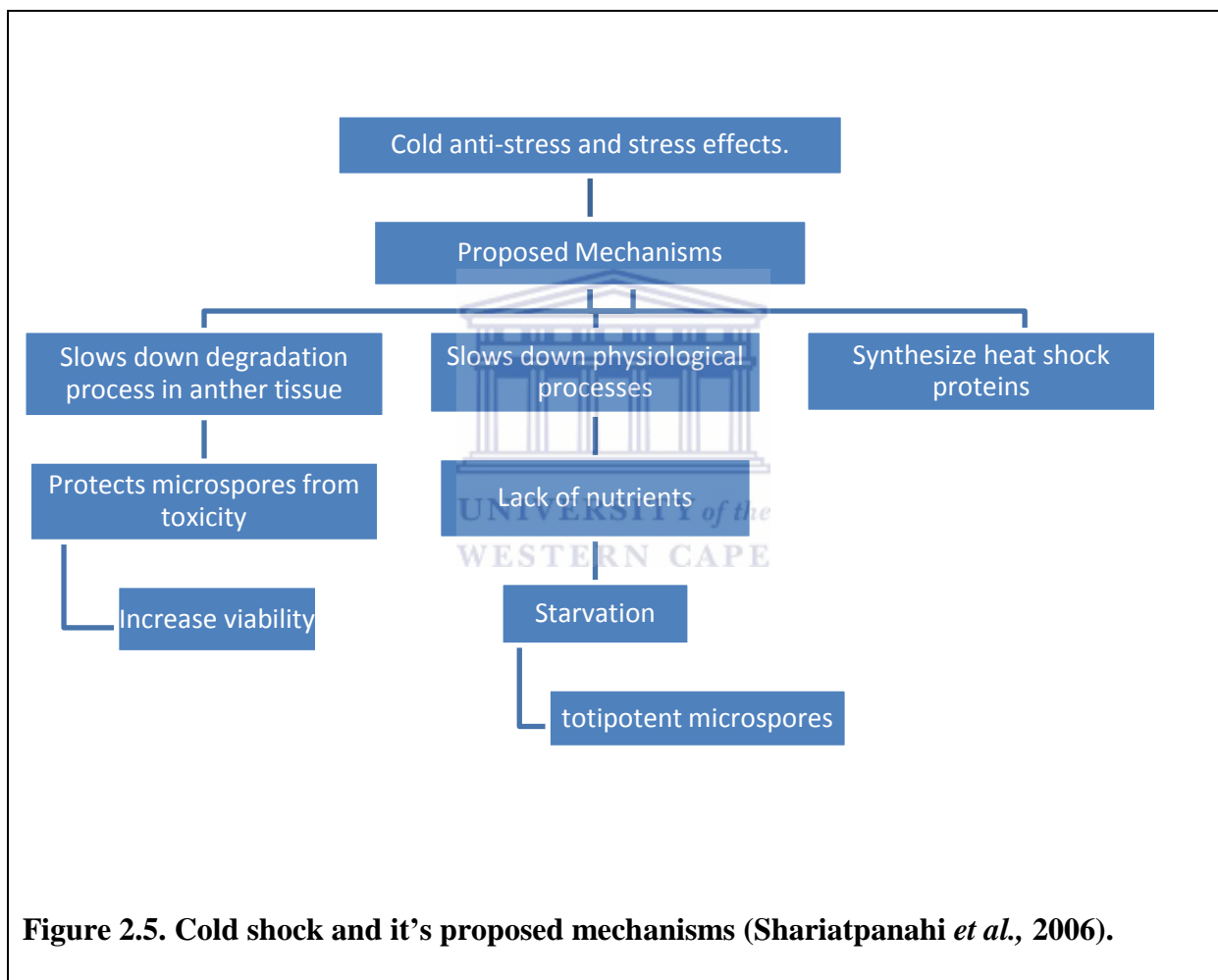
An interesting type of cold treatment was reported in a study published in 2004 by Patel *et al.*. The researchers used a 7 day pre-treatment that consisted of wheat spikes that were harvested at the mid-to late-uninucleate stage. It was placed in the dark at 4°C-5°C, with the basal end in a beaker of water. Their study was based more on the culture conditions than that of pre-treatment used. However, the results obtained from the study were rather impressive. They obtained an average of 296 microspore derived embryoids per 100 anthers and up to 71 green plants per 100 anthers.

Lazaridou *et al.*, (2005) studied the response of barley genotypes to cold pre-treatments and culture media. The cold pre-treatment that was used was fairly simple however the authors did not go into detail when describing the protocol. However, the spikes were kept at 4°C for 14 or 28 days. A surface sterilization protocol was used before the anthers were excised and transferred to the induction media. They used two different induction media, FHG and N6. They found that in all of the genotypes studied there were more embryoids produced from anthers cultured on FHG medium after a 28 day cold pre-treatment compared to those that were on a 14 day pre-treatment. The number of embryoids formed on N6 media, regardless of the time period on pre-treatment was far less than that on the FHG medium. This study suggested that FHG medium with a cold-treatment of 28 days at 4°C was the best pre-treatment to use for anther culture of barley.

In 2006, Shariatpanahi *et al.*, reviewed the effects of stress on embryogenesis. Shariatpanahi *et al.*, suggested that cold treatment slowed down the degradation processes in anther tissues. Thus protecting the microspores from the toxic compounds released in decaying anthers and ensuring a greater survival percentage of embryogenic pollen grains. The pre-treatment increases the frequency of endo-reduplication which leads to the increase of spontaneous doubled haploid plants. It was suggested that an increase in free amino acid may contribute to the alteration of microspores.

During cold shock two heat shock protein HSP genes, *tom66* and *tom111* are expressed to protect cells against chilling injuries. It is thought that an increase of green plant production may be due to the delay of plastid senescence. This is the state where the cells are aging and is still metabolically active, but can no longer divide (Shariatpanahi *et al.*, 2006). The authors summarized the proposed mechanisms in a flow diagram (Fig 2.5).

Jaquard *et al.*, (2009) stated that transcriptome changes were observed in barley microspores following cold/mannitol stress treatment of the anthers. Jaquard *et al.*, stated that it was shown that stress treatment blocked the expression of pollen-related genes. However, the stress treatment would stimulate expression of genes related to sugar metabolism, stress response and proteolysis. The results from the study led to the identification of molecular markers associated with the induction of microspore embryogenesis in barley.



In general it has been shown by numerous scientists (Oleszczuk *et al.*, 2006 and Shariatpanahi *et al.*, 2006) that a cold pre-treatment is an effective stressor. However, the time period associated to it may vary depending on the plant species. Kruczkowska *et al.*, (2002) discussed the various conflicting reports on the optimum length of cold pre-treatment.

The chilling periods reported varied from 14 days up to 42 days. Some concluded that a period longer than four weeks dramatically decreased the regeneration capacity. Other scientists state that a chilling period of 42 days had no negative effect on the regeneration capacity.

2.3.1.2. Mannitol pre-treatment

Jähne and Lörz (1995) states that a 0.3 M Mannitol pre-treatment for 4 days were found to be far superior than cold treatment. One of the reasons why it is considered to be superior is because mannitol creates osmotic stress which enhances callus formation (Hoekstra *et al.*, 1997).

In 1995 Hu *et al.*, published their work on isolated microspore culture of wheat. They tested the effects of pre-treatment, hormones and isolation methods on regeneration frequencies. They found that a 7 day treatment of anthers with a 0.4 M Mannitol solution with the macronutrients of FHG medium performed better than that of 4 days. The result was an increase of both microspore viability and the induction of cell division. Thus a large number of multicellular structures were observed.

Cistué, *et al.*, (1999) found that mannitol performed better than cold pre-treatment for low responding cultivars. It was determined that low responding cultivars or genotypes needed a higher concentration of mannitol. It was found that a 4 day pre-treatment of 0.3 M Mannitol on culture medium prior to a maltose medium was effective in regeneration of green plants. Also, it was found that 0.7 M Mannitol pre-treatment increased the number of dividing microspores as well as the ratio of green plants to albino plants. However, Cistué, *et al.*, (1999) reported that a mannitol concentration higher than 0.7 M Mannitol was not always more effective. This suggests that 0.7 M Mannitol is the optimal pre-treatment for anther

culture, because it had the highest response from all cultivars. However for low responding cultivars such as cv. 'Reinette' an increase in mannitol concentration from 0.7 M Mannitol to 1.0 M increased its percentage green plants regenerated for the cultivar. The results of their study show that it is possible to replace a longer pre-treatment with a mannitol pre-treatment with a higher concentration. Thus the study confirmed that the optimal concentration for a mannitol pre-treatment is genotype-dependent.

In 2001, Kasha *et al.*, published their research on isolated microspore culture of barley. They described in detail the various protocols they used for pre-treatment that they considered to be the most effective for isolated microspore culture. The first pre-treatment was the use of ice-cold (4°C) 0.3 M Mannitol. For this pre-treatment, harvested spikes were placed in a large petri dish and partially covered with 15 ml ice-cold 0.3 M Mannitol. The plates were sealed and kept in the dark at 4°C for 3 to 5 days. The second pre-treatment that they believed to be effective was to place the harvested spikes with 0.5 ml sterile water in a petri dish at 4°C for 3 to 4 weeks. They stated that they found the combination of cold (4°C) plus 0.3 M Mannitol to be very effective and results in a higher green plant regeneration than that of other pre-treatments.

Jacquard *et al.*, (2006) added mannitol and copper sulphate during anther pre-treatment and culture, which separates their study from the rest. For the pre-treatment they placed the anthers of the winter barley variety 'Igri' in a 5 cm diameter petri dish with 10 ml media containing mannitol (62 g/l). This provided an osmotic pressure of 180 mosm/l. Anthers were incubated in the dark at 4°C, 80% relative humidity for 3-4 days. After pre-treatment the anthers were transferred to modified culture medium containing mannitol (36 g/l) and copper sulfate (2.5 g/l). It was found that the addition of the mannitol and copper sulphate to the modified culture medium increased the yield of doubled haploids. The use of mannitol for 4 days seemed to remove the formation of callus during anther culture and enhance embryo

formation instead. The addition of copper sulphate enhanced both the quantity and quality of the regenerated plantlets. It was found that 72.3% of the anthers responded to the pre-treatment. They obtained 1245 plantlets (albinos and green plants) that generated per 100 responding anthers and 1111.1 green plantlets per 100 responding anthers. Thus they obtained an 89.2% green plantlet production per 100 responding anthers. The results obtained for the winter type barley fluctuated significantly throughout the year and they obtained better results during spring and early summer, even though the growth conditions were maintained throughout the year.

Cistué *et al.*, (2003) also took an alternative approach to anther culture in barley by using an unconventional pre-treatment method. They used mannitol as a substitute for a metabolizable sugar. The anthers extracted from the harvested spikes were placed in a 65 mm petri dish on a pre-treatment medium. The medium contained 0.7 M Mannitol, 40mM CaCl₂ and 8 g/L Sea Plaque agarose. The researchers increased the concentration of mannitol from 0.7 M to a 1-1.5 M for the recalcitrant or resistant genotypes. The plates were incubated at 24 °C in the dark for 4 days. The results obtained from using this protocol were disappointing. The researchers planted 26.6 green plants per 100 anthers in soil. From those planted, 10% died, 43% were sterile and 10-20% was haploids. The researchers believed that one of the reasons for the poor performance was due to the difficulties they experienced controlling the photoperiod and temperature. They stated that 15-20% of the sterile plants they obtained were due to the difficulties controlling the growing conditions during the hot season.

Kasha *et al.*, (2003 b) compared the effects of three pre-treatments on isolated microspore culture. The pre-treatments that was compared was as follow; (1) harvested spikes were partially immersed in ice-cold 0.3 M Mannitol and incubated in the dark for 4 days at 4°C. The second group (2) of spikes were partially immersed in 0.3 M Mannitol and incubated at room temperature for 4 days; and (3) the harvested spikes were placed in a Petri dish with 0.5

ml of sterile water at 4°C for 3-4 weeks. The researchers observed an embryo count per plate that had a range of 5.5-15000. They found that the regeneration frequency of albino plantlets were high, however they believed that the frequency of embryos and the regeneration percentage was high enough that the albino percentage did not negatively influence the production of doubled haploids.

Muñoz-Amatriaín *et al.*, (2006) suggested that the optimal pre-treatment for barley is carbohydrate starvation with or without a combination of cold. The treatment was based on the incubation of anthers in a medium with a non-metabolizable carbohydrate such as mannitol. Thus during mannitol treatment there is a decrease of nutrient availability due to the lack of photosynthesis.

In 2006, researchers Oleszczuk, Sowa and Zimny found in their study that the highest number of green plants was obtained on 0.3 M Mannitol at 32°C for 24 hours by microspore culture. The review by Shariatpanahi *et al.*, in 2006 agrees with previous research which stated that carbohydrate starvation, like through mannitol, and is an effective inducer of embryogenesis in isolated microspores for a variety of crops. Cytoplasmic and nuclear changes have been observed. These changes include dedifferentiation of plastids, dilation of the generative cell wall appearance of the large vacuole, loss of nuclear pores in the vegetative nucleus, and changes in chromatin and nuclear structure. Also phospholipid composition and a decrease in the size of the nucleolus were observed. During treatment qualitative- and quantitative changes in protein kinase activity can be observed. In short they proposed three mechanisms to starvation; the synthesis of (HSP), change in protein kinase activity and change in the plasmalemma phosphoprotein composition.

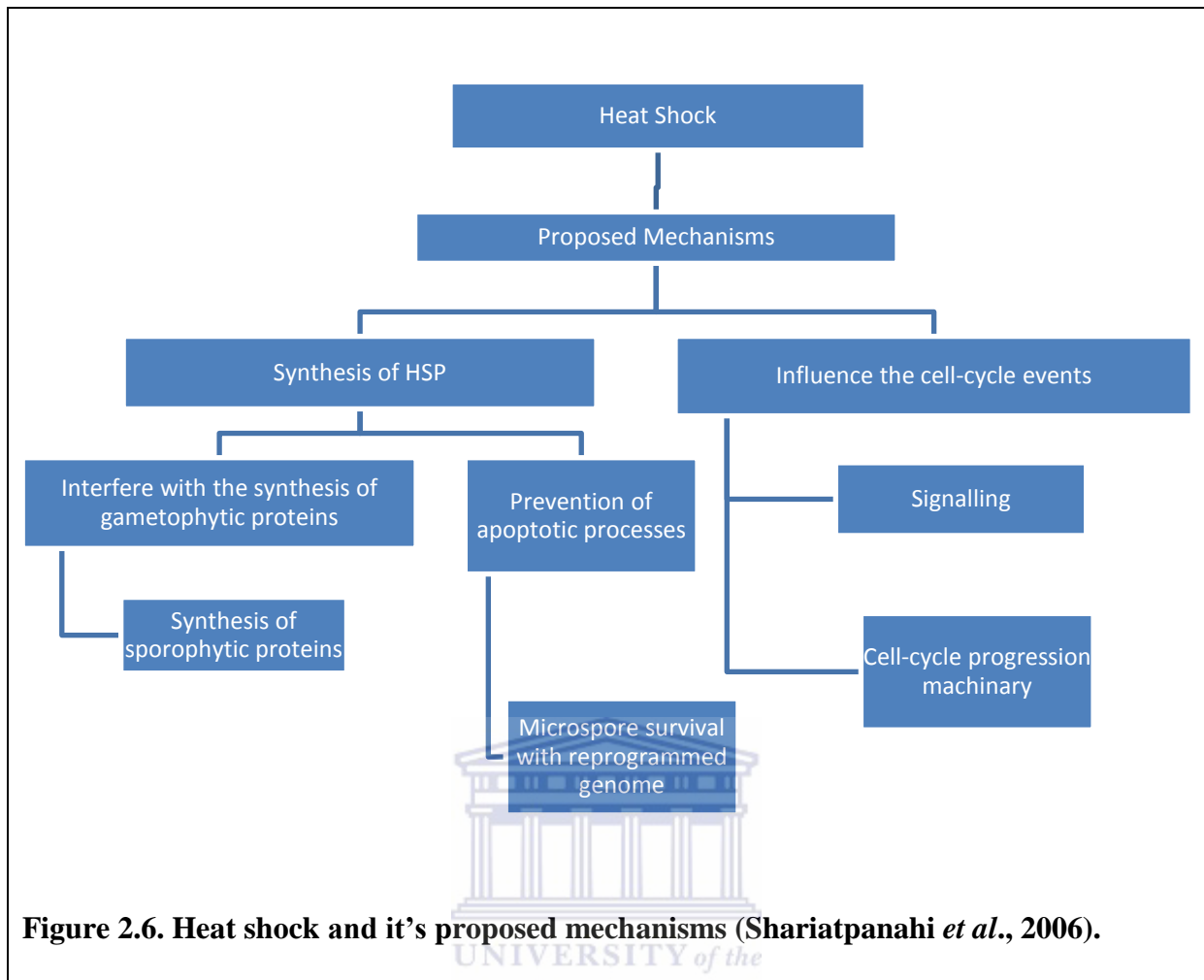
In 2008, Asakaviciute tested androgenesis in anther culture of Lithuanian spring barley and potato cultivars. For the pre-treatment the researcher placed 30 anthers into 5 cm diameter

petri dish. The dish contained 10 ml of Mannitol (62g/L). The plate was incubated at 4°C in the dark for 4 days at 80% relative humidity. After the pre-treatment phase the anthers were transferred to the induction media, without rinsing, and incubated as per protocol. Asakaviciute found that the resulting responding anthers ranged from 0.3-22.7%. They had an average of approximately 200 embryos per 100 anthers, with a high of 580 embryos per 100 anthers and the lowest 162.5 embryos per 100 responding anthers. Asakaviciute's research suggests that the anther response is predetermined by genotype.

2.3.1.3. Heat pre-treatment

The advantage to using heat pre-treatment is that the incubation period is shorter compared to cold pre-treatment. The heat pre-treatment incubation is usually at 26°C to 32°C for several hours or a few days, whereas cold treatment is carried out, on average, from 2-4 weeks depending on the protocol used (Shariatpanahi *et al.*, 2006).

Heat pre-treatment has been used to trigger embryogenesis of various crops such as rapeseed, wheat, tobacco, eggplant and other crops. It has been shown to initiate changes in the microtubule and cytoskeleton in cultured *Brassica* microspores. Shariatpanahi *et al.*, (2006) further state that heat shock affects the appearance of pre-prophase bands and that structural changes occur. These structural changes include electron-dense deposits at the plasma membrane or cell wall interface, vesicle-like structures in the cell walls and organelle-free regions in the cytoplasm. Several heat shock proteins are synthesized, like *HSP20* that may inhibit apoptosis. Figure 2.6 by Shariatpanahi *et al.*, (2006) summarize the proposed mechanisms for heat shock.



In 1996, Touraev *et al.*, reported that a combination starvation and heat stress improved the induction of embryonic microspores in excised wheat anthers. They claimed that using their method improved the production of doubled haploids in recalcitrant species which would eradicate genotype dependency on crops such as wheat. They stated that their method was simple, effective and reproducible. The highest frequencies of embryonic microspores were obtained after spike treatment. The spike treatment that showed the highest embryonic microspore frequency was where the whole spike were starved at 33°C for 4 days in a 10 cm petri dish. These were kept under humid conditions and to achieve that the researchers placed a 3 cm petri dish containing 2 ml of sterile water into the larger dish containing the spikes.

2.3.2. The importance of media composition in Anther Culture.

It has been shown in previous research (Hussein *et al.*, 2004) that the media composition for the induction as well as the regeneration media plays a vital role in green plant regeneration. Another important factor to consider is how the medium is prepared, i.e. whether it is filter sterilized or autoclaved.

In 2002, Mendoza and Kaeppler published an article based on the research they performed on the effects of auxins and sugar on callus induction and plant regeneration on wheat (*Triticum aestivum* L). They found that the type of sugar used in the media had a significant effect on callus production. They stated that the replacement of sucrose with maltose increased the mean callus fresh weight and that it promoted callus development activity.

Hussein *et al.*, (2004) investigated plant regeneration on four different media compositions using six barley genotypes. They found that regeneration was improved separately preparing certain components of the culture media and using maltose as the carbon source. In their article they discussed the effect of genotype-dependency on cereals and how it affects the ability of the tissue to regenerate whole plants. They believed that certain genotypes negatively impact the genetic transformation of most barley cultivars, but that this could be overcome if media composition was optimized. They also believed that commercial barley was one crop that is particularly difficult to transform genetically due to the absence of efficient regeneration systems or culturing protocols.

Hussein *et al.*, (2004) also discussed previous research done on media composition. They stated that an increase in copper concentration, the addition of 6-Benzylaminopurine solution (BAP) and maltose substitution to the regeneration media enhanced the regeneration frequency of barley.

In 2008, Redha and Talaat attempted to increase their green plant regeneration in wheat (*Triticum aestivum* L) genotypes with high albino regeneration frequencies. This was done through supplementing the culture media with various supplements. Maltose and sucrose were compared to each other. The use of maltose in the media induced higher frequencies in producing calli than sucrose. A small percentage of anthers produced green plants but had an increase in yield of green plants per 100 anthers than sucrose. They found that the beneficial effect of maltose on calli induction may be due to the starvation effect on microspores in culture. They found that this was due to the slow hydrolysis of maltose and maintenance of a high osmolality of maltose in medium.

Another supplement to the induction media that was tested was colchicine ($C_{22}H_{25}NO_6$) (Redha and Talaat, 2008). Colchicine is often used to induce polyploidy in plants. It inhibits microtubule polymerization or formation during cell division by binding to tubulin. Supplementation with Colchicine in the culture media showed fewer calli produced per 100 anthers but an increase in frequency of green plants per 100 calli. They deduced that the increase in the green plants produced per 100 calli may have been due to a higher quality of the calli induced. Thus, the ability to regenerate green plants versus albinos is suspected to be dependent on the quality of the calli or embryos developed during induction and not the quantity thereof.

Zamani *et al.*, (2000) tested the effect of colchicine supplementation in wheat (*Triticum aestivum* L.) anther culture media. They treated genotypes with 0.03% colchicine for three days at the beginning of microspore induction. In some of the genotypes it caused a significant reduction in the development of embryos. Colchicine also decreased the plant regeneration frequency in some of the genotypes. However, a significant increase in the fertile plant regeneration percentage was observed in all genotypes. Thus in summary, the

addition of colchicine in the culture media may decrease the formation of embryos and plant regeneration, but it increases the number of fertile plants regeneration in wheat.

Various reports have shown that there are differences between barley genotypes with regards to spontaneous chromosome doubling. It's been reported that the addition of copper sulphate and zinc sulphate to anther pre-treatment increases regeneration capacity for recalcitrant species (Hussein *et al.*, 2004 and Ferrie and Caswell, 2011).

An increase in regeneration frequency was observed when modifying standard MS medium composition (Dahleen and Bregitzer, 2002). The scientists observed the improvement when they increased the concentration of H_3BO_3 (0.75mM) and decreased $FeSO_4$ to (0.5mM). The changes to the medium increased the regeneration frequency between 4 to 40 times as much as the original MS medium.

In 1998, Bregitzer *et al.*, published their study that suggested that genotype dependency can be overcome by developing and using genotype-specific protocols to enhance plant regeneration in barley. Even though their results were ground breaking it is however difficult to implement in production laboratories. It would require an experimental phase to determine the optimal pre-treatment, induction medium composition and regeneration medium composition for each genotype before it can be implemented on a full scale basis. A lot of time would be wasted if such a system would be implemented.

For the purpose of our research we chose to use filtered induction and regeneration media as it has proven to increase the frequency of green plant regeneration.

2.4. Albinism in Anther Culture

Albinism is defined as a lack of pigmentation and it can be seen in various forms depending on the severity of pigment loss, as well as the nature of the missing pigments. In plants, albinism is characterized by a lack of chlorophyll in normally green tissue. The tissue of the plant will appear white in colour due to the lack of chlorophyll. Without chlorophyll, plants die prematurely due to rapid exhaustion of food reserves because chlorophyll is required for the capture of light energy for photosynthesis (Kumari *et al.*, 2009).

Albinism is often encountered by plant breeders when they create wide hybrids or when using tissue culture technology such as anther culture or isolated microspore culture. Breeders use wide hybridization of distant relatives to produce doubled haploid plants for rapid development of homozygous lines, but because albinos are so common in doubled haploid techniques it hampers the production on a commercial scale (Kumari *et al.*, 2009).

Albinism has been reported in anther culture of many plant species such as barley, wheat, soybean and tobacco, to name a few (Kumari *et al.*, 2009). Jacquard *et al.*, (2006) obtained albinos during their study on barley, regardless of optimizing their anther culture protocol by adding mannitol and copper sulphate. This suggests that barley microspores and derived structures are particularly sensitive to albinism.

2.4.1. Factors affecting Albino plant regeneration.

There are many factors that can influence the regeneration of albino plants. Factors include genotype, environment, meiotic abnormalities, hormonal imbalances and various others. Incompatibility between the plastid- and nuclear genome is one of the major factors inhibiting chlorophyll formation (Yao *et al.*, 2000). Many researchers have reported that the growth conditions of the donor plant, the developmental stage of the microspores at isolation,

the composition of the nutrient media as well as the culture conditions play a vital role for regeneration capacity of green plantlets (Kumari *et al.*, 2009). Green and albino plantlet regeneration depends on a number of factors that include donor plant growth conditions, culture temperature, cold pre-treatment, sucrose concentration in combination of growth hormones and the development of the microspore. In anther culture the age and size of the embryos or calli have an effect on regeneration capacity and it has been found that older embryos regenerate fewer green plantlets (Kumari *et al.*, 2009).

2.4.1.1. Environmental factors

There are various environmental factors that influence the regeneration of albino plantlets. These environmental factors include, but are not limited to, temperature, light intensity and media composition. Previous research has shown that the ratio of green to albino plants in tissue culture can be altered by temperature. In 1927, Collins reported that low temperature (<15°C) induced albinism in oat and barley whereas Hallqvist (1923) showed that high temperature (>15°C) induced pigment formation in albinos of barley.

Low light intensities have promoted albinism in certain fruits such as strawberries although it has also shown a decrease in frequency of albinos during the regeneration of Timothy grass (*Phleum pratense* L.) anther culture (Kumari *et al.*, 2009).

Culture medium composition plays a vital role in green plant regeneration as it affects chlorophyll formation in leaves. It is however, difficult to identify the individual effects of different media components. One such component is sucrose. Sucrose is essential for any tissue culture medium. The quantity of the sugar content affects both the amount of carbon available to cells as well as the osmotic environment. The type of sugar used, whether it is sucrose, maltose or mannitol, have an effect on metabolism and it was found that by

increasing the sugar requirements of *in vitro* plants, one can increase the chance of survival (Kumari *et al.*, 2009). Cistué *et al.*, (1994) found that there is a positive correlation between mannitol concentration and green plant regeneration in barley anther culture. Other research show that adding mannitol, glucose and indole acetic acid to the induction media promotes chlorophyll formation, resulting in more green plants (Kumari *et al.*, 2009).

Although studies show how different environmental conditions influence the recovery of albino and green plants, there is not enough substantial evidence to establish a specific relationship between media composition and the occurrence of albinism.

2.4.1.2. The effect of spike and tiller position.

In barley anther culture, the frequency of albinism is dependent on genotype as established by Larsen *et al.*, (1991). The production of green- and albino plants from microspore culture is also affected significantly by the annual cycle of donor plants and the spike position (Jacquard *et al.*, 2006). A particular hormonal balance in the spike of the second tiller influence plastid behaviour in microspores. Spikes on the main stem produce fewer albinos than that from the late tillers furthest from the main stem. This may be due to competition for nutrients or because some hormones are more concentrated towards the centre of the root zone (Kumari *et al.*, 2009).

2.4.2. The genetics of albinism.

Considering the research done on albinos so far, research suggests that albinism is a genetic trait that is recessive in nature. Albinism will persist in populations at a low frequency and cannot be completely eradicated from a population. Researchers have found that albinism is

governed by one or two genes each with two alleles. Two more loci have been reported in banana hybrids in 1994 by Ortiz and Vuylsteke.

When albinism occurs chlorophyll is not synthesized. This results in the plastids losing their internal membranes, accumulated lipids and prolamellar globules. It has been shown that microspore plastids are affected as early as the anther sampling stage. The normal pattern of plastid development is not recovered during the androgenic process (Caredda *et al.*, 2000).

It has been reported that albinism may be correlated to the physiological state of plastids in the microspore at the time of donor plant sampling.

In 2005, Lazaridou *et al.*, published a study based on the response of barley to cold treatments and culture media. They tested the response of anthers to two types of induction media (FHG and N6) after cold-treatments at two different time periods (14 days or 28 days). They found that green plant and albino regeneration was absent after induction on N6 medium. However, high green plant and albino regeneration frequency was observed on FHG medium especially after the 28 day pre-treatment. The researchers believed that the high percentage of albino plants was genotype dependent and that the type of induction media also plays a role in the regeneration frequency of albino plantlets.

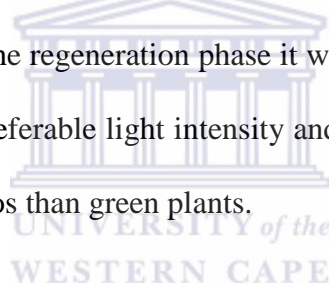
2.4.3. Methods for decreasing the albino population.

Albinism can be overcome, or managed on a cellular level by manipulating ploidy in hybrids (Kumari *et al.*, 2009). This can be done through polyploidization of the genome following hybridization. Thus studies suggest that plastome-nuclear genome incompatibility can be overcome through manipulation.

Acquaah (2007) stated that the regeneration frequency of albinos can be reduced through a high nitrogen content of the donor plant as well as exposure to a low temperature during meiosis. Acquaah (2007) also believed that the above mentioned factors would enhance the chance of green plant regeneration in anther culture.

The frequency of albinism can be amplified through favourable environmental conditions. Thus the frequency of albinism can be decreased by manipulating the *in vitro* culture conditions through appropriate application of plant nutrients and growth regulators. It can also be decreased through deletions in the plastid genome (Kumari *et al.*, 2009).

The most cost effective way of controlling the albino population and increasing the green plant population would be to grow the donor plants in a stress free environment and to control the culture temperature. During the regeneration phase it would be ideal to grow the embryos at a higher temperature with a preferable light intensity and discarding the older calli as they generate higher volumes of albinos than green plants.



CHAPTER 3

METHODOLOGY

Hordeum vulgare L. F₁ hybrids were chosen by the barley breeders from the South African Barley Breeding Institute for their excellent agronomic characteristic potential, to be used as the doubled haploid donor plants. The cross combination for the F₁ hybrids cannot be disclosed as it would infringe upon the intellectual property agreement between the South African Barley Breeding Institute and the University of the Western Cape. Therefore the F₁ hybrids were given a genotype code (CM 01-CM 05) which was used throughout the project and during the discussion of the results.

The protocol used for barley anther culture is relatively easy, and a brief overview of the experimental design is shown in Figure 3.1. The donor plant material were harvested within the glasshouse and placed on a pre-treatment. Three pre-treatments were used (1) Pre-treatment A: 0.3 M Mannitol; (2) Pre-treatment B: 0.7 M Mannitol and (3) a cold treatment with a moist cloth (CMC). After the respective incubation periods were complete for each of the pre-treatments used, the anthers were transferred to an induction medium. The induced calli formed on the induction medium was transferred to a regeneration medium until roots and shoots were formed. Albino plantlets was recorded and discarded during the selection process of green plants to be transferred. These small green plantlets were transferred to a rooting medium until the plantlets were developed well enough to be transferred to the glasshouse where it matured and ripened. The ripened barley plants were harvested, and each plant that produced seed was counted as a doubled haploid plant. The green plants that did not produce a seed set were counted as a sterile plant.

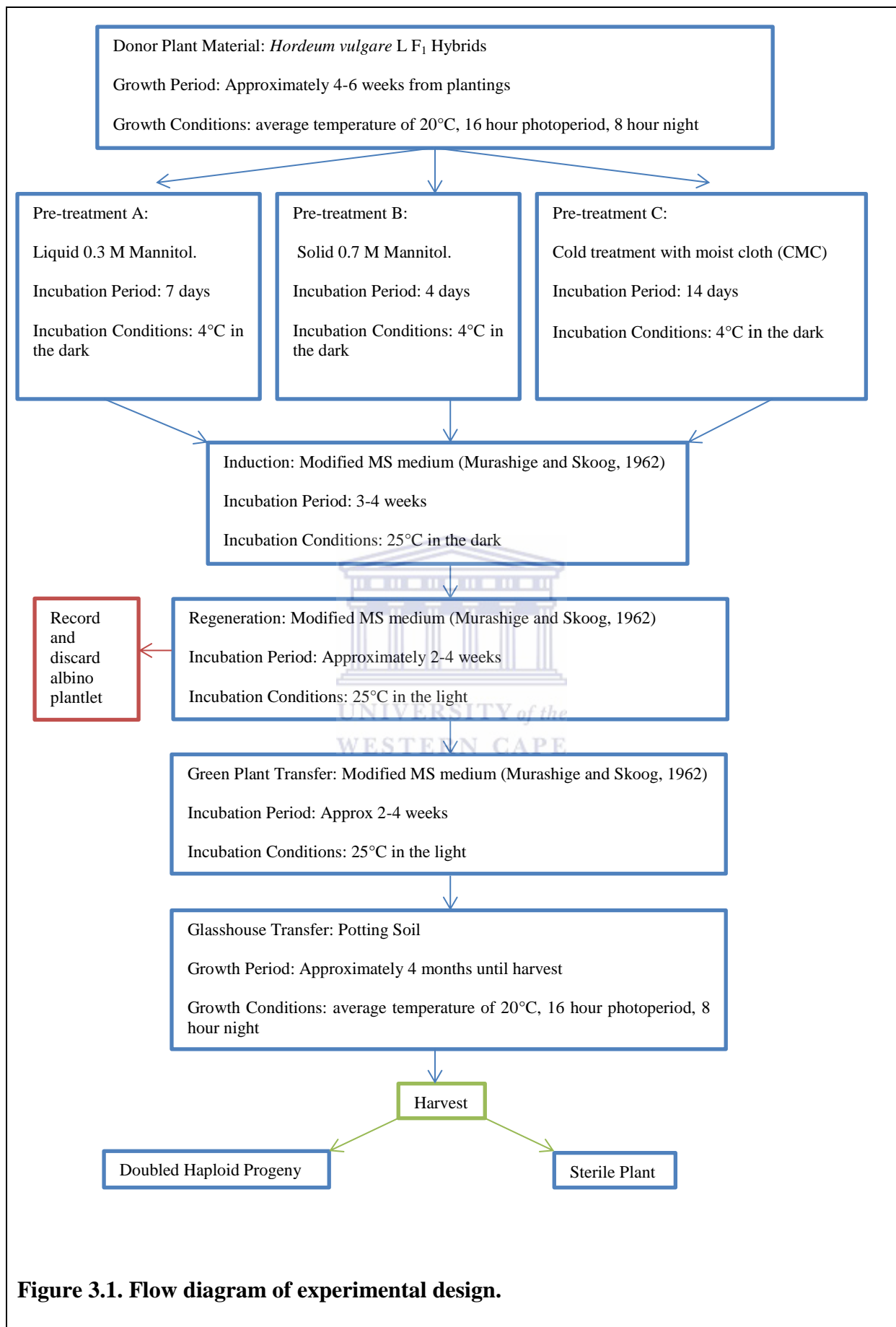


Figure 3.1. Flow diagram of experimental design.

3.1. Plant Material and Growth Conditions.

Hordeum vulgare L. F₁ were grown in the greenhouse of the research institute. The donor plants were grown in potting soil with a 16 hour photoperiod and an 8 hour night in a greenhouse kept at an average temperature of 20°C and 80% relative humidity. The donor plants were watered only until they reached the three-four leaf stage (± 3 weeks), after which they were fertilized with a combination of 2 ml Polaris (2×10^{-3} ml/L) (N 8.6%, Ca 7.3%, Mg 2.3%) per litre, 2.5 ml Orion-plus (3×10^{-3} ml/L) (N 1.0%, P 1.0%, K 10.1%, Mg 0.4%, S 0.5%) and 5 ml Trelmix (Kompel) trace element solution (1×10^{-5} ml/L) (Fe 21.3 g/L, Cu 3.0 g/L, Mn 3.1 g/L, Zn 2.3g/L, B 1.0 g/L, Mo 0.3 g/L, Mg 0.3 g/L). The fertilizer application frequency was determined by the season, thus during the spring and summer season the donor plants received fertigation twice a week with one application of water; and during the fall and winter season the donor plants received fertigation once a week with one additional application of water.

The spikes were harvested when the microspores were at the mid- to late-uninucleate phase. This developmental phase is indicated when the distance between the flag leaf and the penultimate leaf is 3 to 6 cm and the awns are visible just above the flag leaf. However, using the former mentioned criteria is not the most accurate way to determine whether donor plant is in the correct development phase as each genotype of barley shows different agronomical properties. Figure 3.2 is an example of how developmental phase's accuracy was ensured for each genotype used over the harvesting period. The initial harvested spikes were labelled by genotype and their size (small, medium and large) photocopied and measured Fig 3.2A). The phase was verified by staining the anthers of the mid florets with a 1% acetocarmine stain, utilizing the squash method, and looking at the microspore development phase under a standard microscope. The microspores on the slide were then compared to Figure 3.2B and Figure 3.2C (Maluszynski *et al.*, 2003). All donor plants harvested afterwards were compared

to the photocopied donor plant to ensure uniformity. The spikes were kept in water after harvesting to prevent wilting, surface sterilized with an aerosol of 70% ethanol in a laminar flow bench and then placed on the various pre-treatment options.



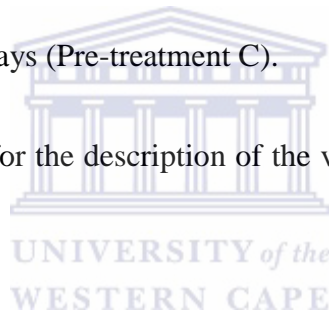
Figure 3.2 displays the process taken to select the donor plant material. Figure 3.2A: Donor plant material from the same genotype cut at different physiological stages to determine the correct pollen developmental stage. Figure 3.2B: Mid-late uninucleate microspore development stage in barley. Figure 3.2C: Late uninucleate microspore development stage in barley (Maluszynski *et al.*, 2003).

3.2. Pre-treatment

The spike harvesting period occurred during mid-April until mid-September. This time period provides the optimal growing conditions for spring type barley in the Southern Cape.

Three pre-treatment methods were tested: Sterilized spikes (Fig 3.3A), covered partially with liquid 0.3 M Mannitol (54.651 g/L) in a 65 mm diameter petri dish that was sealed with parafilm and incubated in the dark at 4°C for a duration of 7 days (Pre-treatment A), excised anthers (Fig 3.3B) placed on a solid 0.7 M Mannitol plate (127.51 g/L; 15 g Bacteriologic Agar), 65 mm diameter, sealed with parafilm and incubated in the dark at 4°C for a duration of 4 days (Pre-treatment B) and spikes (Fig 3.3C) placed in a 90 mm diameter Petri dish with a sterile moist cloth (approximately 2 cm² in size) sealed with parafilm and incubated in the dark at 4°C for a duration of 14 days (Pre-treatment C).

Figure 3.3 provides a visual aid for the description of the various pre-treatment methods that were used in this study.



The plant material used in Pre-treatment A and B were sterilised using the sterilizing method mentioned below before being placed on the respective pre-treatments, thus not requiring sterilization before it was placed on the induction medium.

The spikes on Pre-treatment C were sterilized before the excised anthers were placed on the induction medium. The ears were sterilized by placing it in 70% ethanol for two minutes, then in a 15% sodium hypochlorite (commercial bleach) solution for 8 to 10 minutes, rinsing with double distilled water for 1 minute followed by rinsing in 70% ethanol. Pre-treatment C was used as the control for the experiment as this method has been used extensively at our research institution for the production of doubled haploid barley, and has proven to be a successful method. The performance of the other pre-treatment methods was measured against it.

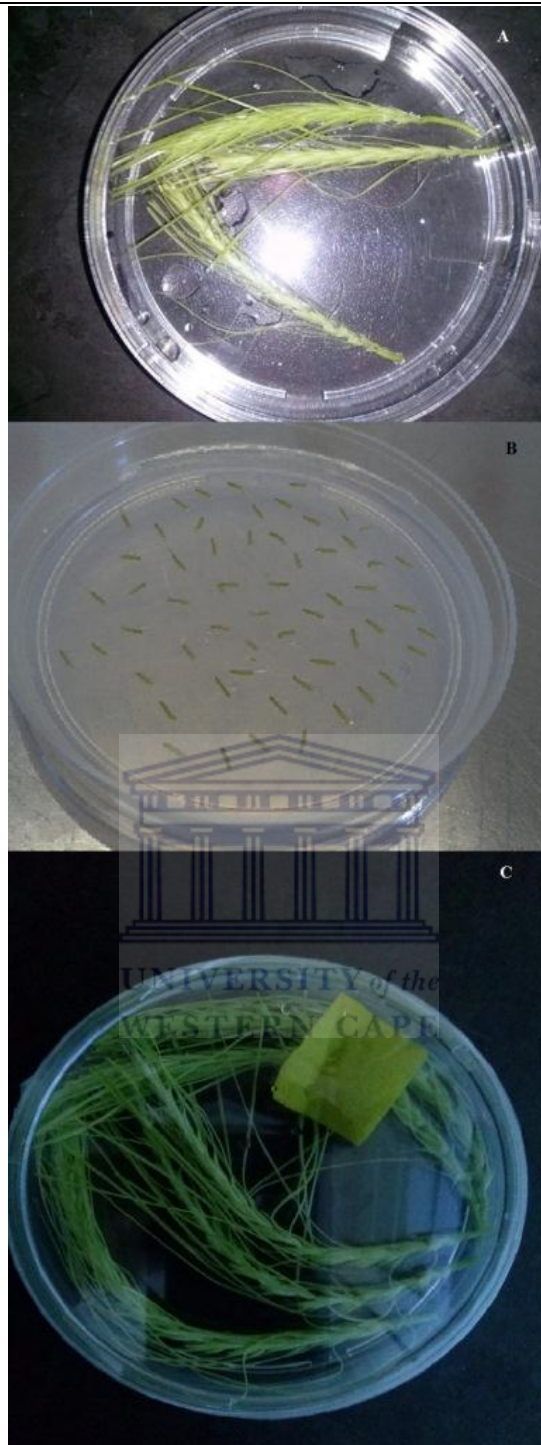


Figure 3.3. Comparison of pre-treatment methods. Figure 3.3A: liquid 0.3 M Mannitol. Figure 3.3B: solid 0.7 M Mannitol. Figure 3.3C: Cold treatment with moist cloth (CMC)

3.3. Induction, Regeneration, Transfer and Harvest

The protocol used for anther culture media was Dr. G. Daniel's (1993) modified MS medium (Murashige and Skoog, 1962) provided by the organisation Sensako (Table 3.1). The stock solutions for the media are listed in Table 3.2.

After pre-treatment the excised anthers were placed on the filtered induction medium (65 mm diameter Petri dish, modified MS medium (Table 3.1 and Table 3.2). The culture plates were sealed with parafilm and incubated at 25°C in the dark for 4 weeks. During this period, embryo-like structures (calli) grew on the induction medium which was transferred to the regeneration media after the four week incubation period.

Table 3.1. Dr. G. Daniel's (1993) modified MS medium (Murashige and Skoog, 1962)

	Induction Media	Regeneration Media	Rooting Media
In-organic Fraction	g/L		
KNO ₃	1.900 g	1.900 g	1.900 g
NH ₄ NO ₃	0.165 g	0.165 g	0.165 g
KH ₂ PO ₄	0.170 g	0.170 g	0.170 g
MgSO ₄ .7H ₂ O	0.370 g	0.370 g	0.370 g
CaCl ₂ . 2H ₂ O	0.440 g	0.440 g	0.440 g
FeNa ₂ .EDTA	0.040 g	0.040 g	0.040 g
Micro Stock 1	10 ml	10 ml	10 ml
Micro Stock 2	1 ml	1 ml	1 ml
Organic Fraction			
Thiamine hydrochloride stock	1 ml	1 ml	1 ml
α-naphthaleneacetic acid solution	1 ml	-	-
6-Benzylaminopurine solution (BAP)	1 ml	-	-
Maltose	63 g	-	-
Sucrose	-	20 g	20 g
Myo-Inositol	0.100 g	-	-
Phytigel	3 g	3 g	3 g
KOH	pH 5.8 - pH 5.9	pH 5.8 - pH 5.9	pH 5.8 - pH 5.9

Table 3.2. Stock solutions

Micro Stock 1	g/500 ml ddH₂O
MnSO ₄ .4H ₂ O	0.825
ZnSO ₄ .7H ₂ O	0.43
H ₃ BO ₄	0.31
Micro Stock 2	g/500 ml ddH₂O
KI	0.415
CuSO ₄ .5H ₂ O	0.0125
Na ₂ M ₀ O ₄ .2H ₂ O	0.125
Thiamine hydrochloride stock	mg/500 ml ddH₂O
Thiamine HCL	80

For the regeneration phase, 30 calli was placed on a solid filtered regeneration media (90 mm diameter Petri dish, modified MS medium) (Table 3.1), sealed with parafilm, and incubated under light at 25°C until shoots and or roots were formed.

These green plantlets were then transferred to solid non-filtered rooting media (7 ml regeneration media in 30 ml McCartney bottles, Table 3.1) and incubated under light at 25°C until the green plantlets have grown to a height of 6 cm to 8 cm. From there it was transferred to the greenhouse and planted in soil. For the first three days after transferring the green plants to the greenhouse, the plantlets were covered with vented plastic bags to facilitate the acclimatization of the plantlets. The plantlets were fertilized with approximately 300 ml of kelp (Effekto Wondersol kelp, 25 ml/L) per pot for the first two weeks after transfer, after which the plants received the same fertilizer blend mentioned above for the donor plants. Plant fertility was evaluated on the basis of seed set.

Albinos generated from the study was recorded and used for comparing the ratio between albinos and green plants per genotype.

The following parameters were monitored ; (i). The number of calli induced per treatment per genotype, (ii). The number of green plants and albinos generated per treatment per genotype and (iii) the ratio of doubled haploids to haploids.

The software, STATISTICA version 12, was used to analyse the data. Basic statistical analysis as well as a t-test was performed. Data analyses were performed on the following:

Responding anthers per treatment

Number of calli induced per treatment

Number of plants regenerated per treatment

Number of albinos regenerated per treatment

Number of green plants regenerated per treatment

Number of doubled haploid plants regenerated per treatment

Number of sterile plants per treatment.



3.3. Media Preparation The induction media as well as the regeneration media was prepared in two phases as it contains an organic fraction and an in-organic fraction. The organic fraction was filter sterilized and the in-organic fraction was sterilized through autoclaving (refer to Table 3.2) The two fractions were mixed together to make up a final volume of 1000 ml, and the pH adjusted between the range of 5.8 – 5.9 with potassium hydroxide (KOH) pellets. After pH adjustment the media were poured into 65 mm and 90 mm diameter Petri dishes, respectively.

The rooting media has the same chemical content as the regeneration media but is prepared differently. The organic fraction was not separated from the in-organic fraction. Both parts were added together and made up to a volume of 1000 ml with distilled water. The solution was sterilized by autoclaving for 15 minutes at 121°C. After sterilization, the media was allowed to cool off to 75°C before adjusting the pH. The pH was adjusted between 5.8 – 5.9 using potassium hydroxide pellets. After adjustment the media was dispensed into 30 ml McCartney bottles (7 ml per bottle). The caps were replaced on the bottles and the bottles were sterilized again for 15 minutes at 121°C.



CHAPTER 4

RESULTS AND DISCUSSION

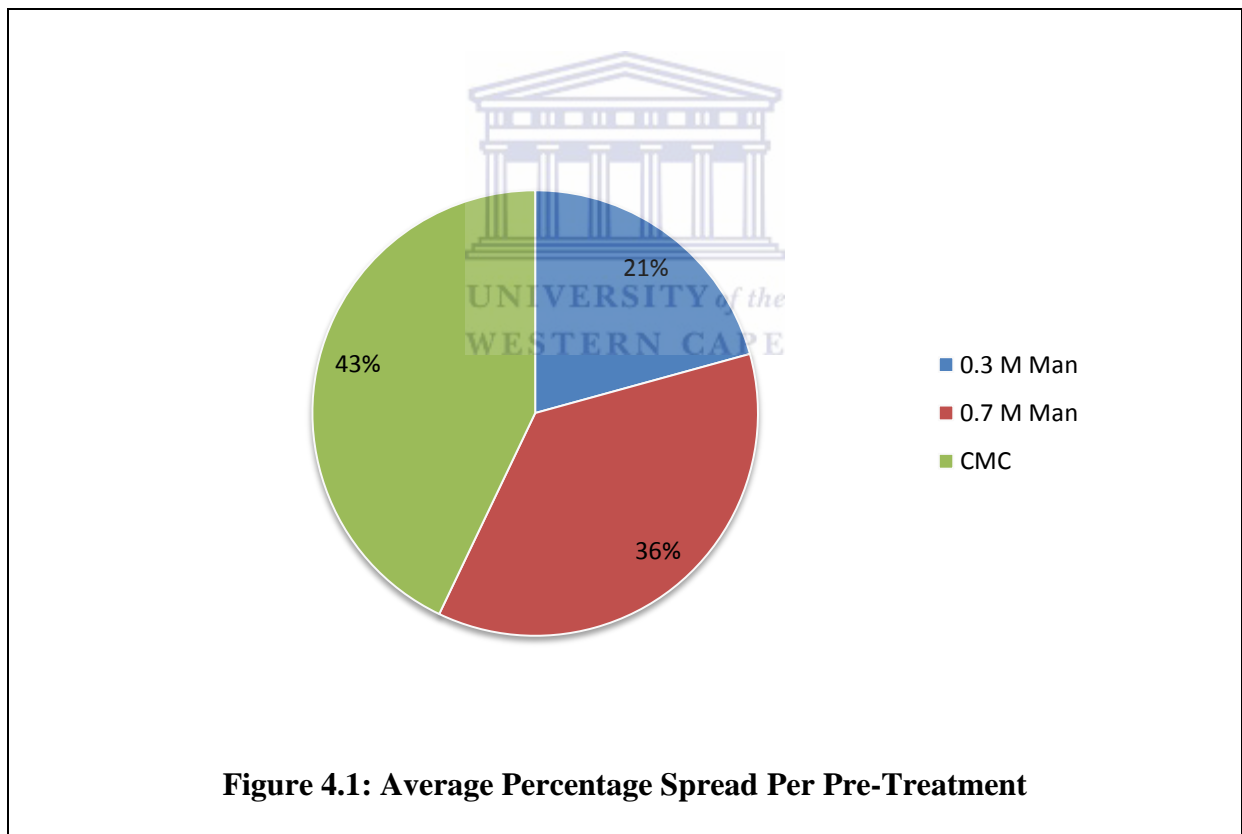
In this study three different pre-treatment methods (0.7 M Mannitol, 0.3 M Mannitol and Cold treatment with moist cloth) were explored that are used in anther culture, to determine which one is the optimal pre-treatment. Pre-treatment C (cold treatment with moist cloth) was used as the control treatment as it has been used extensively in the laboratory of SABBI. The mannitol pre-treatments were used as the test treatments. The performance of the test treatments were compared to the control. The following variables were analysed to assess efficiency of the pre-treatment techniques; (1) Anthers responding to the treatments (2) Calli per 100 anthers, (3) green plants per 100 anthers, (4) percentage of doubled haploid plants per total regenerated green plants.

The amount of calli induced per treatment per genotype, the number of green plants as well as the number of albinos generated per treatment per genotype and the number of doubled haploids produced versus the number of haploids produced was compared.

The performance of five varieties (CM 01-CM 05) of barley against the three protocols was compared with each other; the results were both compared between the different genotypes as well as within a given genotype. It is well known that anther culture is genotype specific (Jähne and Lörz, 1995); hence it was expected to obtain different levels of performance between the varieties. This is also one of the reasons why it was important to test the protocols on different varieties instead of just one, so as to make an informed decision.

4.1. Pre-treatment

Pre-treatment A (0.3 M Mannitol) and Pre-treatment B (0.7 M Mannitol) were spread equally across the five barley varieties (CM 01-CM 05). Initially Pre-treatment C (CMC) which was the control had a higher amount of spikes on the pre-treatment, due to production requirements; the ratio for the division was 2:1:1. Due to contamination, the percentage spread was altered and the effect thereof can be seen in Figure 4.1 as it shows the actual average percentage spread of the plant material across the pre-treatments used. Pre-treatment A had a contamination percentage of 40.0 %, Pre-treatment B 23% and Pre-treatment C was 41%.



Reducing the contamination of the plant material, induction plates and regeneration plates is very important. In this study we observed yeast and bacterial contamination during the induction phase and the regeneration phase of the protocol. There were protocols in place to

minimize contamination. Some of the protocols used were the surface sterilisation (70% ethanol aerosol) of the plant material before placing it on the pre-treatments and induction media, ensuring that the work place was sterile by wiping the benches with 70% ethanol and using sterile tools. Also, surgical masks and latex gloves were worn to prevent any bacterial contamination that may originate from the laboratory technician performing the tasks. Unfortunately these methods can only prevent further contamination from occurring after the spike has been harvested. Most of the contamination observed during this study was due to yeast and/or bacterial contamination originated from the anther. Unfortunately contamination originating from the anther cannot be eliminated completely as harsher protocols may destroy the anther which would render anther culture as inadequate. It is important to ensure that if there are contaminated material present that it is disposed of properly to prevent it from spreading to other culture plates.

No further tests were done to identify the cause of contamination. However, it would be valuable to identify the bacterial, yeast and fungal species that cause the contamination. As the information obtained from the results would help to eradicate and prevent future contamination incidences.

4.2. Induction

An average of 60 anthers was placed on the induction media per plate (Figure 4.2A). Each plate represented a spike. Calli formation usually occurs within 3-6 weeks of induction (Figure 4.2B); in some instances it may take up to 8 weeks before the genotype will respond to the treatment. The quality and quantity of calli induced varies from genotype to genotype as well as the treatment it was exposed to. It was observed that the calli induced from the

anthers that were treated with 0.3 M Mannitol were fewer compared to those induced from 0.7 M Mannitol or the cold treatment with a moist cloth.

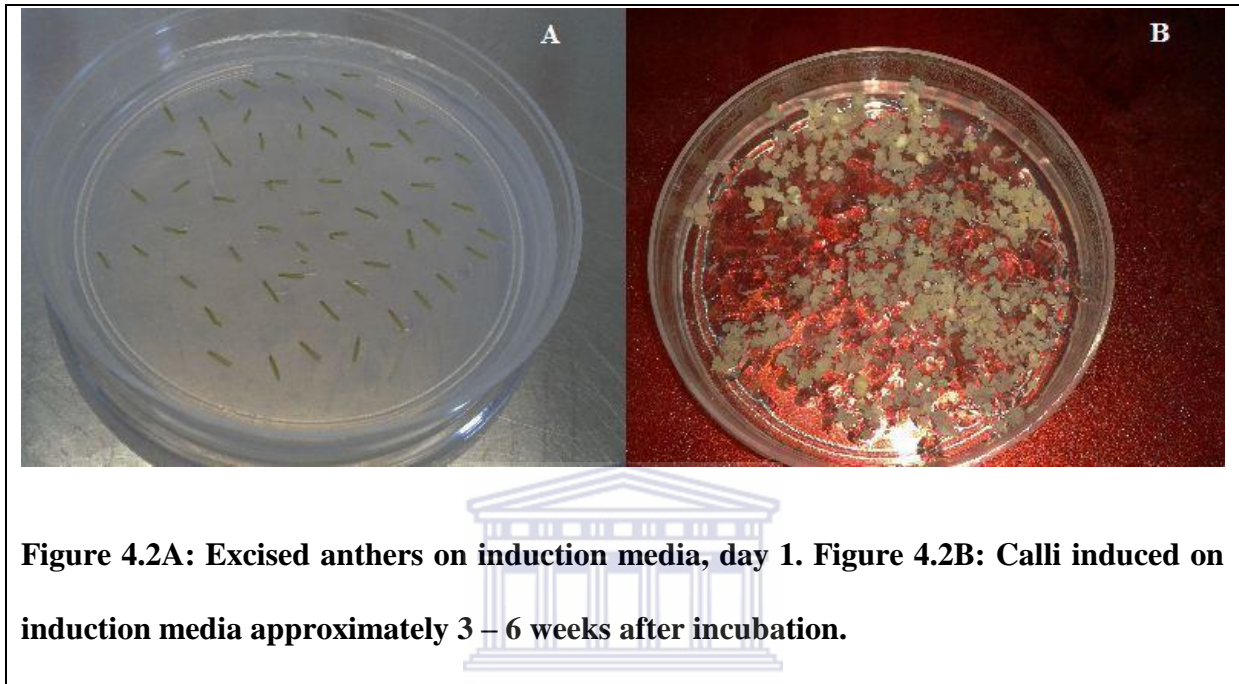
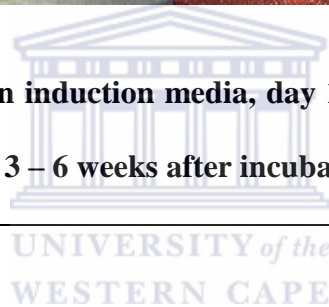


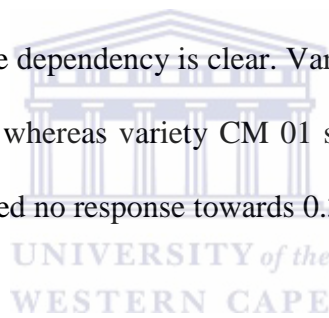
Figure 4.2A: Excised anthers on induction media, day 1. Figure 4.2B: Calli induced on induction media approximately 3 – 6 weeks after incubation.



The amount of anthers that formed calli from the various barley varieties (Codes CM 01-05) was compared and the percentage of responding and non-responding plates/spikes per treatment per genotype was calculated. Figure 4.3 shows how well the spikes responded to the various treatments it was exposed to. On average CMC and 0.7 M Mannitol had the highest percentage spikes responding to their treatment, with 32.5% and 31.3% respectively. This means that at least 31% of the spikes on CMC and 0.7 M Mannitol have induced callus formation, where 0.3 M Mannitol have an induction percentage of 2.4%. When investigating the individual pedigrees (Figure 4.3), it is noticeable that certain genotypes respond better on certain treatments, again confirming that the treatments are genotype dependent. For example, genotype combination CM 01 had a higher induction percentage in callus formation (62.1%) on CMC than CM 05 where only 8% of the spikes responded to the same pre-

treatment. The highest percentage for 0.7 M Mannitol was seen in CM 02 with 46.7% and the lowest was seen in CM 05 with 10.5%. For 0.3 M Mannitol the highest was seen in CM 05 with 10%, with multiple genotypes (CM 01, CM 02 and CM 04) not responding to the pre-treatment at all. Thus suggesting that 0.3 M Mannitol was the least reliable pre-treatment option. The genotype CM 05 was also the genotype with the lowest responding percentage, showing that this genotype is the least suitable to use for doubled haploid production through anther culture. The results conflicted with most researchers whom believe that Mannitol pre-treatment is superior to that of cold treatment as well as those who suggest that mannitol treatment used in combination with cold is better (Jähne and Lörz, 1995; Hoekstra *et al.*, 1997 and Cistué, Ramos and Catillo, 1999).

In Figure 4.3, the role of genotype dependency is clear. Variety CM 05 showed to be the least responsive across all treatments, whereas variety CM 01 showed to be the most responsive even though variety CM 01 showed no response towards 0.3 M Mannitol.



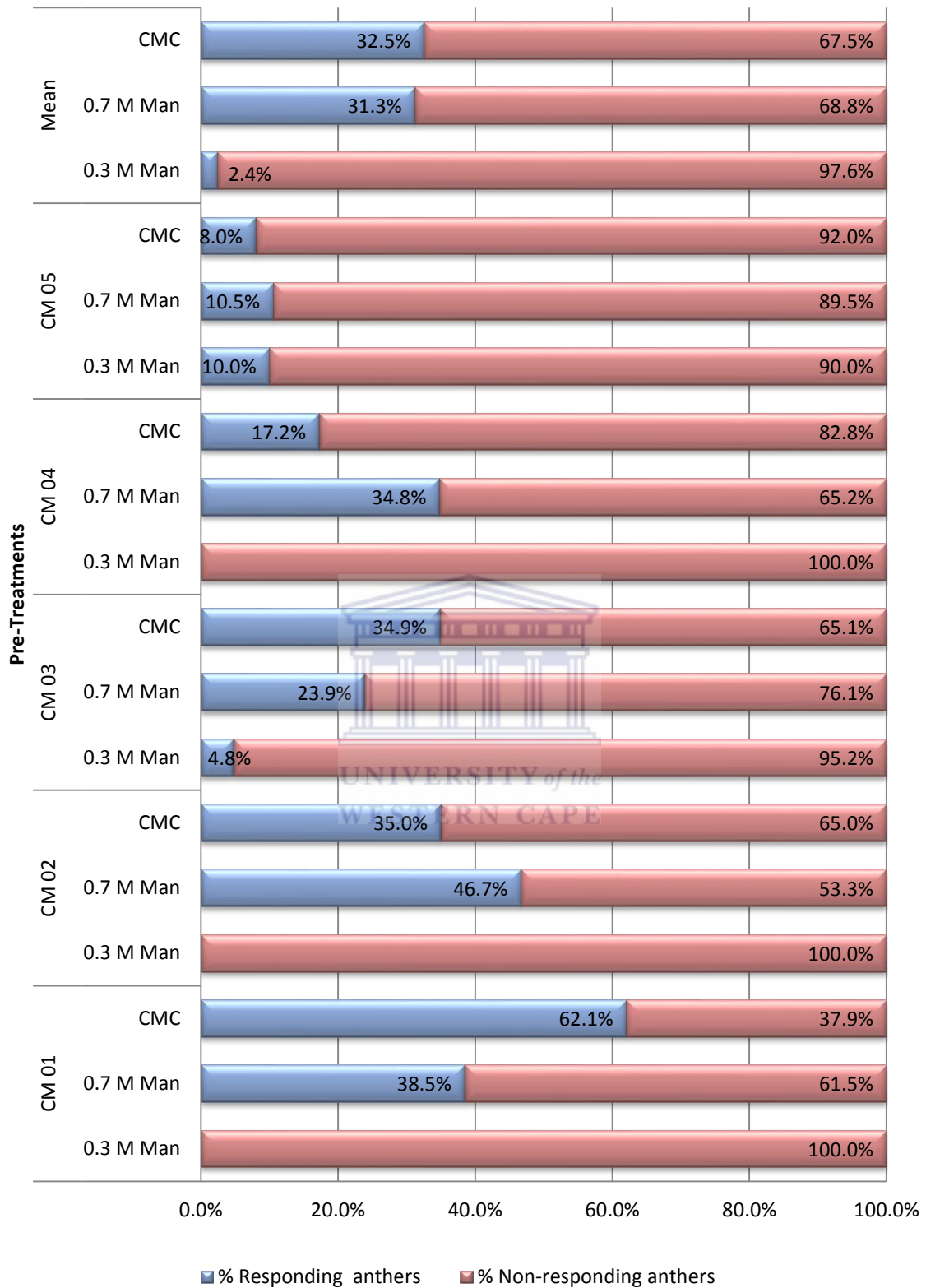


Figure 4.3: The Percentage Of Responding Anthers Versus Non-Responding Plates Per Genotype.

Table 4.1. Comparison Of The Responding Anthers Per Treatment.

Variable	Responding Anthers (RA) per Treatment			
	Valid N	Mean	Std.Dev.	% Responding Anthers
0.3 M	82	1.46341	9.31240	2.4
0.7 M	144	18.75000	27.90782	31.3
CMC	166	19.51807	28.19429	32.5

On average CMC showed to have the highest percentage of anthers responding to the treatment, 0.7 M Mannitol had the second highest percentage and 0.3 M Mannitol had the lowest response to the treatment. The pre-treatment with the lowest responding mean was 0.3 M Mannitol and the highest responding pre-treatment was CMC (Table 4.1).

A t-test was performed on the results obtained. Significance was established at a 95% Confidence interval ($p < 0.05$). There were no significant difference between the results obtained from 0.7 M Mannitol and that of CMC (Table 4.2.). The results suggest that the anthers responded equally to both pre-treatments. However, there were a significant difference observed between 0.3 M Mannitol and 0.7 M Mannitol as well as 0.3 M Mannitol and CMC. Those who showed significance are highlighted in red in Table 4.2.

This suggests that cold treatment with a moist cloth and 0.7 M Mannitol pre-treatment is the optimal pre-treatment to use to induce calli formation regardless of the genotype used in barley anther culture. The results agrees with other researchers that suggested that cold-treatment out performs mannitol, specifically 0.3 M Mannitol (Hou *et al.*, 1993). Another researcher (Cistué, Ramos and Castillo, 1999) suggested that the higher the molarity of

Mannitol in the pre-treatment solution the better the results for induction. Suggesting that 0.7 M Mannitol is better than 0.3 M Mannitol. However, in that study the researchers observed that the results obtained between 0.7 M Mannitol and 1.0 M Mannitol had no significance.

Table 4.2. T-Test For Responding Anthers.

Group 1 vs. Group 2	T-test for Independent Samples (Responding Anthers)						
	Note: Variables were treated as independent samples						
	Mean Group 1	Mean Group 2	t-value	df	p	F-ratio Variances	p Variances
0.3 M vs. 0.3 M	1.46341	1.46341	0.00000	162	1.000000	1.000000	1.000000
0.3 M vs. 0.7 M	1.46341	18.75000	-5.43491	224	0.000000	8.981070	0.000000
0.3 M vs. CMC	1.46341	19.51807	-5.64365	246	0.000000	9.166396	0.000000
0.7 M vs. 0.7 M	18.75000	18.75000	0.00000	286	1.000000	1.000000	1.000000
0.7 M vs. CMC	18.75000	19.51807	-0.24035	308	0.810219	1.020635	0.902643
CMC vs. CMC	19.51807	19.51807	0.00000	330	1.000000	1.000000	1.000000

We compared the number of calli induced per pre-treatment (Table 4.3.). CMC induced the highest amount of calli, with a mean of 94.1 calli per spike. The pre-treatment, 0.7 M Mannitol, came in second (mean = 39.1 calli per spike) and 0.3 M Mannitol induced the lowest amount of calli per spike (mean=5.9). The results of the t-tests (Table 4.4) indicated

that there was a significant difference in the amount of calli formed on the induction medium between the pre-treatments. The results suggest that CMC will induce higher callus formation frequency than mannitol pre-treatment, even though the anthers have responded equally to the 0.7 M Mannitol and CMC pre-treatment. Thus, the quantity of calli induced on CMC is higher than that produced on the 0.7 M Mannitol pre-treatment.

Table 4.3. Comparison Of The Number Of Calli Induced Per Treatment

Variable	Induced Calli per Treatment		
	Valid N	Mean	Std.Dev.
0.3 M	82	5.85366	49.7637
0.7 M	144	39.08333	112.6447
CMC	166	94.08434	248.2780

Table 4.4. T-Test For Calli Induced

Group 1 vs. Group 2	T-test for Independent Samples (Calli)						
	Note: Variables were treated as independent samples						
	Mean Group 1	Mean Group 2	t-value	df	p	F-ratio Variances	p Variances
0.3 M vs. 0.3 M	5.85366	5.85366	0.00000	162	1.000000	1.00000	1.000000
0.3 M vs. 0.7 M	5.85366	39.08333	-2.53243	224	0.012012	5.12385	0.000000
0.3 M vs. CMC	5.85366	94.08434	-3.18347	246	0.001643	24.89153	0.000000
0.7 M vs. 0.7 M	39.08333	39.08333	0.00000	286	1.000000	1.00000	1.000000
0.7 M vs. CMC	39.08333	94.08434	-2.44835	308	0.014909	4.85797	0.000000
CMC vs. CMC	94.08434	94.08434	0.00000	330	1.000000	1.00000	1.000000

Table 4.5 gives a comprehensive view of the results obtained during the induction phase of anther culture with regards to callus formation. On average Pre-treatment A gave the least amount of calli formed per anther and per plate, 0.1 and 4.9 respectively, even in the genotype CM 05 where it showed a higher percentage of spikes responding to it than in Pre-treatment C (control) which had a lower percentage. Pre-treatment C had a higher amount of calli forming per anther for the abovementioned genotype. In some varieties (CM 04 and CM 05) 0.7 M Mannitol outperformed the control by inducing higher calli formation in total, however CMC produced the highest amount of calli overall. These results support the

hypothesis that anther culture is genotype dependent as described by so many other researchers such as Bregitzer *et al.*, (1998), Ward and Jordan (2001), Hussein *et al.*, (2004) and Jaquard *et al.*, (2009). Also, the results suggest that even though mannitol pre-treatment, specifically 0.7 M Mannitol pre-treatment, is an effective treatment in inducing calli, CMC seems to be effective across all varieties. Thus CMC can be used as a broad spectrum pre-treatment that guarantees results irrespective of the genotype or variety involved.

Pre-treatment B proved to be more successful than Pre-treatment A with an average of 0.7 calli forming per anther and an average of 39.5 calli forming per plate. Pre-treatment C, which had the same induction percentage as Pre-treatment B, had the highest callus formation per anther and per plate, 1.6 and 97.6 respectively. Pre-treatment C had more than double the callus formation capacity per anther than Pre-treatment B.



Table 4.5: Comparison Of Average Callus Formation Per Treatment Per Genotype

Genotype	Pre-treatment	Total number of Calli	Calli per spike	Calli per anther
CM 01	0.3 M Mannitol	0	0.0	0.0
	0.7 M Mannitol	1955	75.2	1.3
	CMC	8902	307.0	5.1
CM 02	0.3 M Mannitol	0	0.0	0.0
	0.7 M Mannitol	1140	38.0	0.6
	CMC	3828	95.7	1.6
CM 03	0.3 M Mannitol	450	21.4	0.4
	0.7 M Mannitol	1283	27.9	0.5
	CMC	2547	59.2	1.0
CM 04	0.3 M Mannitol	0	0.0	0.0
	0.7 M Mannitol	1034	45.0	0.7
	CMC	648	22.3	0.4
CM 05	0.3 M Mannitol	30	3.0	0.1
	0.7 M Mannitol	216	11.4	0.2
	CMC	93	3.7	0.1
AVERAGE	<i>0.3 M Mannitol</i>	<i>96.0</i>	<i>4.9</i>	<i>0.1</i>
	<i>0.7 M Mannitol</i>	<i>1125.6</i>	<i>39.5</i>	<i>0.7</i>
	<i>CMC</i>	<i>3203.6</i>	<i>97.6</i>	<i>1.6</i>

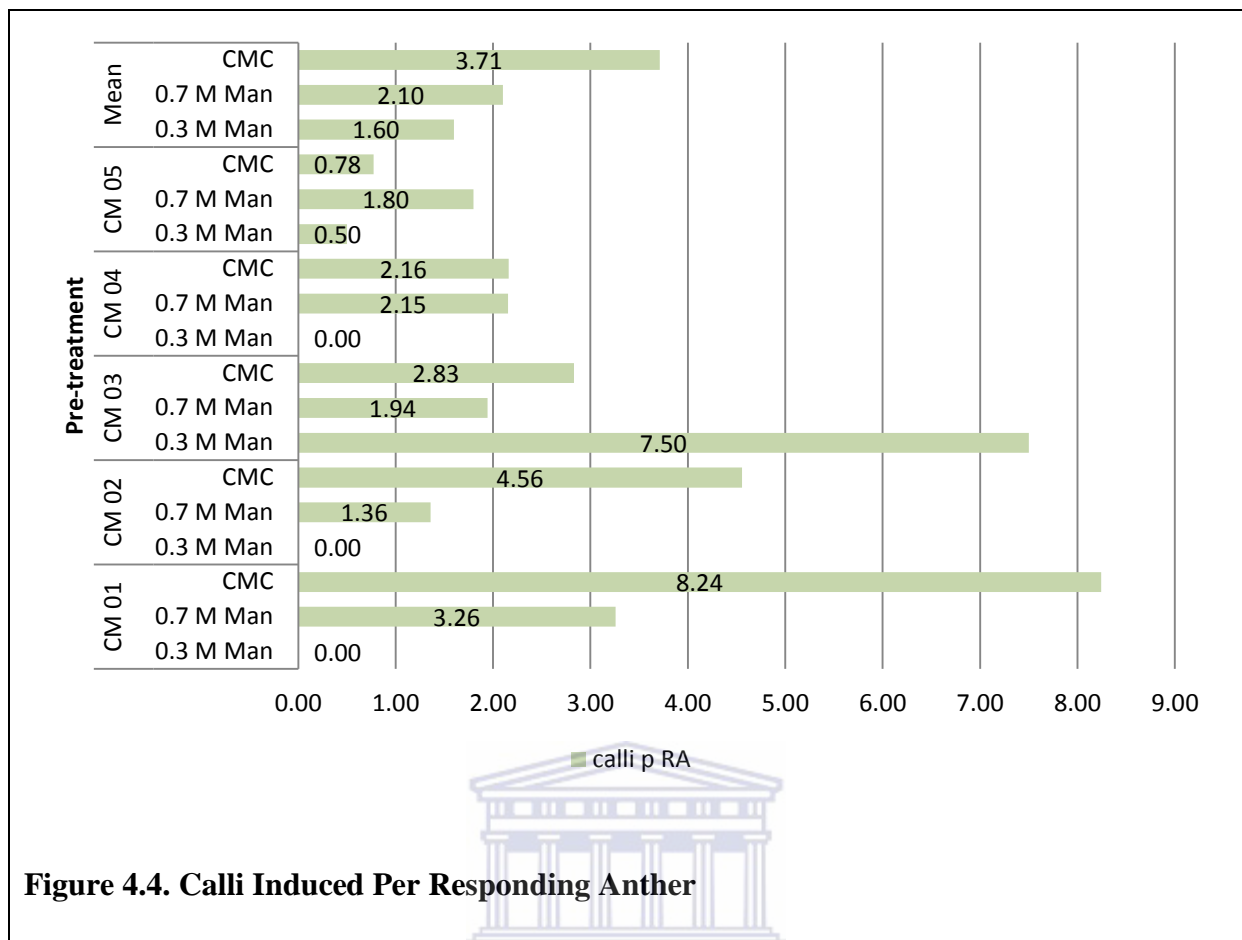
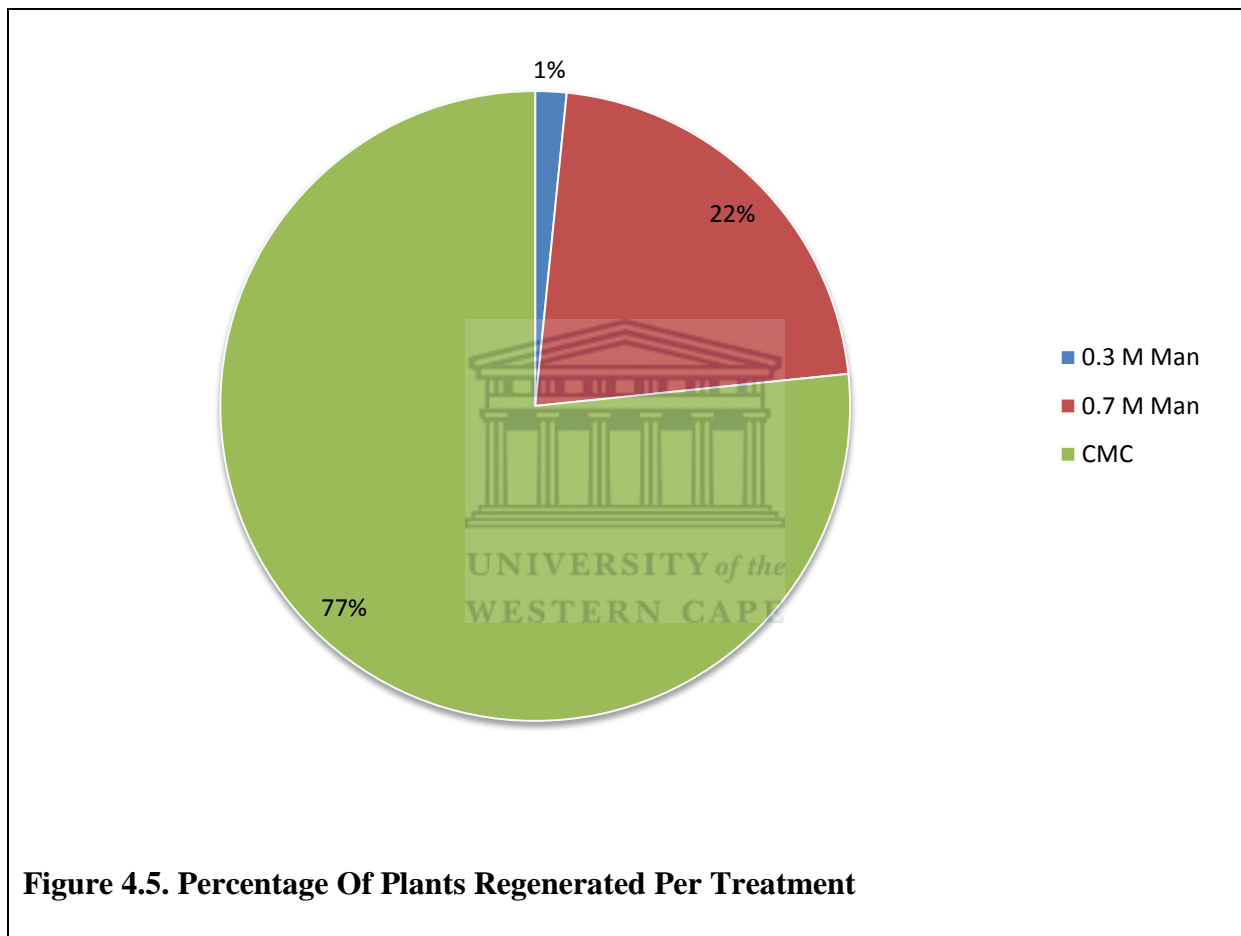


Figure 4.4. Calli Induced Per Responding Anther

4.3. Regeneration, Transfers and Harvest

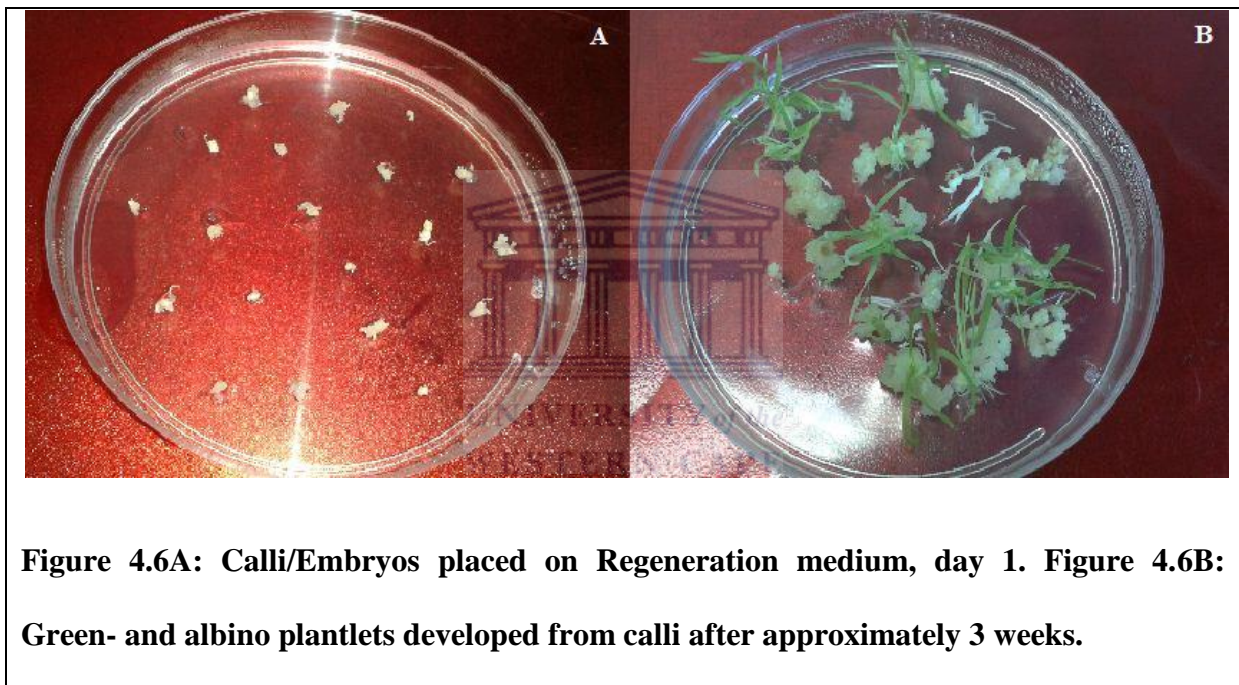
Callus formation is an important indicator with regards to doubled haploid production as every single callus has the potential to become a green plantlet and develop into a doubled haploid plant. Thus the higher the amount of calli formed, the higher the number of doubled haploids that can be produced. However, it is clear that the quantity of calli formed is not the only factor that determines the success of green plant regeneration. The quality of the calli plays a vital role as well. It has been observed that calli that has been transferred to the regeneration medium within the first two weeks of formation has a higher likelihood of regenerating into a green plant compared to older calli (4 weeks and older) that is more likely to regenerate into an albino plant.

The highest plant regeneration (green plant and albino plant) was obtained from Pre-treatment C (CMC). The second highest was obtained from Pre-treatment B (0.7 M Man) and the lowest regeneration frequency was obtained from 0.3 M Mannitol (Figure 4.5). The results obtained were 77%, 22% and 1% respectively



The regeneration phase of barley anther culture is visualised in Figure 4.6. Figure 4.6A displays the embryos/calli placed on regeneration medium; Figure 4.6B shows the regenerated plantlets that grew on the regeneration medium. In Figure 4.6B albino plantlets and green plantlets can be observed. The growth phase of the green plants to be transferred to the rooting media can be observed in Figure 4.6B. The Rooting Media phase of anther culture

can be observed in Figure 4.7. The green plants that were developed during the regeneration phase of the protocol were transferred to the rooting media. The green plants grew stronger whilst they were on the rooting media and the weaker green plants that would die off in the rooting media. However, the green plants that reached the optimal size for transplanting to the greenhouse were moved to the greenhouse (Figure 4.8) and matured to be harvested (Figure 4.10A).

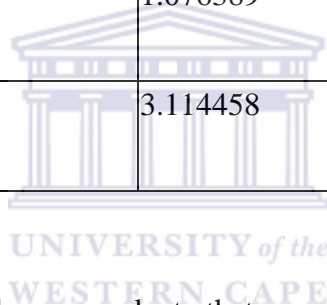


The highest green plant regeneration (Table 4.6) was observed in CMC (mean = 3.1 green plants per spike). 0.3 M Mannitol had the lowest green plant regeneration frequency (mean = 0.1 green plants per spike). There was a significant difference (Table 4.7) observed between the results obtained between CMC and both mannitol pre-treatments. However, there was no significant difference between the two mannitol pre-treatments. Thus, the results suggest that the mannitol pre-treatments would produce similar green plant volumes even though the number of calli for 0.3 M Mannitol would be less. Thus suggesting that the quality/ green

plant regeneration frequency ability of the calli induced would be higher than that of the 0.7 M Mannitol.

Table 4.6. Comparison Of Green Plants Per Treatment

Variable	Green Plants per Treatment		
	Valid N	Mean	Std.Dev.
0.3 M	82	0.146341	1.325178
0.7 M	144	1.076389	5.146905
CMC	166	3.114458	9.522804



After approximately ± 3 weeks the green plants that were transferred to the Rooting Media (Figure 4.7) was transferred to the greenhouse (Figure 4.8). The plantlets were grown to full maturity before the doubled haploids were harvested and the sterile plants were recorded and discarded. Unfortunately, not all of the green plants survived the transfer to the greenhouse. The green plants that died after being transferred to the greenhouse were recorded. Approximately 38.53% of the green plants died within the greenhouse (Figure 4.9). Further research has to be done to ensure higher survival percentages after the greenhouse transfer.

Table 4.7. T-Test For Green Plants Regenerated.

Group 1 vs. Group 2	T-test for Independent Samples (Green Plants)						
	Note: Variables were treated as independent samples						
	Mean Group 1	Mean Group 2	t-value	df	p	F-ratio Variances	p Variances
0.3 M vs. 0.3 M	0.146341	0.146341	0.00000	162	1.000000	1.00000	1.000000
0.3 M vs. 0.7 M	0.146341	1.076389	-1.60489	224	0.109928	15.08494	0.000000
0.3 M vs. CMC	0.146341	3.114458	-2.80622	246	0.005414	51.63938	0.000000
0.7 M vs. 0.7 M	1.076389	1.076389	0.00000	286	1.000000	1.00000	1.000000
0.7 M vs. CMC	1.076389	3.114458	-2.29370	308	0.022481	3.42324	0.000000
CMC vs. CMC	3.114458	3.114458	0.00000	330	1.000000	1.00000	1.000000

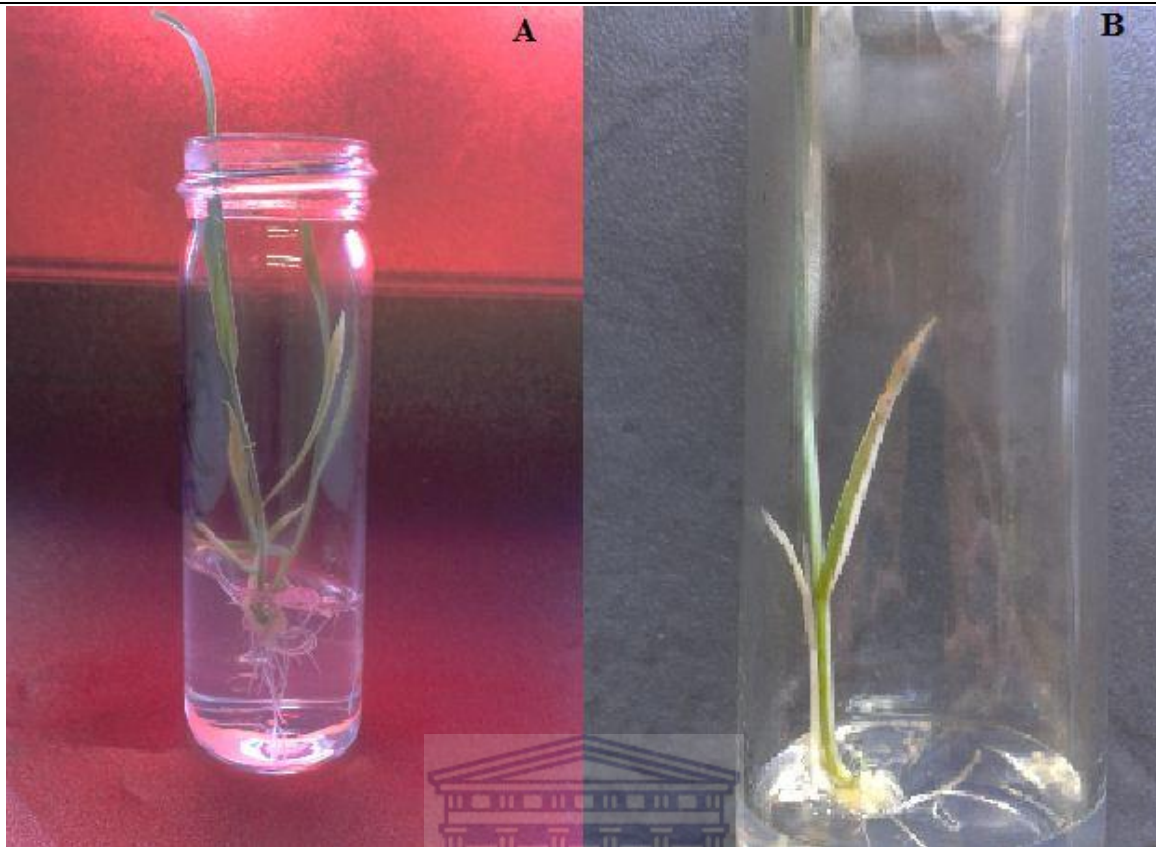


Figure 4.7A: Green plantlet growing in rooting media, approximately 3 weeks after transfer. Figure 4.7B: Partial albinism shown in plantlet, approximately 3 weeks after transfer.



Figure 4.8: Green plantlets transferred to potting soil in the greenhouse.

Figure 4.9 shows that there were a higher percentage of sterile plants for CMC than that of 0.3 M Mannitol and 0.7 M Mannitol. However, the number of green plants obtained for 0.3 M Mannitol (total = 7) was too low to confidently conclude that it performed better in generating doubled haploids than CMC.

In Figure 4.10, the difference between a doubled haploid plant and a sterile plant can be seen. The doubled haploid plant (Figure 4.10A) will have a full seed set and the sterile plant (Figure 4.10B) will have no seed set at maturity.

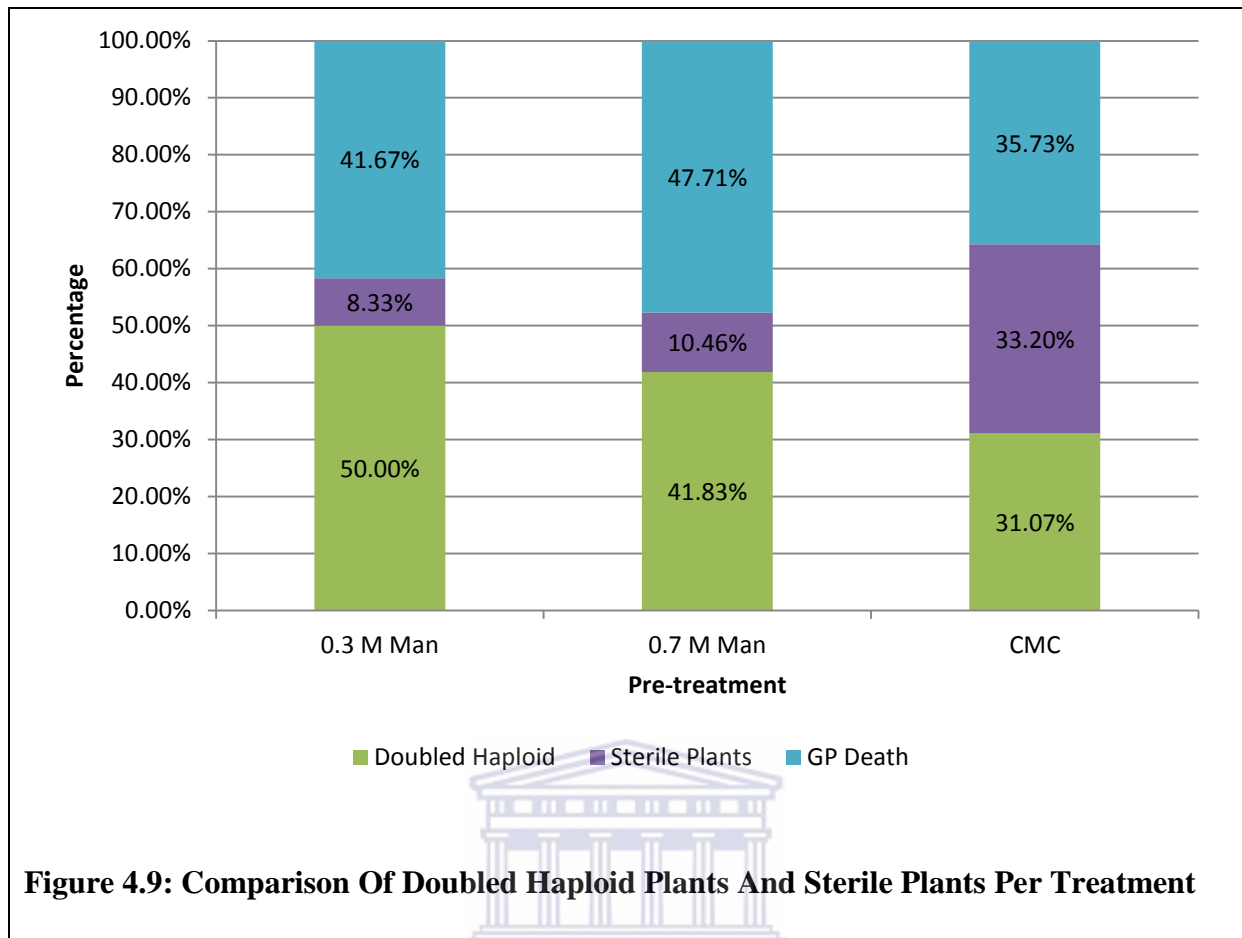


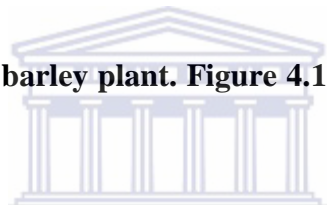
Figure 4.9: Comparison Of Doubled Haploid Plants And Sterile Plants Per Treatment

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The number of doubled haploid plants that were developed through anther culture was low for each pre-treatment (Table 4.8). However, CMC had the highest doubled haploid mean (1) per anther. 0.3 M Mannitol produced the lowest number of doubled haploids per anther (mean = 0.1) and 0.7 M Mannitol had a mean of 0.4 doubled haploid plants per anther. The t-test revealed that there was a significant difference between the doubled haploid plant regeneration frequency of CMC and 0.3 M Mannitol, and no significant difference between 0.7 M Mannitol and 0.3 M Mannitol. The t-test also revealed a slight significance between 0.7 M Mannitol and CMC with a p value of 0.0509.



Figure 4.10A: Doubled haploid barley plant. Figure 4.10B: Sterile barley plant, no seed set on mature spike.



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Table 4.8. Comparison Of Doubled Haploids Per Treatment

Variable	Doubled Haploids per Treatment			
	Valid N	Mean	Std.Dev.	% DH
0.3 M	82	0.073171	0.662589	50
0.7 M	144	0.444444	1.741223	41.83
CMC	166	0.963855	2.736712	31.07

Table 4.9. T-Test For Doubled Haploids

Group 1 vs. Group 2	T-test for Independent Samples (DH)						
	Note: Variables were treated as independent samples						
	Mean Group 1	Mean Group 2	t-value	df	p	F-ratio Variances	p Variances
0.3 M vs. 0.3 M	0.073171	0.073171	0.00000	162	1.000000	1.00000	1.000000
0.3 M vs. 0.7 M	0.073171	0.444444	-1.85444	224	0.064991	6.90590	0.000000
0.3 M vs. CMC	0.073171	0.963855	-2.90265	246	0.004036	17.05963	0.000000
0.7 M vs. 0.7 M	0.444444	0.444444	0.00000	286	1.000000	1.00000	1.000000
0.7 M vs. CMC	0.444444	0.963855	-1.95915	308	0.050997	2.47030	0.000000
CMC vs. CMC	0.963855	0.963855	0.00000	330	1.000000	1.00000	1.000000

T-tests (Table 4.10 and Table 4.11) were performed to establish significance between the pre-treatments based on the number of sterile plants regenerated per responding anthers. The results show that CMC delivered the highest number of sterile plants per anther (mean = 1.03), 0.7 M Mannitol produced the second highest with a mean of 0.1 plant per anther and 0.3 M Mannitol produced the least amount of sterile plants (mean = 0.01) for this experiment. The p-values for the comparison between 0.3 M Mannitol with 0.7 M Mannitol was 0.18 (95% C.I) which suggest that the results obtained was not significantly different. Comparison between 0.3 M Mannitol and CMC was $p=0.008$ which also suggests significance. The

results obtained from comparing 0.7 M Mannitol and CM was $p=0.002$ suggesting a significant difference between the results obtained. The results suggest that CMC have produced the highest number of sterile plants out of all three pre-treatments tested.

Table 4.10. Comparison Of Sterile Plants Per Treatment

Variable	Sterile plants per Treatment			
	Valid N	Mean	Std.Dev.	% Sterile
0.3 M	82	0.012195	0.110432	8.33
0.7 M	144	0.111111	0.670154	10.46
CMC	166	1.030120	3.489247	33.2

The results for all the above mentioned (responding anthers, calli induction, regeneration, doubled haploid and sterile plants) suggests that CMC will produce the highest volumes for all the parameters tested (responding anthers, calli induction, regeneration, doubled haploid and sterile plants). However, in Figure 4.9 it shows that CMC produced the lowest percentage of doubled haploids (31%) compared to 0.3 M Mannitol (50%). The t-test performed on the results show that CMC produced higher doubled haploid plantlets, thus producing conflicting results. The different perspectives the two conflictive results were taken from has to be considered. Figure 4.9 were taken from the holistic perspective, from all the green plants that were planted that were produced from CMC, 31% were doubled haploid plants and 33% were sterile. On the other hand for 0.3 M Mannitol, 50% of the green plants that were planted in

the greenhouse were doubled haploid plants. These results suggest that 0.3 M Mannitol would be a better pre-treatment option as it would yield a higher percentage of doubled haploid plants. The results are flawed due to low population counts for 0.3 M Mannitol. The pre-treatment, 0.3 M Mannitol produced 12 green plants in total compared to CMC that produced 515 green plants in total. Clearly the number of doubled haploids and sterile plants produced by the pre-treatments mentioned above would not be a true reflection on the doubled haploid production capability of either of the pre-treatments. Further research should be done to determine the optimal pre-treatment for doubled haploid production.

Table 4.11. T-Test For Sterile Plants

Group 1 vs. Group 2	T-test for Independent Samples (Sterile)						
	Note: Variables were treated as independent samples						
	Mean Group 1	Mean Group 2	t-value	df	p	F-ratio Variances	p Variances
0.3 M vs. 0.3 M	0.012195	0.012195	0.00000	162	1.000000	1.0000	1.000000
0.3 M vs. 0.7 M	0.012195	0.111111	-1.32516	224	0.186469	36.8267	0.000000
0.3 M vs. CMC	0.012195	1.030120	-2.63839	246	0.008862	998.3373	0.000000
0.7 M vs. 0.7 M	0.111111	0.111111	0.00000	286	1.000000	1.0000	1.000000
0.7 M vs. CMC	0.111111	1.030120	-3.11059	308	0.002042	27.1090	0.000000
CMC vs. CMC	1.030120	1.030120	0.00000	330	1.000000	1.0000	1.000000

4.4. Albinism

As mentioned above, callus formation has a direct impact on the formation of green plant regeneration as well as the regeneration of albinos. Albinism in barley through anther culture is very common (Kumari *et al.*, 2009) and further research has to be done to determine how to lower the percentage of albinos generated, or completely eradicate it.

The percentage of green plants obtained versus that of the albinos regenerated per treatment is displayed by Figure 4.11. In all pre-treatments used, there was a higher percentage of albino plantlets regenerated than green plants (0.3 M Man: 57.1% Alb & 42.9% GP; 0.7 M Man: 59.8% Alb & 40.2% GP; CMC: 61.6% Alb & 38.4% GP). The results suggest that the albino regeneration frequency is higher than green plant regeneration frequency for all the pre-treatment tested. However, it also suggests that 0.3 M Mannitol has the lowest albino regeneration frequency. The pre-treatment, 0.3 M Mannitol is the optimal a pre-treatment for a low albino regeneration frequency.

As mentioned before there are different environmental factors that influences albino regeneration frequency. Acquaah (2007) suggested that the regeneration frequency of albino plants can be reduced if there is a higher concentration of nitrogen present in the donor material. Thus, it would be advised to study the effects of different fertilizer components on the production of plants through anther culture. If the fertilizer components have an effect on albino and green plant regeneration frequency it would be an easy solution to implement in breeding programs to reduce albinism.

Acquaah (2007) also stated that colder temperatures will reduce green plant regeneration; however it was unclear whether the author meant that colder temperatures were to be introduced during the growth period of the donor plant material or during pre-treatment of the anthers.

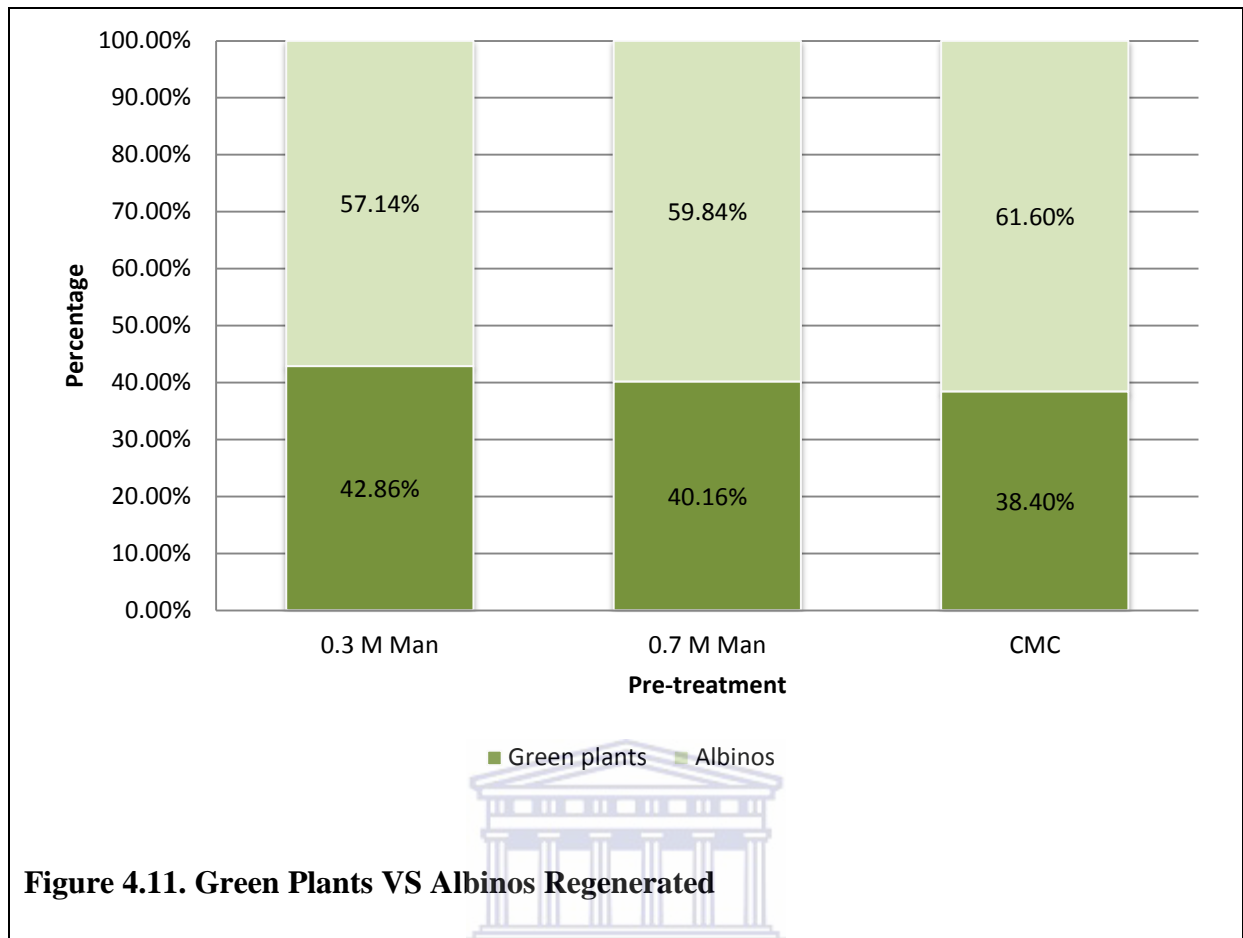


Figure 4.11. Green Plants VS Albinos Regenerated

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Table 4.12. Comparison Of Albino Plants Regenerated Per Treatment

Variable	Albinos per Treatment		
	Valid N	Mean	Std.Dev.
0.3 M	82	0.195122	1.65879
0.7 M	144	1.583333	7.03786
CMC	166	4.915663	11.87481

Table 4.13. T-Test For Albinos Regenerated

Group 1 vs. Group 2	T-test for Independent Samples (Albino)						
	Note: Variables were treated as independent samples						
	Mean Group 1	Mean Group 2	t-value	df	p	F-ratio Variances	p Variances
0.3 M vs. 0.3 M	0.195122	0.195122	0.00000	162	1.000000	1.00000	1.000000
0.3 M vs. 0.7 M	0.195122	1.583333	-1.75702	224	0.080280	18.00110	0.000000
0.3 M vs. CMC	0.195122	4.915663	-3.57895	246	0.000415	51.24728	0.000000
0.7 M vs. 0.3 M	1.583333	0.195122	1.75702	224	0.080280	18.00110	0.000000
0.7 M vs. 0.7 M	1.583333	1.583333	0.00000	286	1.000000	1.00000	1.000000
0.7 M vs. CMC	1.583333	4.915663	-2.94781	308	0.003445	2.84690	0.000000
CMC vs. 0.3 M	4.915663	0.195122	3.57895	246	0.000415	51.24728	0.000000
CMC vs. 0.7 M	4.915663	1.583333	2.94781	308	0.003445	2.84690	0.000000
CMC vs. CMC	4.915663	4.915663	0.00000	330	1.000000	1.00000	1.000000

Table 4.14 compares the amount of albinos and green plants regenerated per anther and per plate/spike per genotype and per pre-treatment. On average Pre-treatment C produced a higher amount of albinos and green plants compared to Pre-treatment A and B, this however is expected due to the higher number of calli formed per treatment as shown in Table 4.14.

What is most important to note here is that the average number of albinos regenerated is almost double that of the green plants generated across the pre-treatments used which concur with previous research.

A total number of 22126 calli have developed across the different varieties and all the pre-treatments used. From the 22126 calli, 1070 albinos and 522 green plants were developed. The number of albinos regenerated was almost double the amount of green plants regenerated. Thus there's an average of a 2:1 ratio in the albino: green plant regeneration capacity throughout the different varieties. Green plants have the potential of developing into a doubled haploid, or alternatively a haploid. It is well known that barley has a 70% chromosome doubling capacity, meaning that 70% of the green plants regenerated from embryos have the potential of becoming a doubled haploid plant. With the 2:1 ratio mentioned above, in theory it would also mean that there would be twice as many albino plantlets generated than doubled haploids, and in some instances the number would be even greater.

Very limited literature is available on effective reduction of albinism in anther culture. There is a unanimous concern about the general high volumes of albino plantlet regeneration in anther culture as well as microspore culture. Further research needs to be performed to reduce the number of albinos regenerated through anther culture.

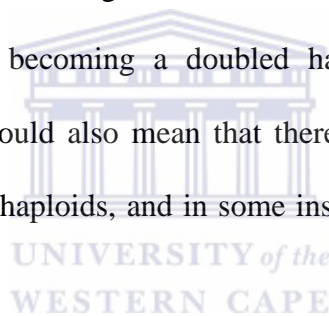


Table 4.14: Comparison Of Albino And Green Plant Regeneration Per Pre-Treatment.

Genotype	Pre-treatment	Albino per anther	Albino per plate	Green plant per anther	Green plant per plate
CM 01	0.3 M Mannitol	0.0	0.0	0.0	0.0
	0.7 M Mannitol	1.4	8.3	0.4	2.3
	CMC	5.8	19.3	1.7	5.6
CM 02	0.3 M Mannitol	0.0	0.0	0.0	0.0
	0.7 M Mannitol	0.5	2.0	1.6	7.0
	CMC	4.2	17.8	5.0	21.5
CM 03	0.3 M Mannitol	0.3	15.0	0.2	12.0
	0.7 M Mannitol	1.2	6.4	0.4	2.4
	CMC	2.5	9.9	1.6	6.2
CM 04	0.3 M Mannitol	0.0	0.0	0.0	0.0
	0.7 M Mannitol	0.7	5.0	0.1	0.5
	CMC	1.3	15.8	0.2	2.6
CM 05	0.3 M Mannitol	0.0	1.0	0.0	0.0
	0.7 M Mannitol	0.1	3.5	0.0	0.5
	CMC	0.0	0.5	0.1	3.5
<i>Mean</i>	<i>0.3 M Mannitol</i>	<i>0.1</i>	<i>3.2</i>	<i>0.0</i>	<i>2.4</i>
	<i>0.7 M Mannitol</i>	<i>0.8</i>	<i>5.0</i>	<i>0.5</i>	<i>2.5</i>
	<i>CMC</i>	<i>2.8</i>	<i>12.7</i>	<i>1.7</i>	<i>7.9</i>

CHAPTER 5

CONCLUSION

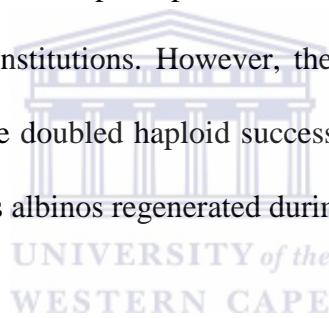
The aim of the study was to determine the optimal pre-treatment method for green plant production through barley anther culture. The results showed that anthers treated with CMC had a higher response percentage than anthers treated with 0.3 M Mannitol. The results show that the cold treatment with a moist cloth (CMC) induced a higher embryo formation frequency per 100 anthers than mannitol starvation combined with temperature. However, when comparing the two mannitol variations it is shown that the mannitol pre-treatment with a higher molarity (0.7 M Mannitol) shows a higher embryo formation frequency per 100 anthers than that of 0.3 M Mannitol. These findings are consistent to that of Cistué, Ramos and Catillo's findings in 1999, where the authors stated that 0.7 M Mannitol pre-treatment induced higher callus and green plant formation than that of 0.3 M Mannitol.

The results for green plant regeneration capacity after callus formation obtained in this study conclude that CMC will produce higher green plants per spike than the 0.3 M Mannitol pre-treatment. However, 0.3 M Mannitol produced a higher percentage green plant yield (50%) from the total number of calli formed. This suggests that if the total number of calli formed from the anthers treated with 0.3 M Mannitol were higher, then 0.3 M Mannitol could be considered to be a superior treatment than CMC.

It was also seen that the success of barley anther culture is highly genotype dependent. The pre-treatment CMC performed well across all genotypes used as donor material where 0.3 M Mannitol and 0.7 M Mannitol did not induce calli formation in all of the genotypes used.

This leads us to conclude that the success of green plant regeneration through anther culture is dependent on two things. The first is the genotype of the donor plant, as recalcitrant donor plants will not give the desired outcome. The second is the pre-treatment used to induce callus formation. CMC showed to be the most effective pre-treatment option on a broad spectrum of genotypes. However, further research needs to be conducted, because the results are inconclusive. The results obtained from this study conflicts with results obtained from other researchers such as Kasha *et al.* (2003 b) and Jähne and Lörz (1995) that stated that 0.3 M Mannitol pre-treatment is the optimal pre-treatment to use for doubled haploid production in barley.

The use of anther culture for doubled haploid production, even though it is labour intensive, has its use for barley breeding institutions. However, there is a need to optimise the pre-treatment methods to improve the doubled haploid success rate percentage and decrease the number of sterile plants as well as albinos regenerated during the process.



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Addendum A

List of Materials And Suppliers

Item	Suppliers
6-Benzylaminopurine solution	Sigma-Aldrich
Ammonium nitrate	Merck
Bacteriologic agar	Merck
Boric acid	Merck
Calcium chloride	Merck
Cloth, wipe	Spar supermarket
Copper (II) sulphate pentahydrate	Merck
D-Maltose	Sigma-Aldrich
d-Mannitol	Merck
Ethylenedinitrilotetraacetic acid iron sodium salt	Merck
Glacial acetic acid 100%	Merck
Indigo carmine solution (C.I. 73015)	Merck
Kelp (Effekto Wondersol)	Overberg Agri
Magnesium sulphate heptahydrate	Merck
Manganese (II) sulphate monohydrate	Merck
Myo-Inositol	Sigma-Aldrich
Nitrocellulose filter membrane	Sigma-Aldrich
Orion plus	Yara
Phytigel	Sigma-Aldrich
Polaris	Yara
Potassium di-hydrogen phosphate	Merck
Potassium hydroxide	Sigma-Aldrich
Potassium iodide	Merck
Potassium nitrate	Merck
Sodium hypochlorite	Spar supermarket
Sodium molybdate dehydrate	Merck
Sugar	Spar supermarket
Thiamine hydrochloride	Merck
Trelmix (Kompel trace element mix 200)	Overberg Agri
Zinc sulphate heptahydrate	Merck
α -naphthaleneacetic acid solution	Sigma-Aldrich