In vitro methods to accelerate oat breeding

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Abstract

Crop breeding is an endeavour which began over ten thousand years ago. Initially new variants would have been found by chance in the wild or selected from cultivated fields where uncontrolled crossing may have occurred. Over time, controlled crossing procedures have been developed for almost all crops leading to major improvements in performance. Choice of parents and selection of progeny has now become a precise art with the advent of genomic technologies and refined phenotyping. However, a major restriction remains in the number of generations required for stable varieties to be created by normal segregation from the heterozygous offspring of the initial crosses. Two artificial techniques may avoid the need for repeated selfing or back-crosses. Double haploid (DH) techniques manipulate gamete development to regenerate fully homozygous plants in a single generation, while genetic modification (GM) introduces desirable genes directly without the need for crossing. Both DH and GM are valuable tools for crop researchers investigating gene function and carrying out trait dissection as well as for breeders creating new varieties directly. However, both are reliant on efficient tissue culture methods. Oat is a significant crop, approaching 1M tonnes of production annually in the UK, with an increasing demand for high quality milling varieties whose breeding must keep pace with that of competing crops. Oat has proved recalcitrant to tissue culture and efficient protocols are lacking for both DH and GM. The aim of this project was to address this lack. Media optimisation steps improving DH efficiency are reported. F2 progeny of a cross between responsive and non-responsive parents were analysed with flow cytometry and SSR markers to confirm that haploids and spontaneous dihaploids were produced by the method. Analysis of donor plant phenotypic traits and tissue culture response suggest ways to further optimise the DH protocol to reduce labour and increase efficiency. Attempts to transform oat were not successful, however improvements in regeneration efficiency will support further optimisation of the transformation procedure. Overcoming oat recalcitrance to tissue culture methods is an incremental process. The improvements described here provide a reliable baseline from which to test further modifications.

Keywords: *Avena sativa* • Double haploid • GM • Tissue culture

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List of Abbreviations

°C	Celsius
AC	anther culture
AGP	Arabinogalactan proteins
Bar	Bialaphos resistance
CaMV	Cauliflower mosaic virus
DH	Double haploid
DNA	Deoxyribonucleic acid
DP	Donor plant
EDTA	Ethylenediaminetetraacetic acid
ELS	Embryo like structure
FAEA	Ferulic acid esterase
FIN	Kiviharju & Tauriainen AC media
GM	Genetic modification
IBERS	Institute of Biological, Environmental and Rural Sciences
IMC	Isolated microspore culture
Kb	Kilo base
L	Litre
M	Molar
MDHM	Modified double haploid *Miscanthus*
MIMI	Modified isolated microspore induction
mmHg	Millimeter of mercury
MS	Microspore
no.	number
pH	Potential of hydrogen
POL	Ponitka & Slusarkiewicz-Jarzina AC media
PPT	Phosphinothricin
QTL	Quantitative Trait Locus
RNAi	RNA interference
RO	Reverse osmosis
SSR	Simple sequence repeats
T-DNA	Transfer DNA
Ti	Tumour inducing
TR	Tandem repeats
VIGS	Virus-induced gene silencing
µM	Micro molar

Chapter 1: General introduction

1.1. Introduction

With increasing pressures on agriculture from climate change, the need for greater sustainability and consumer demands for healthier products, there are urgent needs to accelerate crop breeding cycles and to improve the efficiency of methods to identify and introduce novel genetic variation. Recent advances in genomics and sequencing technology have greatly improved breeders' ability to carry out genotyping, develop genetic markers, and predict likely phenotypes of elite crosses, but there are still major limitations in the speed at which new varieties may be produced. Significant reductions in breeding cycle times have been achieved for some crops by development of doubled haploid (DH) methods which generate homozygous plants in a single generation, rather than the five or more needed to produce a potential new variety from an initial cross using conventional methods. Genetic modification (GM) may bypass the need for crosses altogether by introducing the desired gene or allele directly into an elite variety. Even where GM may not be of direct interest for commercial variety production, it may be of great value in confirming the potential value of a novel breeding target. Both DH and GM are critically dependent on the development of efficient tissue culture protocols, which allow redirection of normal plant development and regeneration of fertile plants. Typically these protocols must be adjusted for each species, with strong genotype dependent variations in efficiency being seen even between varieties. Large numbers of variables may need to be optimised, requiring the establishment of a reliable initial protocol from which incremental improvements may be made. For the major temperate cereals, wheat and barley, such activities have been carried out in academic and commercial laboratories since the first release of DH varieties in the early 1980s and the first GM experiments in the 1990s. Oats have proved to be more recalcitrant and no reliable DH or GM protocols are currently available.

Haploid cells are generated during gametogenesis, and both male and female reproductive tissues may be used as a source of cells for DH. In addition, incompatibilities of alien chromatin in wide crosses may lead to breakdown of the chromosomes from the alien parent, generating haploid cells. Haploid tissue is grown under conditions which encourage embryogenesis, followed by transfer to media

which promotes the regeneration of plants. Variable rates of spontaneous doubling of chromosomes may occur at any stage, but can be artificially induced by anti-microtubule agents such as colchicine. DH plants are 100 % homozygous, avoiding the problems of phenotypic instability between generations caused by segregation of recessive alleles and new recombination events in heterozygotes. The DH method shortens the breeding process by up to 5 years compared to conventional breeding (Buyser *et al.,* 1987; Manninen 2008) making it very attractive to plant breeders as it allows them to react faster to changes in commercial markets. However, for DH technology to be deployed routinely by breeders there must be a high frequency of green plant regeneration without marked genotype dependence. Important factors for efficient production of DH include; donor plant (DP) genotype, DP growth conditions, DP pre-treatment, and growth medium composition (Kiviharju & Pehu 1998; Kiviharju *et al.,* 2005; Cesaro *et al.,* 2009). Double haploid technology has been incorporated into breeding programmes for a number of important crops including barley (*Hordeum vulgare* L.), oilseed rape (*Brassica napus* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.) (Wędzony *et al.,* 2009) but oat (*Avena sativa*) has so far proven to be largely recalcitrant (EM Kiviharju, 2009). Tissue culture methods in general are still inefficient for oat; in addition, wide crosses using maize pollen were found to yield partial aneuploids with significant retention of the alien chromosomes, complicating use of this approach (Kynast et al., 2001).

Transformation of oat was first reported in 1992 (Somers *et al.,* 1992) but has not been applied since, despite attempts by a number of laboratories to develop efficient protocols. *Agrobacterium*-mediated transformation has now been adopted as the method of choice by many cereal facilities while advances in genomic resources have increased the interest in a system to validate candidate genes *in vivo*.

1.2. Taxonomy and morphology of oat

Avena sativa L. (the common oat, 2n = 6x = 42) is an important temperate cereal crop grown for grain, silage, forage and as a cover crop. Traditional use frequently combined several rounds of grazing with a final grain or hay harvest Growth as a mixture with other cereals or legumes was also common. The genus *Avena* L. is nested within the tribe Aveneae, part of the subfamily Pooideae (Family Poaceae). All but one

species of *Avena* are inbreeding annuals. *Avena macrostachya* Bal. ex Coss. et Dur. is an outbreeding autotetraploid, perennial species (B. Baum, 1977; B. R. Baum & Rajhathy, 1976). The classification of the genus is still debated. The most comprehensive classification by Baum (1977) divided the genus into 29 species based on morphology. However Ladizinsky & Zohary (1971) recognised only 7 species based on interfertility of the taxa. A more recent review of the genus can be found in Ladizinsky (2012). Oats, as a monocotyledon, have seedlings with a single cotyledon (single leaf seed). They grow branches, called tillers, from their stem base. The narrow and parallel veined leaves differentiate from the tiller at points called nodes. The floral structure is complex. The inflorescence is compound, made up of a series of branches called spikelets (**figure 1.1**). In oats the spikelets are arranged as a panicle, branching off from the rachis. This contrasts with barley and wheat where the inflorescences are arranged directly on rachis nodes along the spike.

Each spikelet has one to several flowers, called florets (**figure 1.2**.). The floret is made up of the male and female parts; the three stamens and the superior ovary, respectively. The floret is enclosed within two bracts, the outer lemma and the inner palea. At the base of each floret there are additional bracts, called the glumes (E. White, 1995).

1.3. Evolution and cytogenetics

The genus *Avena* in the Poaceae family has a basic haploid chromosome number of n = 7 and is made up of three ploidy groups: diploid (2n = =2x = 14), tetraploid (2n = 4x = 28) and hexaploid (2n = 6x = 42). With the exception of the autopolyploid *Avena machrostachya* (a basal, outbreeding sp.), all tetraploid and hexaploid *Avena* species are allopolyploid. There are four karyotypes (genomes) (A, B, C, D) recognised in the genus. Genomes A and C are highly diverged (Legget & Thomas, 1995; Rajhathy & D., 1974). Genomes A and D are related, with evidence suggesting the D genome could be derived from the A genome (Li *et al.,* 2000). Yan *et al. (*2016) used a high density marker approach to show that *A. longiglumis* was the A genome species most closely related to ACD genome species. More recently, a study by (Fu, 2018) used a

multiplexed shotgun sequencing procedure to look at phylogenetic signals from chloroplast and mitochondrial genomes of 25 *Avena* species. The study suggested that the sub-genomes of the cultivated hexaploid (*A. sativa*) were mostly closely related to three of the diploid species (*A. ventricosa*, A. *canariensis* and *A. longiglumis*) and two of the tetraploids (*A. insularis* and *A. agadiriana*). It further demonstrated that *A. sativa* acquired its maternal genome from a DC genome tetraploid closely related to *A. insularis*. Understanding the evolution of the oat genome is important for effective germplasm conservation and its utilization in plant breeding.

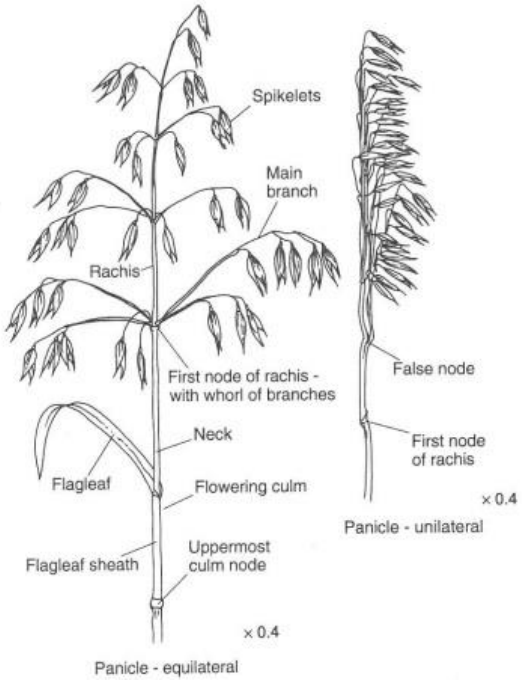


Figure 1.1. *An oat panicle* (E. White, 1995)

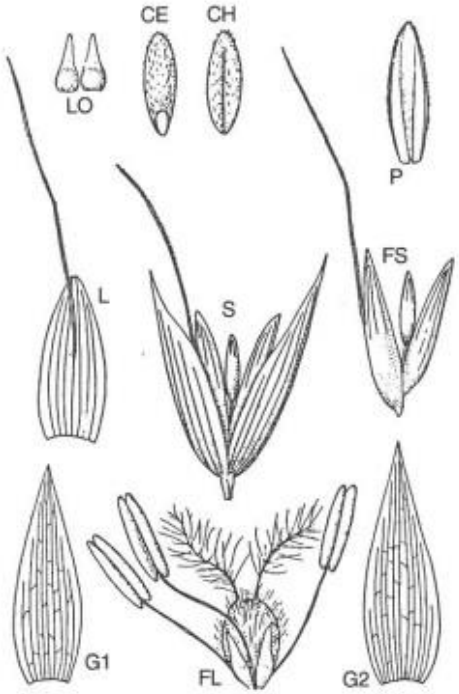


Figure 1.2. *A spikelet of Avena sativa. LO, locule; P, Palea; L, Lemma; S, Spikelet; FS, Florets; G1, Lower Glume; G2,Upper Glume; FL, Flower; CE, Caryopsis, dorsal view with embryo; CH, caryopsis, ventral view showing groove* (E. White, 1995).

1.4. Origin, distribution and domestication

Cultivated oats most likely originates from wild progenitors in Turkey and the Fertile Crescent. At least two origins have been suggested on the basis of distinct karyotypes shared with wild *A. sterilis* lineages (Jellen & Beard, 2000). The two cultivar types have been considered as separate species (*A. sativa* and *A. byzantina*), although they are interfertile and morphologically very similar. Compared with other Old World cereal crops, such as wheat and barley, the common oat was domesticated much later, being first seen as a significant crop in Western Europe, but some 5000 years after the initial wheat and barley (Ladizinsky, 1998). The weed species *A. fatua* is still common in cereal fields through Western Europe surviving through vegetative and seed mimicry. The cultivated oat is most likely a secondary domesticate which spread in a similar fashion.

1.5. Commercial uses

Oat is the fifth largest cereal crop in Europe after wheat, barley, maize and triticale, producing 23 million metric tonnes in 2016 (FAOSTAT, 2016). Oats are a low input cereal crop which were traditionally grown primarily as livestock feed. However more recently oats have been grown for human consumption due to the discovery of health benefits of the grain (Valentine *et al.,* 2011), namely the high levels of soluble dietary fibre β-glucan which have been shown to have positive health benefits including reducing blood cholesterol (Braaten *et al.,* 1994), postprandial blood glucose level (Braaten *et al.,* 1994) and the effect of retarded absorption of nutrients as a deterrent to numerous gastrointestinal tract disorders (Virtanenb & Mälkkia, 2001). The demand for oats, particularly for human and industrial use has been growing steadily over the last 20 years (**figure 1.3.**).

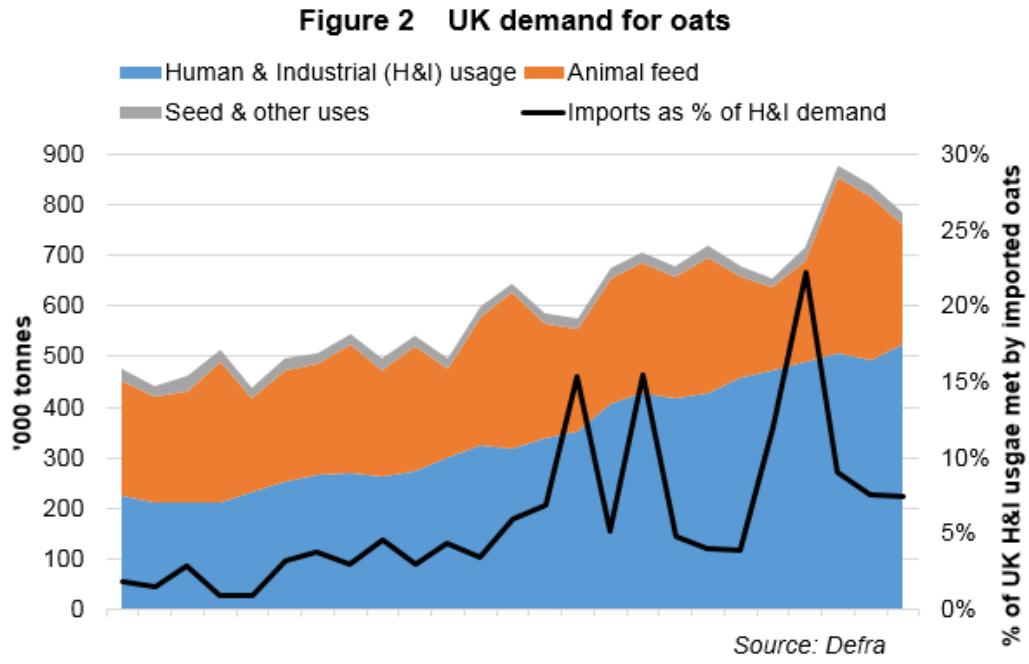


Figure 1.3. *UK demand for oat between 1992/93 and 2016/17.*

While oats have traditional been used as an animal feed, the fibrous husk of the grain lowers the energy of the grain. Naked oat cultivars have been developed as a specialist feed for horses. Due to the high oil content, naked oats are also valuable to the poultry industry due to the high levels of protein (Marshall *et al.,* 2010). The low lignin husk has also been found to be more digestible by rumen microbes, which could therefore have potential to lower methane emissions (Marshall *et al.,* 2010).

1.6. Tissue culture

Plant tissue culture, also commonly referred to as *in vitro* culture is a blanket term for the culture of cell, tissue and organ culture under sterile conditions, which has become a powerful tool in plant breeding. This section provides a brief history and highlights some key uses.

1.6.1. A short history of plant tissue culture

Plant tissue culture techniques became very important after the discovery of cellular totipotency in plants. Totipotency is the genetic potential of a plant cell to produce an entire plant. Totipotency was first suggested by Gottlieb Haberlandt (1902). The basis of plant tissue culture is to take plant cells from the root, shoot or any part of the plant, and culture them on a medium containing mineral nutrients, vitamins and hormones to encourage cell division and growth. This manifests over time as a ball of undifferentiated, unorganized cells which is called callus tissue. These cells are totipotent, and under the right conditions with the application of the right hormones in the correct concentration, whole plants can be regenerated.

In the 1950s Skoog and Miller discovered the plant growth hormone kinetin from the DNA of herring sperm (Miller *et al.,* 1955). In 1957, they presented the concept of hormone control of organ formation (Skoog & Miller, 1957). They showed that the differentiation of roots and shoots in tobacco pith tissue cultures was controlled by the ratio of auxin and cytokinins, and that organ differentiation could be regulated by changing the relative concentrations of the two hormones in the culture medium. This model has been shown to operate in many plant species (Evans *et al.,* 1981). Endogenous plant hormones were later discovered, including in coconut water (Letham, 1974) which had been used 30 years earlier for the culture of young embryos and other recalcitrant tissues (Van Overbeek *et al.,* 1941). The production of whole plants in tissue culture was found to occur via shoot or root differentiation, and also by embryogenesis. Shoots and roots are monopolar, while somatic embryos (different from zygotic embryos) are bipolar, meaning they can develop into a whole plant without needing to be cultured on a multitude of media. The first publications about somatic embryos came in the late 1950s (Reinert 1959; Steward *et al.,* 1958).

A nutritious medium is vital for successful, long term tissue growth. The nutritional requirements of different species can vary considerably, and the same is true for different plant tissues (Murashige & Skoog, 1962). There is no single medium which is optimal for all situations. Tissue culture medium optimization is therefore a crucial step for any new

experiment which relies on a tissue culture phase. The earliest tissue culture media were developed from nutrient solutions used to culture whole plants. The root culture medium developed by White (1943) was based on the algae medium of Uspenski and Uspenskaia (1925). All subsequent media is now based on the two formulas by Gautheret (1939) and White (1943). White's medium added iron in the form of $Fe2(SO_4)_3$, which was then replaced with $FeCl_2$ due to metallic impurities. However this form was succeeded by Fe-EDTA, as $FeCl_2$ is not available to roots above pH 5.2 (root culture pH is usually 5.8-6.0) leading to iron deficiency. Fe-EDTA could be used up to pH 6.0 due to roots secreting chelates which bind with the iron (Heller, 1953). This was further improved by Nitsch and Nitsch (1956) with their work on artichoke. Despite this, the addition of yeast extract, protein hydrolysate and coconut water were often necessary, although their constituents and action were not fully understood. Tissue culture became possible for more and more species as the medium continued to be improved. Work on tobacco callus by Murashige and Skoog (1962) found that supplementing White's medium with kinetin and indoleacetic acid led to a four-fivefold increase when an aqueous solution from tobacco leaves was used. Their media used a much higher concentration of salts, particularly NO_3^- and NH_4^+ as well as increasing the range of micronutrients.

1.6.2. Clonal propagation

Ball (1946) was the first to produce rooted shoots, by culturing shoot tips with primordia of *Lupinus* and *Tropaeolum*. The importance of this discovery was not realised, with meristem culture only being of interest to plant pathologists, until Morel (1960), used the approach to produce virus-free *Cymbidium* plants. This was due to the viruses inhabiting the vascular tissue, which does not extend all the way into the root or shoot apex. This method has been refined and is now used routinely (Houten *et al.,* 1968).

1.6.3. In vitro fertilisation and embryo rescue

A major goal for crop improvement is to overcome the difficulty of transferring desirable genetic material across divergent species, where laborious wide hybridisation must be attempted using conventional crossing. Tissue culture techniques have provided more

efficient alternatives. *In vitro* fertilisation (IVF) was developed by Kanta, Rangaswamy and Maheshwari (1962) with *Papavar somniferum*, the opium poppy. The method involved culturing excised ovules and pollen grains together. This technique has allowed breeders to overcome sexual incompatibility in the pre- and post- zygotic stages, such as failures in pollen tube growth. IVF has been used to create many important interspecific and intergeneric hybrids. These include important crops such as tobacco, corn, rice and poppy. A very common cause of post-zygotic failure during wide hybridization is abnormal endosperm development. Embryo rescue overcomes problems with low seed set, seed dormancy and germination, and embryo growth in the absence of symbiotic partners (Aragão *et al.,* 2002; Acebedo *et al.,* 1997; Emershad & Ramming 1994; Cisneros & Tel-Zur 2010). IVF has also been very useful as a tool to directly observe and analyse fertilization and post-fertilization processes in angiosperms (Wang *et al.,* 2006) which was previously confined to *Arabidopsis* mutants.

1.6.4. Protoplast fusion

Plant somatic hybridization through protoplast fusion is another powerful tool for developing hybrid plants which cannot be made by traditional crossing techniques. The method allows the fusing of somatic cells from different cultivars, which is otherwise halted by sexual incompatibility and sterility.

Plant somatic hybridization via protoplast fusion has become an important tool for ploidy manipulation in plant improvement schemes, allowing researchers to combine somatic cells from different cultivars, species, or genera, resulting in novel allotetraploid and autotetraploid genetic combinations. This technique can facilitate conventional breeding, gene transfer, and cultivar development by bypassing some problems associated with conventional sexual hybridization including sexual incompatibility, nucellar embryogenesis, and male or female sterility.

1.6.5. Genetic transformation

Tissue culture is vital for reliable regeneration and selection of transformed plants, and is a necessary step in the vast majority of transformation protocols, which were first

reported for tobacco (Bevan, Flavell, & Chilton, 1983). Tissue culture medium can be optimised for the various needs of the plant species or tissue, and creates a closed environment where selection agents such as antibiotics can be used safely and in small amounts. Transformation strategies that do not require tissue culture or regeneration have been published (Clough & Bent, 1998) but for the majority of plant species these techniques are not possible, particularly with monocots which are confined to regeneration from meristematic tissue.

1.6.6. Haploids and their production

Haploid plant cells are produced during gametogenesis. Normally, a haploid pollen grain fertilises a haploid egg cell, the gametes fuse and a diploid embryo then develops (together with a more complex pathway to generate the supportive endosperm tissue). However embryogenesis can also occur in somatic and gametic tissue, spontaneously or triggered *in vitro*. In somatic embryogenesis, embryos are initiated from already differentiated cells. This requires reprogramming of gene expression, causing structural changes similar to those in zygotic embryogenesis (Sharma & Millam, 2004). In gametic embryogenesis, gametes are diverted from their gametic pathway for microspore and ovule development to a sporophytic pathway, before they are allowed to mature. Somatic embryogenesis will produce clonal diploid embryos of the parent genotype (excluding somaclonal variation (Larkin & Scowcroft, 1981), while gametic embryogenesis will initiate the production of haploid plants which are not identical to the parental genotype, due to the gametes origin following meiosis and subsequent chromosomal segregation. Gametic embryogenesis can occur directly from predetermined cells (without a callus phase), or indirectly from undetermined cells (callus). Doubling of haploid chromosomes may then occur spontaneously or following the application of external or artificial stress, leading to tissue and regenerated plants with a normal diploid composition, known in this context as doubled haploids (DH). The first haploids in angiosperms were identified in *Datura* in 1922 (Belling & Blakeslee A. F. 1922). Haploids have been found to occur spontaneously (but rarely) in numerous plant species (Maluszynski *et al.,* 2003; Harlow *et al.,* 1996) but it was not until Guha and Maheshwari

developed numerous pollen derived plants from anther cultures of *Datura innoxia* (Guha & Maheshwari 1964) that the technique could be reliable, and had value for plant breeders.

There are four main methods for inducing haploidy in plants at high enough efficiency for use in breeding programmes: androgenesis, the culture of microspores which undergo embryogenesis via callus, gynogenesis, the culture of unfertilised isolated ovules which undergo embryogenesis, wide hybridization, the use of interspecific or intergeneric crosses followed by chromosome elimination of the pollinator during cell division, and parthenogenesis, the development of the embryo through pseudogamy, semigamy or apogamy.

1.6.7. Wide hybridization

Where empirical tests have identified suitable conditions, wide hybridization may be the most efficient method to generate haploid plants. During crosses between particular species or genera, chromosomes of the pollen parent are lost during the early cell division stages, resulting in a haploid embryo derived from the female parent only. In most cases the endosperm also fails to develop normally, however. Using embryo rescue, these haploid embryos can survive by being isolated and grown on artificial medium with supplemented nutrients and growth hormones. It was first reported in 1984 (Zenkteler & Nitzsche, 1984) that embryos were formed when hexaploid wheat was pollinated with maize (Zea mays L., 2n=20) and has since been confirmed in numerous experiments (Garciallamas, 2004; Guzy-Wrobelska & Szarejko 2003; Bakos *et al.,* 2005). The approach has also been successful in durum wheat (Almouslem & Jauhar, 1998; Jauhar, 2003). When wheat is fertilised with maize pollen, the hybrid zygote develops, but preferentially eliminates the maize (pollen parent) chromosomes during the early development stage of the hybrid embryo (Almouslem & Jauhar, 1998; Jauhar, 2003), which leads to the formation of a haploid plant.

1.6.8. Gynogenesis

Gynogenesis may be an alternative for haploid production where species are recalcitrant to androgenesis. It is also an important alternative for species which exhibit male sterility or are dioecious (Bhat & Murthy, 2007). The first description of gynogenesis by culturing unfertilised ovules was in barley (Noeum, 1976). Since then recovery of haploid plants through gynogenesis has been successful in a number of species, including *Allium cepa* (Luthar & Bohanec, 1999), *Ipomoea batatas* (Kobayashi *et al.,* 1993), and *Zea mays* (Tang *et al.,* 2006). As with androgenesis, the development stage of the donor tissue plays an important role (San *et al.,* 1986), with most success achieved with almost mature embryos (Lux *et al.,* 1990). In tobacco, ovules with young uninuclear to mature embryos were most responsive (Wu & Cheng, 1982). As plants have many hundred fold more microspores than ovaries, gynogenesis presents greater logistical limits than androgenesis. Its use is mostly restricted to onion and sugar beet.

1.6.9. Parthenogenesis

In this method of haploid formation, the egg cell of the embryo sac develops into an embryo without involvement of the sperm cell nucleus. This is called pseudogamy. Apogamy is where the embryo develops from a haploid cell of the embryo sac other than the egg cell. Parthenogenesis differs from chromosome elimination and gynogenesis which usually trigger endosperm failure and require that the embryo must be cultured in vitro. In parthenogenesis the endosperm develops, and matures in vivo. Parthenogenesis can be induced using inactivated pollen, or by chemical treatment (Khush & Virmani, 1996).

1.6.10. Androgenesis

Androgenesis is also referred to as microspore embryogenesis. It is a form of cellular totipotency, which is defined as the ability for a cell to divide and produce all of the differentiated cells in an organism (Mitalipov & Wolf, 2009). It is the process of induction and regeneration of plants from the male gametic cells; microspores or immature pollen. Androgenesis is thought to be a stress induced survival adaption (Bonet *et al.,* 1998). It is

the most universal and dependable form of gametic embryogenesis, as it does not depend on particular genetic incompatibilities, unlike wide hybridisation, and has a potentially high throughput, unlike gynogenesis or parthenogenesis. It has been successfully developed in a wide range of plant species, and is used for breeding of numerous important crops. The key step in the method is the switching of the gametophytic developmental pathway for mature pollen grain development to a sporophytic pathway generating haploid callus or embryos (Murovec & Bohanec, 2012).

1.7. Project objectives and impact

The main goal of this project is to make progress towards a commercially viable method for the creation of double haploid lines for oat. Double haploid production has become a powerful tool for plant breeders to accelerate the production of homozygous lines (Germanà, 2011), which become even more effective when combined with other technologies such as marker assisted selection (Xu *et al.,* 2017) or GM (Chauhan & Khurana, 2011). Major crops, including barley, wheat and maize, now have commercially available DH derived lines, with many additional crops being studied to make the technology applicable. The ability of these crops to be adapted rapidly by breeders to meet commercial, legislative and environmental conditions presents significant challenges to oat breeders who must maintain the competitive value of oat varieties for producers. While progress in DH has been reported in oat with wide-hybridization (Rines, 2003), anther culture (Tanhuanpää *et al.,* 2012) and isolated microspore culture (Ferrie *et al.,* 2013) protocols still fall a long way short of the levels of efficiency that can be commercially viable, or reliable for research purposes.

The overall objectives of this Ph.D are (i) build on current research and further optimise stages of the anther culture protocol using responsive genotypes. (ii) Apply protocol optimisation to recalcitrant genotypes with the aim of improving the universal oat base line response (iii) and develop double haploid populations from oat crosses and screen for homozygosity with SSR markers.

The second part of this project is to optimise the transformation protocols for oat. While oat has been successfully transformed by biolistics (Maqbool *et al.,* 2004) and *Agrobacterium* (Gasparis *et al.,* 2008) the efficiency is not high enough for routine use due to time consuming optimisation and genotype dependency. Objectives here are (iv) apply *in vitro* advances from oat DH work to recent protocols developed for other species using biolistic and *Agrobacterium* approaches (v) optimise procedures if justified by preliminary experiments.

The double haploid component of the project is a co-operation between Aberystwyth University, UK, the seed marketing and crop development company Senova Ltd, UK, and the joint group of plant breeders Saaten-Union Biotec GmbH Germany.

Chapter 2: Optimization of anther culture and isolated microspore culture

2.1 Introduction

2.1.1. Anther development and embryogenic potential

Male gametes are generated in grasses and cereals by meiosis in young anthers, followed by mitosis of the haploid microspores and development into mature pollen grains. Fertilisation of egg cells by pollen is then followed by an embryogenic pathway leading to development of diploid plants.

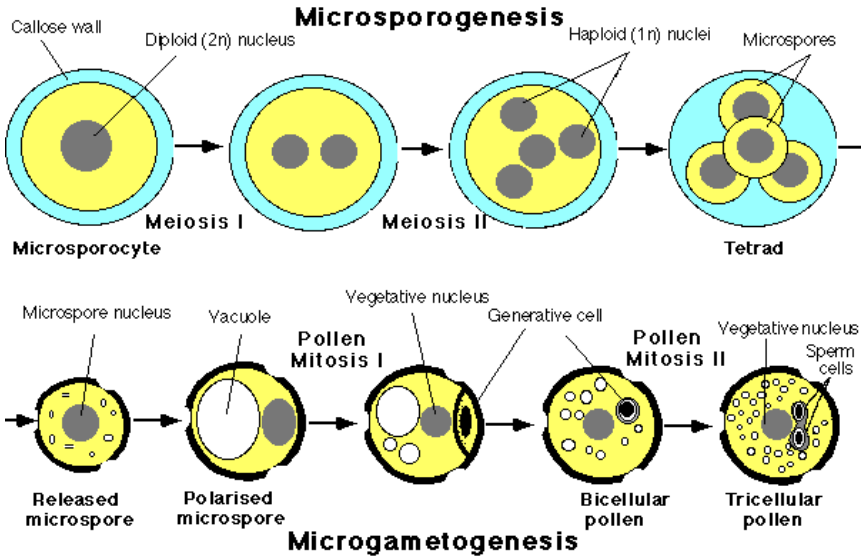


Figure 2.1. *Source* (*Pollen Development — University of Leicester*)

Microspores retain a degree of totipotency, allowing them to switch from a gametophytic to an embryogenic pathway. This switch occurs spontaneously at a very low rate, which may be significantly increased by stress during the critical phase of microspore development (Hosp *et al.,* 2007) (**figure 2.1.**). A range of different challenges have been shown to induce microspores to switch to an embryogenic pathway, including extreme temperature differences, carbohydrate or nitrogen starvation, and chemical, hormonal or irradiation treatments (Würschum *et al.,* 2013) (**figure 2.2.**). By optimising pre-treatment stress and donor plant cultivation conditions, microspore embryogenesis may be increased and mortality decreased to levels where microspore derived embryos maybe be recovered in sufficient numbers to allow efficient regeneration of plants *in vitro* following culture with appropriate nutrients and hormones.

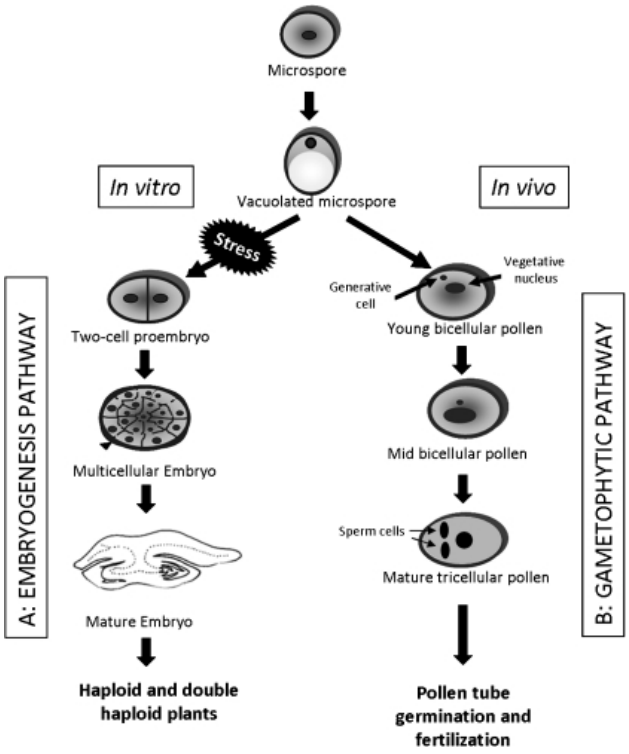


Figure 2.2. *Embryogenic and gametophytic developmental pathways of the microspore. (A) Embryogenic pathway: after stress treatment* in vitro at 4°C in darkness, the *microspore is reprogrammed and follows an embryogenic pathway form an embryo, that can regenerate into a plant. (B) Gametophytic pathway: in vivo, without stress the microspore continues on the gametophytic pathway and develops into a mature pollen which further germinates and carries out fertilization. (Source: María Rodríguez-Serrano, Ivett Bárány, Deepak Prem, María-José Coronado et al., 2012)*

2.1.2. Anther culture and isolated microspore culture

Anther culture (AC) or isolated microspore culture (IMC) are used to overcome the very low frequency of spontaneous embryogenesis by creating a favourable environment and providing additives that increase induction rates. Different plant species, as well as different varieties within species have been shown to respond differently, and it is clear there is no universal protocol. The two approaches differ primarily in the way the

microspores are cultured. In both cases immature flower spikes are harvested, and pre-treated. Culture medium is similar, but the mode of suspension is different. In anther culture, anthers containing microspores are plated directly onto solid state medium, while in IMC anthers are mechanically broken, and the microspores are isolated first by shedding (natural or induced) or destruction of the anther by blending or macerating. The mixture is centrifuged, sieved or both to separate the microspores from other debris, and then suspended in the culture medium. In barley, IMC is more efficient than AC for green plant regeneration (H. Li & Devaux, 2005). Isolating anthers in AC is time consuming, as each flower needs to be taken apart to extract the immature anthers. This is especially laborious in species with small flowers. With IMC, many whole flowers can be blended in seconds, and the microspores can be isolated by centrifugation in minutes. Removal of the anther walls in IMC prevents the somatic tissues of the wall interfering with the microspore. It also removes the possibility of somatic embryogenesis from the anther tissue. Finally, IMC also allows direct observation of the development of the microspore development without needing to sample destructively. However, the isolation of microspores is difficult due to the high risk of damaging microspores, and the media conditions must be fully optimised, as the microspores are not protected by the anther somatic tissue.

2.1.3. Genotype of donor plants

Genotype plays a major role in embryogenic response, with numerous studies finding variation in embryogenic potential. In almost all species studied, genotype has consistently been a key factor, with different cultivars showing great differences in response. In rice, *japonica* varieties were found to be more responsive than *indica* (Yan *et al.,* 1996). Even model species show a strong genotypic response of cultivars within a particular species. The first successful induction of androgenic callus in *Miscanthus sinensis* showed one genotype to be more responsive to three others (Glowacka & Jezowski, 2009). Rudolf *et al.* (1999) found that crossing recalcitrant genotypes of white cabbage with non-recalcitrant genotypes produced a progeny line more responsive than both parent, showing that embryogenesis genes can be effectively transferred to hybrid

crosses. This has also been reported in maize (Petolino & Thompson, 1987), and has potential in other species (Smykal, 2000). In 1998, Bregitzer *et al.,* showed that genotype dependency can be overcome by developing genotype specific protocols, primarily by using concentrations of particular medium components at concentrations tailored to each genotype to maximise regeneration efficiency.

2.1.4. Growth of donor plants

Optimisation of growth conditions have been found to be critical for efficient DH production, with commercial services generally operating in natural growth seasons to provide donor plants in autumn or spring as appropriate. To maximise success, donor plants should be healthy, pest free and grown in rich, fertile soil with optimum temperature and light. Most physiological factors have been investigated, including time of year, plant position and comparisons of greenhouses to growth chambers. Dahleen (1999) studied the effects of the donor plant environment on regeneration in barley. It was found that green plant regeneration was increased and less variable from donor plants grown in growth chambers than from plants growing in a greenhouse. Regeneration efficiency from greenhouse-grown donor plants could match donor plants grown in growth chamber conditions when natural light levels were high and temperatures were moderate. In barley cultivars Igri and Cork, tillers harvested between January-July produced significantly more plants than tillers harvested from August-December (Jacquard *et al.,* 2006) which suggests that light intensity and day length are important factors and that donor plants grow better in their preferred season. They also found that the physiology of the spikes harvested from donor plants was crucial, and that the position of the spike was also an important factor. In perennials the age of the plant was found to be important. *Aesculus carnea* trees older than 60 years gave a higher embryogenic response (20%) compared to younger trees (9%) (Marinkovié & Radojevié, 1992).

2.1.5. Development stage of microspore

The development stage of the microspore is one of the most important endogenous factors for successful induction. For the vast majority of species the first pollen mitosis stage, when the cell vacuolates before the early bicellular stage (Touraev *et al.,* 2001) is the optimum stage for inducement. During this stage the microspore is on its way into developing into a pollen grain that comprises of generative and vegetative nuclei. It is the generative nucleus that develops into two sperm nuclei. At this stage induction can be successful due to semi differentiated transcriptional status (Malik *et al.,* 2007) of the cell, in comparison to mature pollen which are fixed to their development pathway (Honys & Twell, 2004).

2.1.5.1. Development of microspores

Figure 2.3. shows the key steps in microspore development. Under sporophytic development, microspores are surrounded by a primexine wall, with the nucleus in the centre of the cell. This is followed by the formation of the exine and the vacuole, which fills the microspore. The nucleus moves to the edge of the microspore. After the first cell division the vacuole is reabsorbed and the cell accumulates starch grains. When stress is successfully applied, the isolated microspores swell, to become significantly larger than non-induced cells. The cytoplasm structure is rearranged, and the nucleus moves from the edge of the microspore to a more central position with linear cytoplasmic strands coming out from it, forming a 'star-like' structure, as seen in tobacco (Touraev *et al.,* 1996) and rice (Raina & Irfan, 1998), barley, wheat and oat (Gnad, personal communication). No starch grains are present.

2.1.5.2. Identifying microspores at optimum cell stage

Identifying when microspores are at the optimum cell stage for harvesting is difficult due to the inability to observe the microspores without destructive sampling. However, cytological screening is critical to ensure that microspores are stressed and cultured at the optimum cell stage. In oats, florets are sampled from the developing panicles, the anthers dissected, and then squashed onto a microscope slide (Lantos et al., 2013). The microspores are released and can be observed. Once the optimum microspore stage has

been determined (mid to late uni-nucleate), physical characteristics can be used as a non-destructive indicator. The method suggested by Wheatley *et al. (*1986) correlates the length between the flag leaf base and the penultimate leaf base to the microspore stage. The method is not wholly precise as it varies between genotype, and within the genotype as the seasonal/environmental conditions affect plasticity (personal observation). Measurement of the flag leaf base to penultimate leaf base can then be correlated to microspore stage for harvesting, but regular anther squashes are still necessary.

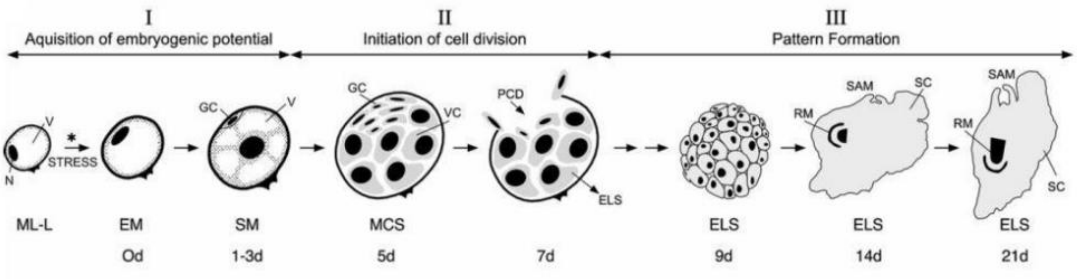


Figure 2.3. *The phases of androgenesis* (Maraschin *et al.,* 2005) *The three phases of androgenesis are: I- The acquisition of embryogenic potential, through by stress inducement, causing a change in the development pathway. II- Multicellular structures develop. III- Embryo-like structures are released from the exine wall. ELS, Embryo-like structure; EM, enlarged microspore; GC, generative cell; MCS, multicellular structure; ML-L, mid-late to late uni-nucleate microspore; N, nucleus; PCD, programmed cell death; RM, root meristem; SAM, shoot apical meristem; SC, scutellum; SM, star-like microspore; V, vacuole; VC, vegetative cell.*

2.1.6. Pre-treatment of donor plants

Microspores retain the ability to differentiate into any other tissue type under appropriate conditions ('totipotency'). Under *in vitro* conditions they may be diverted from normal pollen development to an embryogenic pathway by a number of different physical and physiological stress treatments. Stress treatments during initial microspore development have been found to be beneficial in inducing an embryogenic response

(Touraev *et al.,* 1996). A large number of pre-treatments have been evaluated, both independently and in combination. They include cold stress (Imin *et al.,* 2004; Kiviharju & Pehu, 1998; Oleszczuk *et al.,* 2006; Xie *et al.,* 1997), heat shock (Telmer *et al.,* 1995; Würschum *et al.,* 2013; Parra-Vega *et al.,* 2012,) osmotic pressure (Ochatt *et al.,* 2009), starvation (Touraev *et al.,* 1996) pre-treatment with oligosaccharides (Lemonnier-Le Penhuizic *et al.,* 2001), colchicine (Smykal & Pechan, 2000), and gamma-irradiation (Pechan & Keller,1989). Mejza *et al.* (1993) found incubating wheat spikes at 25 °C instead of 5 °C improved embryogenic responses in wheat.

2.1.7. Composition of Induction medium

The tissue culture media composition is important for maximising embryogenic potential, ensuring as many microspore derived embryos as possible have favourable conditions to develop successfully. Antibiotics can also be used in cases where infection is difficult to avoid (Lantos & Páricsi, 2006). No universal induction media exists for all species. A well-documented list can be found in Kasha & Maluszynsky (2003). Most variation in media composition is in the source of nitrogen, carbohydrate and growth regulators. There is no universal culture media for all species. A number of base media have been used for anther/IMC culture. Murashige & Skoog media (MSO) is the medium used as the base for many plant cell/tissue culture experiments (Murashige & Skoog, 1962). Nitsch & Nitsch medium NPB-99 (Liu *et al.,* 2002), modified as W14 (Ouyang *et al.,* 1989), is a media originally devised for wheat, and has been used as a basis for many species, including oat (Ślusarkiewicz-Jarzina & Ponitka, 2007; Kiviharju & Tauriainen, 1999; Kiviharju, 2009). One of the early discoveries for improving efficiency was the reduction in use of inorganic nitrogen such as ammonium nitrate (NH_4NO_3) in preference to organic sources such as glutamine (Olsen, 1987). In barley, over 500 mg/L of glutamine is now used (Li & Devaux, 2001; Ritala *et al.,* 2014). In triticale, increased levels of glutamine or asparagine (an organic amino acid) have been used (Pauk *et al.,* 2003; Wędzony, 2003), while in *Brassica,* high levels of glutamine with serine (an organic amino acid) (Ferrie, 2003), were both

successful. It is therefore thought that organic sources of nitrogen are better for embryonic development than inorganic nitrate sources.

Carbohydrate is important for providing energy for cell growth, as well as aiding in the regulation of osmotic potential in the media. The switch from sucrose to maltose as a carbohydrate source has made a large improvement in efficiency in embryogenesis. For a number of protocols, maltose has replaced sucrose as the sole carbohydrate source. In wheat, while maltose was reported to improve the efficiency of microspore embryogenesis in general (Mejza *et al.,* 1993), Trottier *et al.* (1993) found that while it improved the embryogenic efficiency in recalcitrant genotypes, genotypes responsive in sucrose were less responsive in maltose. This phenomenon was also reported in rice (Lentini *et al.,* 1995) with some rice cultivars responding well to use of some sucrose (Shahnewaz & Bari, 2004). In oats, Kiviharju & Pehu (1998) found using maltose over sucrose improved embryogenesis efficiency. This is possibly due to its slow hydrolysis by plant cells in comparison to sucrose, which causes starvation conditions early in culture, and stable osmolarity of the media in the long term (Indrianto *et al.,* 1999).

Casein hydrolysate is a widely available compound used in culture media. It is produced by acid hydrolysis of casein, a phosphoprotein found in cow milk. It contains amino acids such as glutamic acid/glutamine, lysine and proline (Wang *et al.,* 2013). It is primarily used as an amino acid source for *Bacillus* spp. but has also found use in plant tissue culture, and is routinely included in induction media for barley (Kumari *et al.,* 2009) and also oat at 300 mg/L in anther culture (Kiviharju & Pehu, 1998) and 500 mg/L in somatic embryogenesis (Hao *et al.,* 2006). In date palm, it was found to improve callus growth 2-3 fold over control cultures (Al-Khayri, 2011).

The application and balance of plant growth regulators is important for morphogenetic processes (Schulze, 2007) including embryogenesis. A large number of different nutrients and hormones have been tested for improving DH efficiency. The most significant are highlighted here. Most microspore culture protocols use either a cytokinin alone or in combination with an auxin. Two of the most widely used are the auxin 2,4-D (2,4,

dichlorophenoxyacetic acid) and cytokinin BAP (6-Benzylaminopurine). 2,4-D is a synthetic auxin (a class of plant hormones which regulate growth), a commonly used systemic herbicide of broad-leaf plants. It has been used as a inducer for microspore differentiation (Xie *et al.,* 1995; Rodrigues & Forte 2004), and in *Brassica napus* has been proven to be an effective pre-treatment (Ardebili & Shariatpanahi, 2011). 2,4-D has also been used successfully as an additive in induction media in small quantities (10 μM) in a wide range of plants (Zhang & Qifeng, 1993; Shariatpanahi *et al.,* 2006; Kiviharju *et al.,* 2004). Kinetin is a type of cytokinin, a class of plant hormones that promotes cell division in the presence of auxin. As with 2,4-D, it is widely used in media to induce embryogenesis (Kiviharju & Tauriainen, 1999; Olszewska *et al.,* 2014; Ibrahim *et al.,* 2014; Lentini *et al.,* 1995). More recently, other auxins and cytokinins have been used to attempt to improve efficiency in recalcitrant genotypes. Ascorbic acid is a naturally occurring compound produced from glucose, and is found in animal and plant organisms. It plays an important role in iron reduction in plants, and has been proven to be produced in embryos (Grillet *et al.,* 2014). In *Brassica napus* the addition of ascorbic acid improved embryogenesis substantially (Hoseini *et al.,* 2013) and in shoot formation of Tobacco (Joy *et al.,* 1988).

Extracellular arabinogalactan proteins (AGPs) are found in plants and bacteria. In plants they are a major component of gums, such as gum arabic. Their basic make up is a core-protein backbone O-glycosylated by one or more complex carbohydrates which are predominately made up of galactan and arabinose. AGPs have many important roles for plant growth and development, including cell division and programmed cell death (Seifert & Roberts, 2007). Van Hengel & Roberts (2003) identified a particular AGP called AtAGP30 in *Arabidopsis* which is only expressed in the roots. Using a mutant they found the wild type product was essential for *in vitro* root regeneration. Prior to this Kreuger & Van Hoist (1993) found AGPs to be essential for somatic embryogenesis in *Daucus carota* L. in microspore culture of maize (Borderies *et al.,* 2004), AGPs are secreted by microspores over the course of culture. When glycosylation is inhibited by tunicamycin, embryo formation is halted. It was also shown that non-stressed microspores transferred to

culture did not produce embryos or AGPs (Shariatpanahi *et al.,* 2006). Similar results have been found in wheat, where the addition of AGPs in the form of Arabic gum and larcoll decreased the death rate of microspores, and improve the induction of embryos and regeneration of green plants (Letarte *et al.,* 2006). Arabinogalactans have been identified and extracted from *A. sativa* (Göllner *et al.,* 2011) but to date there are no published experimental data for their use in microspore culture.

Polyamines are naturally occurring aliphatic nitrogenous compounds that are important in cellular processes such as protein synthesis, abiotic stress response and cell division (Kakkar & Sawhney, 2002). The most well-known polyamines are putrescine, cadaverine, spermidine, and spermine. Putrescine has been used for pre-treatment in wheat anther culture with positive results (Redha & Suleman, 2011) and was found to improve stability in the culture of oat leaf protoplasts (Altman *et al.,* 1977) and also increased the efficiency in somatic embryogenesis of elite oat cultivars (Kelley *et al.,* 2002), inducing significant numbers of embryos even in previously recalcitrant cultivars. However to date putrescine has not been tested in microspore embryogenesis of oat. Glutamine is an amino acid that has an important role in nitrogen metabolism and synthesis of many nitrogen containing compounds. Early reports found that glutamine was important for successful embryogenesis of pollen without the anther present (Wernicke & Kohlenbach, 1977) and the same was seen in carrot suspension culture (Wetherell & Dougall, 1976).

Proline is another amino acid which is often used in tissue culture medium, and plays an important role in cell wall synthesis and plant development (Kavi Kishor *et al.,* 2015). It is associated with plant stress responses (Subhashini & Reddy, 1991) and at optimum concentration improves embryogenic callus formation and plant regeneration (Holme *et al.,* 1997). Proline is routinely added to tissue culture medium for many species including rice (Pawar *et al.,* 2015), oat (Gana *et al.,* 1995), sorghum (Liu & Godwin, 2012) and *Miscanthus* (Holme *et al.,* 1997), and so is a very strong candidate compound for improving anther culture in oat.

Trottier *et al.* (1993) found that liquid media was more efficient at producing green plants than solid or gelatinous media. This is possibly due to the increase in surface area of the anther available to absorb nutrients. In oats, solid, liquid and combinations of both have been tested with varying levels of success (Ślusarkiewicz-Jarzina & Ponitka, 2007; Kiviharju *et al.,* 2000). In barley, filter sterilised media yielded a two fold higher frequency of living microspores, and significantly more green plants, than autoclaved media (Li & Devaux, 2005), possibly due to organic media compounds being damaged by autoclaving.

2.1.8. Co-culture and Inter-culture

The environment provided *in situ* for developing microspores by the surrounding somatic tissue is inevitably far more complex than can be replicated by simple culture media. A requirement for additional factor(s) may often be seen by a density dependent effect on survival and growth of isolated microspores, which is interpreted as an interplay of hormones and micronutrients. DH protocols have been developed to use additional plant tissues in an attempt to provide additional factors. In particular, the use of ovaries to improve anther culture and isolated microspore culture has been successful in improving the efficiency of several plants. Kohler and Wenzel (1985) found that conditioning of IMC medium for winter and spring barley was required for successful callus formation and plant regeneration. Pre-conditioning with 10 ovaries per millilitre of medium for seven days was the most effective method. In wheat, the addition of wheat and barley ovaries were critical for microspore-derived embryos (Mejza *et al.,* 1993). It was also noted that the genotype of the ovary used was significant. Hul and Kasha (1997) found that the use of ovary co-culture improved the quality and quantity of embryogenesis in wheat. Genotypic differences were also reduced with the use of ovaries; however genotype still had a significant effect on regeneration. Broughton (2008) found that for spring barley the addition of ovaries to the induction medium increased the mean number of embryogenic like structures per spike from 7.6 to 50.1, and that of green plants per spike from 0.6 to 8.9.

In wheat, live ovaries increased embryogenesis by up to 4.5-fold over the control for microspores isolated from responsive genotypes (Zheng *et al.,* 2002). Live ovary co-culture alone was not effective for microspores isolated from recalcitrant genotypes, the addition of medium preconditioned by ovaries to microspore cultures increased embryogenesis by more than 100-fold, however some recalcitrant genotypes did not (Zheng *et al.,* 2002). The use of ovaries in wheat IMC has been highly effective for producing DHs of many agronomically important cultivars (Lantos & Páricsi, 2006).

The mechanism by which ovary conditioning enhances microspore growth remains unclear. Metabolites from ovaries potentially responsible for promoting growth were identified using spring barley IMC (Kohler & Wenzel, 1985), including one compound isolated by thin layer chromatography which may be a form of phytohormone. Hul and Kasha (1997) hypothesised that phenylacetic acid (PAA) or an analogue is released from ovaries into the medium, which has a positive effect on embryogenesis while Letarte *et al* (2006) found that successful embryogenesis in wheat could be achieved without ovaries if the medium was supplemented with arabinogalactan and gum arabic. More recently research has been conducted to investigate the genotype dependency of ovaries, and the genes behind their action (Castillo *et al.,* 2015).

Conditioning of media by tissues other than ovaries may also be used. McCabe *et al.* (1997) found that liquid media from embryogenic callus cell suspension cultures of *Daucus carota* could trigger embryoid formation in non-embryogenic callus. This inter-culture approach has largely been neglected as a practical tool, as purified additives with similar effect have been identified for many species (e.g. Letarte *et al.,* 2006). However, given the range of possible additives to test, the use of ovaries, anthers or other tissues to support microspore development remains a potentially productive initial step in development of effective protocols.

2.1.9. Composition of Regeneration medium

The regeneration medium is very similar to the induction medium in virtually all nutrient content for healthy growth. The significant difference is the use of different hormones to induce regeneration. Their origins and mechanisms are reviewed by Ikeuchi *et al. (*2016).

2.1.10. Anther Orientation and Density

The concentration of anthers and their orientation also affects embryogenic efficiency. Keller (1984) found six anthers per ml of medium was optimum for *Brassica*. Similar results were reported in broccoli (Arnison & Donaldson, 1990). These authors suggest that a high density is important for efficient response, as anthers clump together more at higher densities, which potentially improves conditioning of the media. The effect of microspore density in isolated microspore cultures is known to influence the callus induction rate (Castillo *et al.,* 2000; Ferrie *et al.,* 2013; Davies & Morton, 1998).

There have been conflicting reports about the effect of orientation of anthers on media. In barley, Powell *et al. (*1988) found that while the orientation did not affect the percentage of responding anthers, anthers placed in the 'up' position (one lobe in contact with the medium) produced significantly more green plants than anthers placed in the 'flat' position (both lobes in contact with the medium). However, Shannon *et al.,* (1985), reported that fewer calli were produced by anthers in the 'flat' position, which were also slower to respond. Hunter (1985) also reported zero response in barley anthers plated in the 'flat' position. In coconut, higher embryogenic rates were reported in anthers placed in the 'up' position compared with 'flat' (Perera *et al.,* 2008).

2.1.11. Albinism

Albinism in plants is caused by a lack of chlorophyll in plant cells. Chlorophyll is vital for photosynthesis, and without it the plant will inevitably die. Chlorophyll is contained within chloroplasts, plastids which differentiate during cell development. In albino plants, the proplastids fail to differentiate. Albinism can occur naturally through mutation in either the nuclear or the chloroplast genomes, or through incompatibilities between

them (e.g. J. Yao & Cohen, 2000). It is often encountered in plant breeding in wide hybridization and in tissue culture. When producing doubled haploids through wide hybridization as well as androgenesis, albino plants are frequently obtained (Datta, 2005). Albinism has been reported in numerous species including barley, wheat, tobacco, soybean, oat (Jahne & Lorz 1995; Ferrie *et al.,* 2013). The cause of albinism is difficult to identify as there are hundreds if not thousands of genes involved in chloroplast development but factors influencing its rate in androgenesis include genotype, donor plant health (Bullock *et al.,* 1982), culture temperature, and development stage of the microspore (Chen & Lin, 1976). Larsen *et al.* (1991) reported that genotype was responsible for between 62% and 76% of the total variation in barley for albinism. Barley particularly suffers from high rates of albinism (Makowska & Oleszczuk, 2014) and can have an albino regeneration frequency of 100% in some genotypes (Caredda *et al.,* 2000), possibly due to plastid DNA degradation in the microspore inhibiting chloroplast development. In addition, somaclonal variation in tissue culture may result from aberrant chromosome replication and recombination. This can lead to chromosomal deletions, duplication, inversions and translocations in the nuclear genome, while major rearrangements in chloroplast DNA have been observed in albino wheat and barley plants derived from anther culture (Day and Ellis, 1984, 1985).

Albinism frequency can be reduced with appropriate culture conditions, one of which is the addition of copper ions supplemented in the pretreatment solution and the induction media (Wojnarowiez *et al.,* 2002). Cu^{2+} ions are toxic to plants in high concentrations (Maksymiec, 1997), but are required in trace amounts for healthy plant growth and have an important role in photosynthesis (Droppa & Horváth, 1990). Increased copper (5 µM) improved green plant regeneration in barley (Bregitzer *et al.,* 1998). By increasing the Cu^{2+} concentration to 10 µM times in the pre-treatment solution and induction media the androgenic response, plant regeneration and green to albino plant ratio was improved. Increases were seen in the rate of responding anthers (from 57.3 % to 72.3 %), and the number of regenerated plantlets (from 2.4 to 11.1 per responding anther) while the proportion of albino plantlets was reduced from 13 to 10.8 % (Wojnarowiez *et al.,* 2002).

It has also been shown that the addition of copper sulphate in the anther pre-treatment medium improved the rate of green plant regeneration from barley cultivars that had previously produced albino plants exclusively (Jacquard *et al.,* 2009). In plastids, it was observed that the effect of copper was characterized by a decrease in starch, and a parallel increase in internal membranes.

2.1.12. Advances in Doubled Haploid research in Oat

Considerable effort has gone into developing DH production for oat through anther culture, however due to the recalcitrant nature of oat, progress has been slow. This section reviews current progress.

2.1.12.1. Anther culture

The first cultivated *A. sativa* to be recovered by anther culture was reported by Rines (1983), who regenerated three plants, one haploid and two double haploid, of the cultivar 'Stout'. Of the 65,000 anthers plated, the only anther to produce plants was plated on a modified potato extract medium containing 2 mg/L 2, 4-D and 0.5 mg/L kinetin. Plants of naked oat (*A. nuda* L.) were first regenerated from anther cultures by Sun *et al.* (1991). Kiviharju *et al. (*1997) looked at seven genotypes from four *Avena* species including *A. sativa* cvs Stout. The only genotype to produce regenerants was *A. sterilis* acc. CAV 2648. Their results agreed with previous studies, that the genotypic effect was significant (Rines 1983; Gana *et al.,* 1995). Kiviharju & Pehu (1998) tested the effect of heat pre-treatment on *A. sterilis* and *A. sativa*. Their results showed species respond differently, as heat pre-treatment had no effect on embryo frequency in *A. sativa* cvs 'Stout'. Their results also found maltose to be a better carbon source than sucrose for the genotypes tested. The effect of 2,4-D and kinetin were studied in anther culture of cultivated oat *Avena sativa* L., wild oat *A. sterilis* L. and their cross progeny, and found that a high concentration of 2,4-D (5-6 mg/L) increase embryo frequency, while kinetin caused callus browning, but was required in small concentration to initiate embryo production in *A. sativa*. The study also found that W14 salts gave a higher regenerant frequency over Murashige and Skoog

salts in cross progeny (Kiviharju & Tauriainen, 1999). Further testing with 14 genotypes recorded the same results with the use of 2,4-D and kinetin concentrations as Kiviharju & Tauriainen (1999), and also found BAP and zeatin improved embryo frequency when combined with 2,4-D and kinetin (Kiviharju *et al.,* 2000). Green plants were also recovered from oat cv. Katri and naked oat cv. Lisbeth.

The anther culture protocol was further improved by Kiviharju *et al. (*2005) with a medium containing W14 salts and vitamins, supplemented with 2,4-D, BAP, Ethephon, L-cysteine and myo-inositol, compared to medium containing only 2,4-D and kinetin. Plant regenerants were successfully obtained in the cultivars Lisbeth and Aslak at considerably higher rates than previous attempts, with up to 5 green plants per 100 anthers from Aslak x Lisbeth progeny.

2.1.12.2. Isolated Microspore Culture

There has been even less progress in isolated microspore culture. There are currently only two published papers that report oat regeneration through IMC. The first was by Sidhu & Davies (2009), which tested 22 genetically diverse accessions and focused on pH, culture conditions and conditioned media. Of the 22 genotypes, three were responsive. The use of media conditioned with actively growing barley microspores significantly increased results, and a pH of 8.0 produced significantly more multicellular structures than pH 5.0. They found that multicellular structures and embryo like structures of greater than eight cells were observed only in cultures containing conditioned media.

The second study by Ferrie *et al. (*2013) also found a higher pH of 6.5 – 7.5 to be optimum for IMC. They also found that a very high microspore density in culture of 1 x 10^6 MS (much higher than used in other species) produced the largest mean number of embryos. They also found a 0.3 M mannitol pre-treatment to be more effective.

2.1.12.3. The first Doubled Haploid linkage map for oat

The first doubled haploid linkage map of hexaploid oat was produced by Tanhuanpää *et al. (*2008). Prior to this all QTL maps have been constructed from recombinant inbred

lines. The linkage map was constructed from a DH population recovered from an anther culture protocol of F_1 individuals of a cross between the cultivars 'Aslak' and 'Matilda', made up of 137 individual plants. They found 54 % of the mapped markers exhibited segregation distortion. In a subsequent publication (Tanhuanpää *et al.,* 2010) looked at ten agronomic and quality traits, including protein content and *beta*-glucan. Between 1-8 QTLs were discovered for each trait; however they suggest the need for anchor markers, to ensure this QTL map can be compared to the previous study. In their third publication (Tanhuanpää *et al.,* 2012) the DH linkage map was extended with microsatellites and diversity array technology, giving a map with 1058 DNA markers and 34 linkage groups. With the new markers, most of the link age groups could be anchored to the standard 'Kanota' x 'Ogle' oat reference map, providing a useful resource for plant breeders.

2.2. Double haploid materials and methods

2.2.1 Donor plant material

Nine different *A. sativa*, one *A. fatua* and one *A. sterilis* cultivars were tested. The cultivars used are summarized in **table 2.1.**

Species	Genotype	Source/origin
A. sativa	Lisbeth	Boreal Plant Breeding Ltd, Finland
A. sativa	Assiniboia	Agriculture and Agri-Food Canada, Cereal Research Centre, Canada
A. sativa	Canyon	Nordsaat GmbH, Germany
A. sativa	Firth	KWS Ltd, UK
A. sativa	Bajka	Polish cultivar
A. sativa	Aspen	Bauer, Germany
A. sativa	Aslak	Boreal Plant Breeding Ltd, Finland
A. sterilis	B443	Dr. Tim Langdon
A. fatua	Cc8430	Dr. Tim Langdon
A. sativa	N15.1407	Nordsaat Saatzucht GmbH, Germany
A. sativa	N15.1408	Nordsaat Saatzucht GmbH, Germany

Table 2.1*. Oat genotypes used in the various anther cultures and isolated microspore cultures.*

2.2.2. Culture media and materials

2.2.3. Induction media for anther culture and IMC

The modified double haploid *Miscanthus* (MDHM) medium (**Table 2.2.)** was based on C17 (Pei & Yu-rong, 1975) and was designed by Sue Dalton (personal communication). The FIN double-layer induction medium (**Table 2.2**.) based on Kiviharju & Tauriainen (1999). The POL induction media was based on Ponitka & Slusarkiewicz-Jarzina (2009). The modified IMI medium (**Table 2.2.**) was based on Esteves *et al. (*2014). **Table 2.3.** contains the preparations for stock solutions.

The isolated microspore culture medium (**Table 2.2**.) was based on Ferrie *et al. (*2013). **Table 2.3.** contains the preparations for stock solutions.

chemical mg/l		MDHM	MIMI	FIN	POL	IMC Ferrie
based on		C17	IMI	W14	W14	W14
Major Minerals						
NH4NO3	Ammonium nitrate	300.0	165.0	-	-	-
NH4H2PO4	Ammonium dihydrogen phosphate	-	-	380.0	380.0	380.0
KNO3	Potassium nitrate	1400.0	1900.0	2000.0	2000.0	2000.0
CaCl2.2H2O	calcium chloride dihydrate	150.0	440.0	140.0	140.0	140.0
KH2PO4	Monopotassium phosphate	200.0	170.0	-	-	-
Mg(SO4).7H2O	Magnesium sulphate heptohydrate	150.0	370.0	140.0	140.0	140.0
K2SO4	Potassium sulphate	-	-	700.0	700.0	700.0

Minor minerals						
H3BO3	Boric acid	6.2	6.2	3.0	3.0	3.0
MnSO4.4H2O	Manganese(II) sulphate tetrahydrate	11.2	22.3	-	-	-
MnSO4.H2O	Manganese(II) sulphate monohydrate	-	-	8.0	8.0	8.0
ZnSO4.7H2O	Zinc sulphate heptahydrate	8.6	8.6	3.0	3.0	3.0
KI	Potassium Iodide	0.9	0.8	0.5	0.5	0.5
Na2MoO4.2H2O	Sodium molybdate dihydrate	-	0.3	0.0	0.0	0.0
CuSO4.5H2O	Copper(II) sulphate pentahydrate	0.0252	0.0	0.0	0.0	0.0
CoCl2.6H2O	Cobalt(II) chloride hexahydrate	0.0	0.0	0.0	0.0	0.0
FeSO4.7H2O	Iron chelate solution	27.8	-	27.8	27.8	27.8
Na_2EDTA.2H2O		37.3	-	37.3	37.3	37.3
FeNAEDTA		-	43.0	-	-	-
Vitamins						
	Glutamine	-	500.0	-	-	-
	Thiamin HCl	1.0	10.0	2.0	2.0	2.0
	Glycine	2.0	-	2.0	2.0	2.0
	Nicotinic acid	0.5	1.0	0.5	0.5	0.5
	Pyridoxine HCl	0.5	1.0	0.5	0.5	0.5
	L-cysteine	50.0	-	-	-	-
	Biotin	1.0	-	-	-	-
	Folic acid	0.5	-	-	-	-
	Ascorbic acid	-	25.0	-	-	-
	casein hydrolysate	-	500.0	-	-	-
Plant Growth Regulators						
	2,4-D	1.5	-	5.0	5.0	2.0
	BAP	-	1.0	0.5	0.5	0.5
	kinetin	0.5	-	-	-	-
	Dicamba	-	0.6	-	-	-
	Thidiazuron	-	0.3	-	-	-
	myo-Inositol	100.0	300.0	500.0	500.0	100.0

Sugar source						
	maltose	90000.0	60000.0	100000.0	100000.0	100000.0
Gelling agents						
	Gelrite	-	6000.0	6000.0	6000.0	-
	Ficoll 400	-	-	100000.0	-	-
	SeaPlaque agarose	12000.0	-	-	-	-
Media	**Additions to MDHM media**					
GAM	Gum arabic	50 or 200				
	Arabinogalactan	50 or 200				
Casein hydrolysate		500.0				
Glutamine		500 or 1000				
Putrescine		44.0				
Additional Copper	CuSO4*5H20	2.475				
Proline		500 or 750				
/L 2,4-D		3.0				
pyridoxal phosphate		0.5				
ascorbic acid		25.0				
	pH	5.8	5.8	6.0	6.0	7.0

Table 2.2. *Composition of induction media for anther and Isolated microspore culture.*

Petri dishes for anther culture

Induction cultures used 35/10mm petri dishes (Item No.: 627160, Greiner Bio one, Austria). Regeneration cultures used 94/16mm petri dishes (Item No.: 633180, Greiner Bio one, Austria).

Laboratory-blender for IMC

A Waring (USA) blender model 7009 with a stainless steel MC2 container was used to macerate oat inflorescence to release the microspores. The MC2 container was wrapped in tinfoil and autoclaved before each use.

Stock solution	Preparation	Final concentration	Storage conditions
2,4-Dichlorophenoxyacetic acid (2,4-D)	Prepared by dissolving 10 mg in 1-2 mL of 100% (neat) ethanol. Once dissolved it was gradually diluted with 100 mL distilled water	x10	4 °C
Kinetin	Prepared by dissolving 10 mg in 1-2 mL 1M NaOH. Once dissolved it was gradually diluted with 100 mL distilled water		
α – Naphthaleneacetic acid (NAA)			
6- Benzylaminopruine (BAP)			
Fe-EDTA	3.73g of Na_2EDTA was dissolved into 100 mL of distilled water and 2.78g of $FeSO_4.7H_2O$ into 100 mL of distilled water. Both were heated to approx. 60 °C and stirred using a hotplate magnetic stirrer until fully dissolved. They were then combined, by pouring the clear solution into the turbid solution slowly, and maintaining a temperature of approx. 60 °C while stirring using a hotplate magnetic stirrer.	x40	4°C
$CoCl_2.6H_2O$	Prepared by dissolving 10 mg in 100 mL distilled water	x100	4°C
$CuSO_4.5H_2O$			
1M NaOH	Prepared by dissolving 40g NaOH in 1 litre of distilled water. The NaOH was added to the water in small batches every minute while stirring continuously, due to the exothermic reaction caused during dissolving.		Room temp.
Colchicine	Prepared by combining 0.005 g colchicine, 0.2 mL Dimethyl sulfoxide (DMSO), and 9.8 mL distilled water.	0.05%	4°C

***Table 2.3**. Additional stock solutions and how they were prepared and stored.*

2.2.4. Preparation of induction culture media

The MDHM, POL, and MIMI anther culture media were prepared from the stock solutions to create batches of 2x strength media for combination with gelling agent stocks. Appropriate volumes of stocks of major and minor minerals, vitamins, maltose, and growth regulators for a final volume of 100 mL were combined and made up to 50 mL with distilled water after setting the pH to the required level. This nutrient solution was filter sterilised using a 0.2 µm Acrodisc membrane filter (Pall Corporation, USA) and combined with 50 mL of a 2.4 % w/v solution of gelling agent which had been autoclaved at 120 °C for 20 minutes, to create 100 mL of 1x strength solution. The mixture was shaken gently by hand to mix and then approximately 3 mL was added per petri dish using a sterile graduated Pasteur pipette (Star Labs, UK). Petri dishes were stored at 4 °C either wrapped in cling film or their original packaging sealed tightly with tape. The isolated microspore culture media was filter sterilised in the same way. The FIN double-layer induction media was made up of two parts; the filter-sterilized (Phytagel autoclaved) solid phase containing 0.3 % Phytagel (Sigma), and the autoclaved liquid phase contained 10 % Ficoll 400 (Pharmacia Biotech). 2.5 mL of the solid and 1 mL of the liquid medium was put into 3.5 cm diameter Petri dishes.

2.2.5. Preparation of regeneration and rooting media

Regeneration media was made in batches of one litre, combining two double strength solutions of 500 mL distilled 3 g/L gelrite with one litre of media components in 500 mL

chemical mg/L	Regeneration media	Rooting media
Maltose	30000	-
Sucrose	-	30000
M0245	4400	-
M0222	-	4400
BAP	1	-
NAA	1	-
Gelrite	3000	3000

Table 2.4. *Components of the regeneration and rooting media*

distilled water (**table 2.4.**). Both parts were sterilised by autoclaving for 15 min at 120 °C, and the pH adjusted to 5.8 by adding 200 µL sterile 1M NaOH per 1 L of media.

2.2.6. General pre-culture methods

2.2.6.1. Donor plant conditions

All donor oat plants were grown in 13 cm pots in a glasshouse at Saaten-Union Biotec GmbH, operating at 16hr/8hr 16°C/12°C day/night with light intensity above 15kLUX, and sodium lamps (SON-T agro), or at Aberystwyth University in a heated but unlit greenhouse or in a Fitotron® growth chamber operating at 16hr/8hr 16°C/12°C day/night with a light intensity of 600 $\mu M.m^{-2}.s^{-1}$. The leaf tips of the donor plants were cut during the seedling (leaves approximately 10 cm) growth stage to induce tillering.

2.2.6.2. Harvesting of donor plant material

Tillers were harvested when microspores in the mid-section of the panicle were at the mid to late uni-nucleate stage for the majority of microspores. This was screened for by opening up a panicle and removing florets from the middle of the panicle during the booting stage of plant development. The distance between the top of the florescence within the panicle to the flag leaf was measured and used as a guide for harvest (**figure 2.4.**), which was typically between the stages GS43 (flag leaf sheath just visibly swollen) to GS45 (flag leaf sheath swollen) (Zadoks *et al.,* 1974). The panicle was felt through the sheath with bare hands to find the top of the inflorescence which could then be measured with a ruler. A floret from the centre of the panicle was opened and anthers were squashed in a drop of water between a microscope slide and cover slip to release the microspores. A minimum of five or six panicles for each genotype was tested when the first panicles reached the booting stage. This measurement was then used to gauge when the other panicles would be ready to harvest. This sampling was done throughout the harvesting period.

Figure 2.4*. The measurement used to control the harvest time of panicles is labelled A.*

2.2.6.3. Pre-treatment of donor plant material

Tillers were cold treated at 4 °C in the dark for 14-18 days. After 12-14 days panicles were screened to check microspore status. The tillers were stored in autoclaved water and covered with a clear plastic bag to maintain humidity (**figure 2.5.**). The water was changed every 4-5 days to reduce the growth of mould.

Figure 2.5*. Panicles that have been recorded and placed in a plastic beaker of autoclaved tap water ready for pre-treatment.*

2.2.6.4. Sterilisation of donor plant material

Tillers were trimmed just above the bottom culm node so the leaf sheaf could be unravelled to remove the panicle. The panicles were surface sterilized in 250 mL flask, filled with 190 mL of sterile distilled water and 60 mL bleach (DanKlorix, Germany or

Domestos, UK) with 0.5 mL tween for 15 minutes. The flask was shaken gently after the panicles were added, and turned upside half way through at 7 minutes 30 seconds. The panicles were removed using sterilised tweezers, and placed into a new 250 mL flask containing sterile distilled water. The flask was shaken gently by hand. This was repeated three times each with a fresh flask of sterile deionised water.

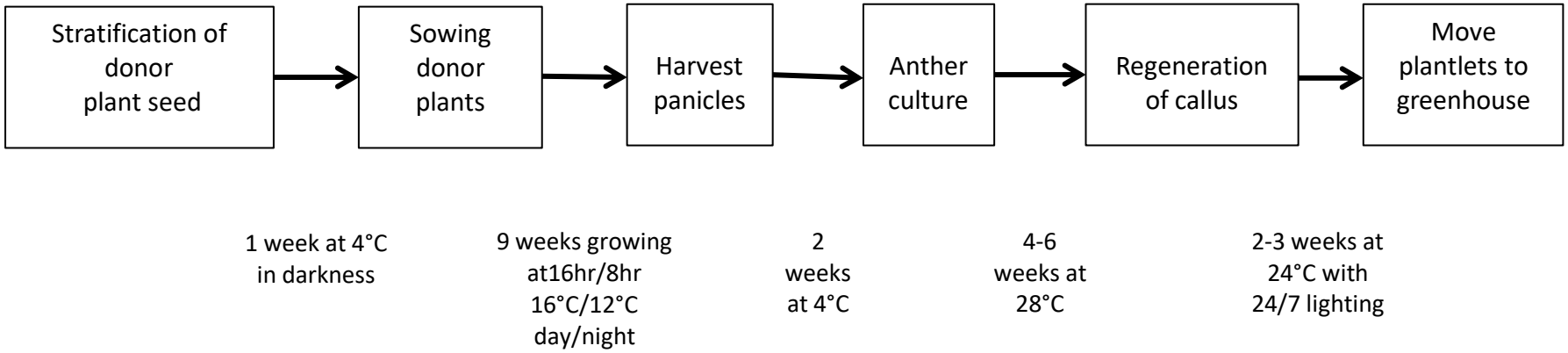


Figure 2.6. *The key steps in the double haploid protocol using anther culture.*

2.2.7. Isolation of anthers for anther culture

After sterilisation and rinsing the panicle was placed on an aseptic surface. Each panicle was used to make one individual anther culture. Fourteen florets per panicle were selected from the mid-section of the panicle **(Figure 2.7)**. The florets were sampled from the panicle from the lowest end of the sample area to the top in order. These florets were cut from the panicle and laid out in order from bottom to top before florets were dissected. Anthers were isolated aseptically using a scalpel and tweezers on a petri dish under a dissecting microscope. The tweezers held the end tips of the first and second glumes, and the scalpel carefully cuts through the glumes and lemmas at the base of the floret, between the pedicel and the lodicule. Then the scalpel was turned so it lay flat to hold the base of the floret against the cutting surface. The tweezers gently pull the lemmas off to reveal the ovary, anthers inside the lower palea. The three anthers were carefully removed using tweezers and transferred to petri dishes containing the induction media. (**figure 2.7**). During the early experiments approximately 30-40 anthers were plated per petri dish, spread evenly across the surface in no particular order. In later experiments anthers were plated in a specific order (**figure 2.8.**). The florets were sampled starting from the bottom of the panicle. The anthers were placed in rows in triplicate, corresponding to individual florets. The first row contained three florets of anthers, the second and third row contained four florets of anthers, and the fourth row contained three florets of anthers, totalling fourteen florets and 42 anthers. Plates were labelled with genotype, media, date of plating and the replicate number (**figure 2.9**).

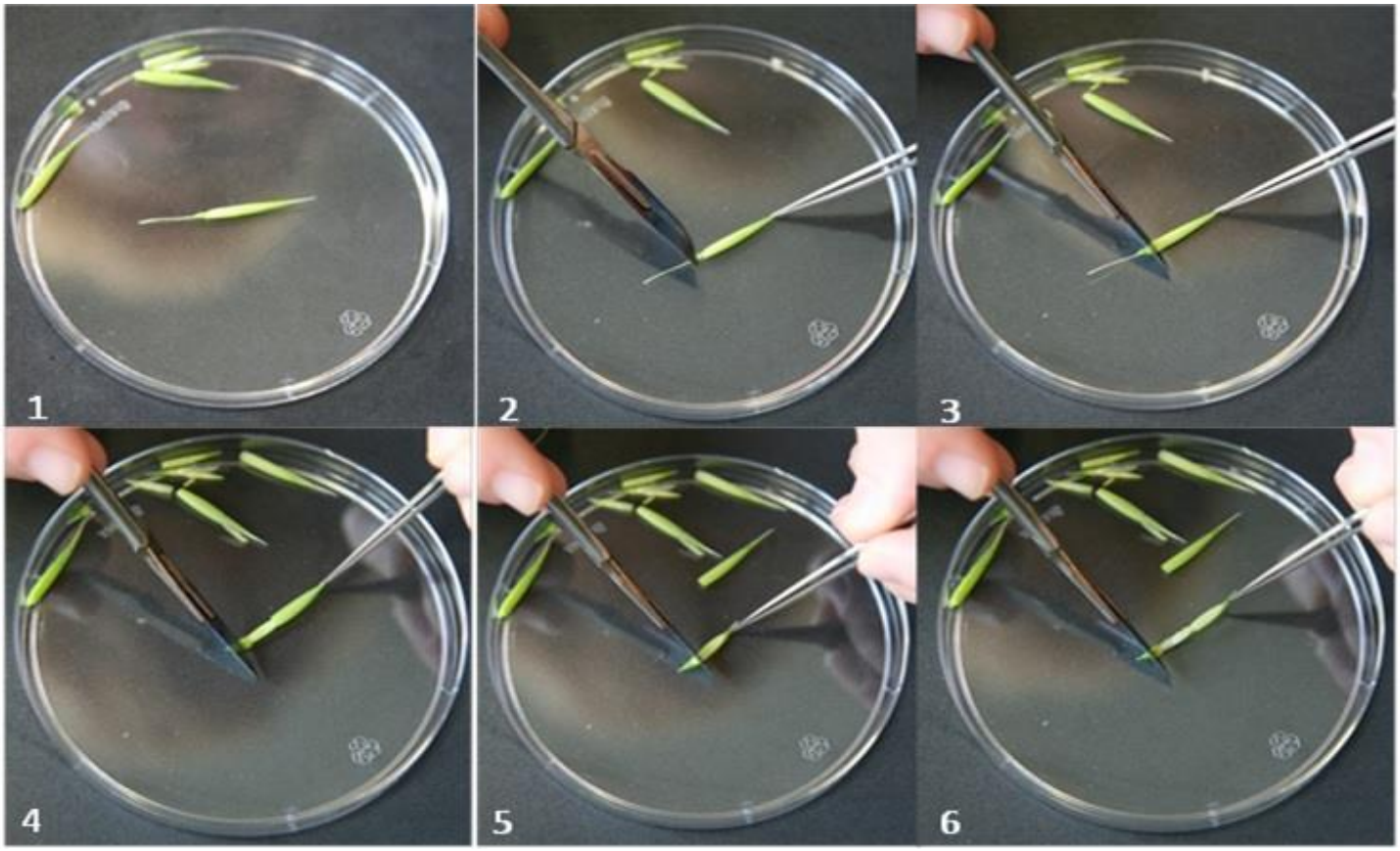


***Figure 2.7**. A step by step guide to dissect an oat floret. 1. The oat florets were dissected on a sterile petri dish. 2. Tweezers hold the tip of the floret 3. The scalpel cuts through the glumes just above the pedicel, without cutting through to the ovary and anthers. 4. The scalpel blade is tilted slightly to hold the floret, and the tweezers pull the glumes away. 5. This is repeated with the lemna, exposing the palea, which can be pulled back to reveal the anthers.*

Figure 2.8*.This diagram shows an example of which florets were selected from the panicle. This area was decided based on anther squashes, and can vary slightly between panicles of the same genotype. When there were a lot of florets, florets were sampled from the along the whole sample area.*

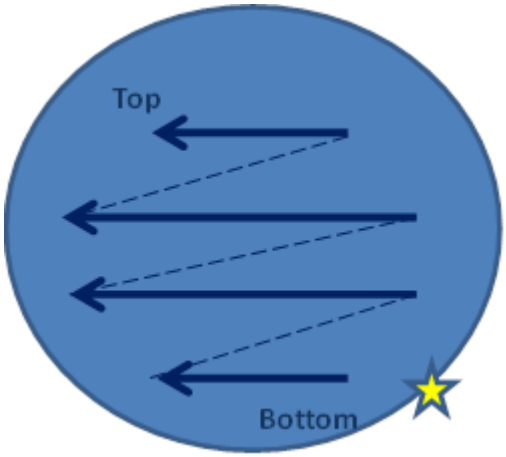


Figure 2.9*.The plan showing how the anthers were laid out on the petri dish. The star is where a mark is made on the petri dish to distinguish the petri dish orientation.*

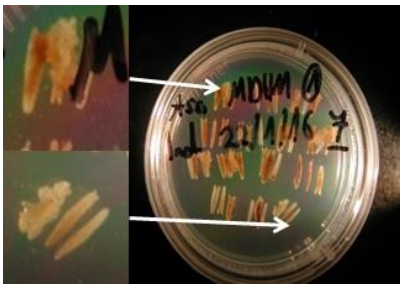


Figure 2.10. *This image shows an example of an induced anther culture plate. Anthers are grouped to distinguish between florets. The two small images show close up the anthers from the top and the bottom of the sampling area.*

2.2.8. Incubation of anther cultures

Anther cultures were sealed with parafilm (Bemis Company, INC., USA) and were incubated in a THERMO Heraeus cupboard at 28°c in complete darkness. Anther cultures were incubated in cardboard boxes containing a small plastic container of sterilised tap water which was checked regularly and refilled as required to raise the humidity and reduce the rate of drying of the induction media.

2.2.9. Regeneration of induced callus from anther culture

Anther cultures were checked periodically (twice a week) under the binocular microscope for signs of callus growth and contamination. Contaminated anther cultures were autoclaved. After 5 to 7 weeks the number of anthers producing callus were recorded, and the callus was transferred to regeneration media. The callus was removed from the anthers where possible. In later experiments the same layout for the anther cultures were used for regeneration plates (**Figure 2.11**). Previously four induction plates were plated onto one regeneration plate, but later experiments found it better to have just two or one induction plates on one regeneration plate which made the plates less crowded.

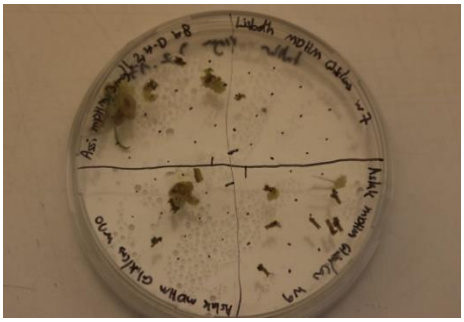


Figure 2.11.*This image shows an example regeneration culture plate. In this case callus from four induction plates were plated on one regeneration plate. The plate was separated, and points were marked on the corner to denote where the first floret is placed. The plate is laid out just like the induction plates, with pen marks to denote the positions. If there is no callus to move the spot was left empty.*

2.2.10. Incubation of anther culture regeneration cultures

Regeneration cultures were incubated at 24-25 °C under lights with a 16hr/8hr day/night photoperiod. The cultures were initially covered with paper towel for the first three or four days under these conditions to acclimatise before being fully exposed to the light.

2.2.11. Isolated microspore culture methods

2 x 15 mL tubes were prepared with 12 mL of 20% maltose solution, and stored in the fridge. 1 x 2 mL tube was prepared with 90 µl RO water, and stored in the fridge. The centrifuge was set to 4°C. Sterile tweezers and scissors were used to cut florets from sterilized panicles and placed into the blender beaker. A maximum of 4-5 panicles per blending were used. 50 mL of filter sterilised mannitol was added to the blender beaker with the florets. The mixture was blended on low speed for 5 seconds and on a high speed setting for 7 seconds. The blended solution was poured through a 500 µm sieve into a conical beaker. The sieve was rinsed with sterilise mannitol to wash through microspores stuck on the sieve or within the detritus. This step was repeated with a 100 µm sieve. The filtrate was poured evenly into 4 x 50 mL tubes. The tubes were centrifuged at g x 498 for three minutes at 4 °C. The slowdown was set to 6. The solution was discarded without disturbing the pellet. The pellets were each re-dissolved in 3-4 mL of sterile 0.3 M mannitol and the solutions were combined into a single 50 mL tube. The solution was then carefully poured evenly between the two 20 % maltose solution tubes, to avoid mixing the two solutions. The tubes were centrifuged at g x 94 for 5 min at 4 °C, set to slow down at 6. A sterile single use pipette was used to extract the microspores from the interface which was added to a new 15 mL tube. 10 µl of the solution was extracted for the microspore count. The total volume of the microspore solution was recorded. The microspores were washed with 15 mL of fresh IMC media by centrifuging at g x 94 for one minute at a temperature of 4 °C and set to slow down 6. During centrifuging, the microspore concentration was calculated using the 10 µl sample. After centrifuging the supernatant was carefully discarded and the microspores retained. An

appropriate amount of media was added to the microspore pellet to make a microspore concentration of 10^6 /mL. A sterile pipette was used to transfer the microspore solution to a pre-coated 35/10mm petri dish.

2.2.12. Incubation of microspore cultures

Anther cultures were sealed with parafilm (Bemis Company, INC., USA) and were incubated in a THERMO Heraeus cupboard at 28 °c in complete darkness. Anther cultures were incubated in cardboard boxes containing a small plastic container of sterilised tap water which was checked regularly and refilled as required.

2.2.13. Rooting of regenerated plants

All regenerated plantlets were moved to plastic magenta GA-7 vessels (Sigma-Aldrich), or a similar vessel containing approximately 30 mL of rooting media. Rooting media was made in batches of one litre, combining two double strength solutions of 500 mL distilled 3 g/L gelrite with one litre of media components in 500 mL distilled water. Both parts were sterilised by autoclave, and the pH adjusted to 5.8 by adding 200 µL sterile 1M NaOH per litre of media. Plants were moved to the rooting media once their shoots were 1-2 cm in length.

2.3. Results

This section looks at the results from the anther culture and isolated microspore culture, first with the preliminary experiments to identify promising oat genotypes and culture medium to use as a basis for DH optimization. **Figure 2.12.** shows factors at various stages of the DH process that could be targeted for protocol optimization.

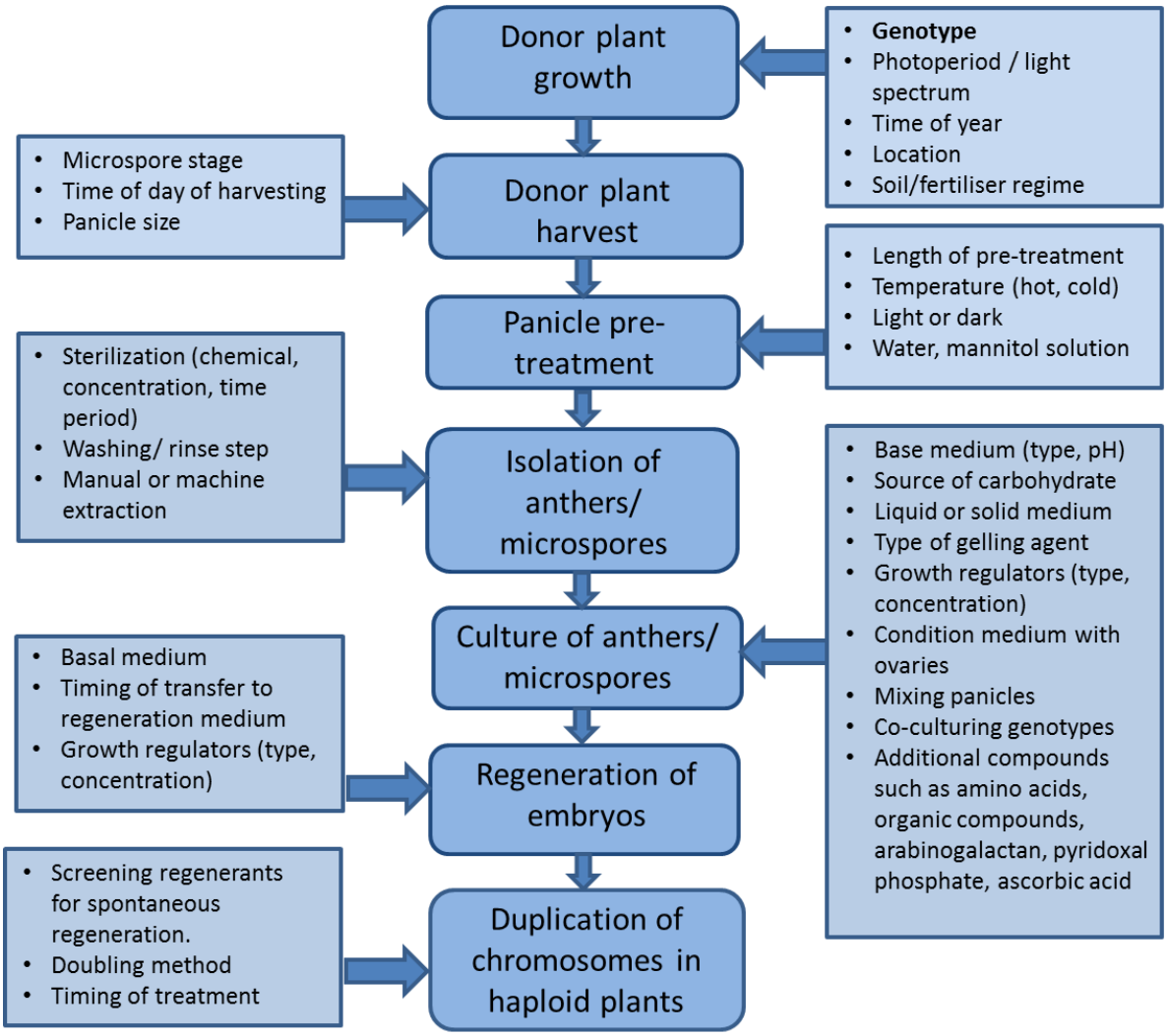


Figure 2.12. *The key steps in anther and IMC culture and potential areas for protocol optimization.*

2.3.1. Preliminary experiments

2.3.1.1. Introduction

The first step of the project was to identify oat genotypes and media which could be used as a basis for protocol improvement as well as to learn the methods and procedures involved in producing double haploids by anther culture and isolated microspore culture (IMC). In order to provide a continuous supply of donor plants at the critical stage for harvest, seedling germination was carried out in staggered blocks so the work could be spread over several months. For the first block experiment, five genotypes were selected for anther culture. Cv. Aslak and cv. Lisbeth are varieties produced by Boreal Plant Breeding Ltd, Finland, and were positive standards in anther culture work published by Kiviharju *et al. (*2005). Cv. Bajka is a Polish positive standard used for double haploid production by wide hybridization with maize (Marcińska *et al.,* 2013). Cv. Assiniboia is an amenable genotype routinely used for anther culture experiments at Saaten-Union Biotec GmbH (Germany). Cv. Canyon is a cultivar produced by Nordsaat (Germany) and at the time of experimentation was widely grown across Europe. Having a DH responsive commercially relevant cultivar would be beneficial as it would potentially decrease the lead time to having commercially viable DH lines for breeding. Three different media were selected to see which would work best with the selected genotypes and act as a basis for further optimisation. The modified double haploid *Miscanthus* (MDHM) media was based on C17 (Pei & Yu-rong, 1975) and designed by Sue Dalton (personal communication), the FIN double-layer induction media was based on Kiviharju & Tauriainen (1999), and the POL induction media was based on Ponitka & Slusarkiewicz-Jarzina (2009). Each media was tested at two different pH levels. The standard pH for plant tissue culture is 5.8, however Sidhu & Davies, (2009), found microspores were induced when a higher pH was applied (pH8). A minimum of 90 anthers were cultured per condition (an average of 30 anthers per anther culture plate).

2.3.1.2. Block one anther culture: five genotypes

Genotype	Media	pH	no. of calli	no. of calli moved to regen media	no. of regenerated calli	Green plants
Bajka	MDHM	5.8	9	2	0	0
		7.5	0	0	0	0
	POL	5.8	0	0	0	0
		7.5	0	0	0	0
	FIN	5.8	2	1	0	0
		7.5	0	0	0	0
Aslak	MDHM	5.8	17	0	0	0
		7.5	0	0	0	0
	POL	5.8	0	0	0	0
		7.5	0	0	0	0
	FIN	5.8	0	0	0	0
		7.5	0	0	0	0
Assiniboia	MDHM	5.8	2	2	0	0
		7.5	1	6	0	0
	POL	5.8	5	4	1	1
		7.5	1	0	0	0
	FIN	5.8	1	0	0	0
		7.5	0	0	0	0
Lisbeth	MDHM	5.8	5	0	0	0
		7.5	0	0	0	0
	POL	5.8	1	0	0	0
		7.5	0	0	0	0
	FIN	5.8	1	0	0	0
		7.5	2	0	0	0
Canyon	MDHM	5.8	0	0	0	0
		7.5	0	0	0	0
	POL	5.8	0	0	0	0
		7.5	0	0	0	0
	FIN	5.8	0	0	0	0
		7.5	0	0	0	0

Table 2.5*. Number (no.) of anthers producing calli for each scenario, the number of calli moved to regeneration media, and the number of plants regenerated.*

Overall the callus induction response was low for all genotypes (**Table 2.5.**). The genotypes Bajka, Aslak, Lisbeth and Assiniboia induced callus on at least one media condition, however Canyon failed to produce any callus. The pH 5.8 media produced far

more callus (43 calli) than the pH 7.5 (four calli). Only genotypes Assiniboia and Lisbeth induced callus on the pH 7.5 media.

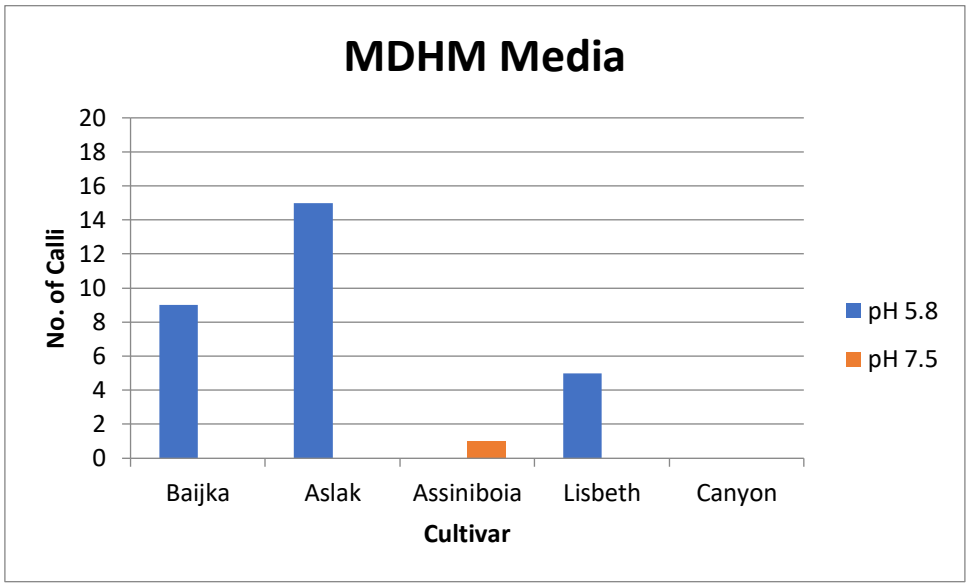


Figure 2.13. *Total numbers of calli produced on MDHM medium with different genotypes and pH.*

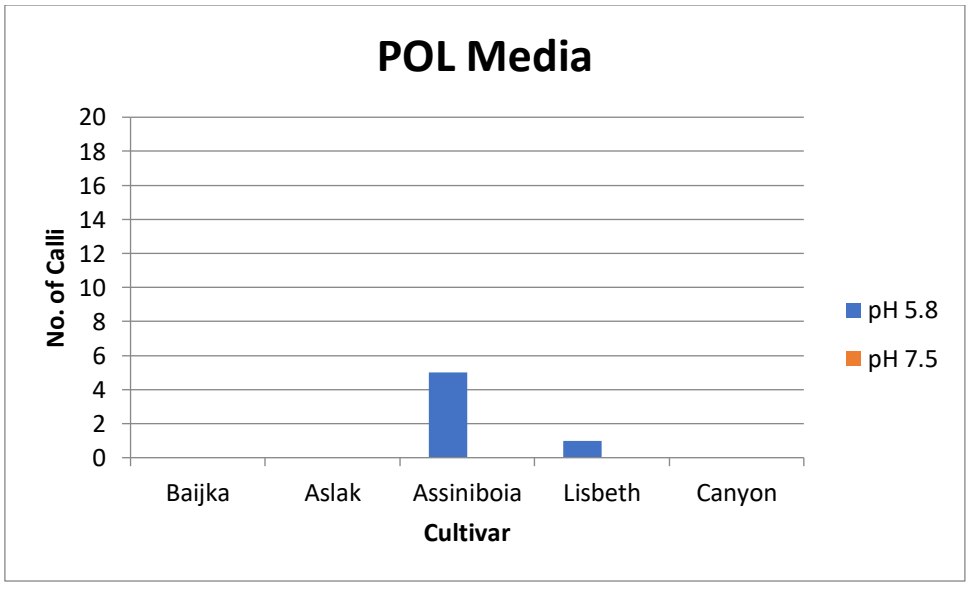


Figure 2.14. *Total numbers of calli produced on POL medium with different genotypes and pH.*

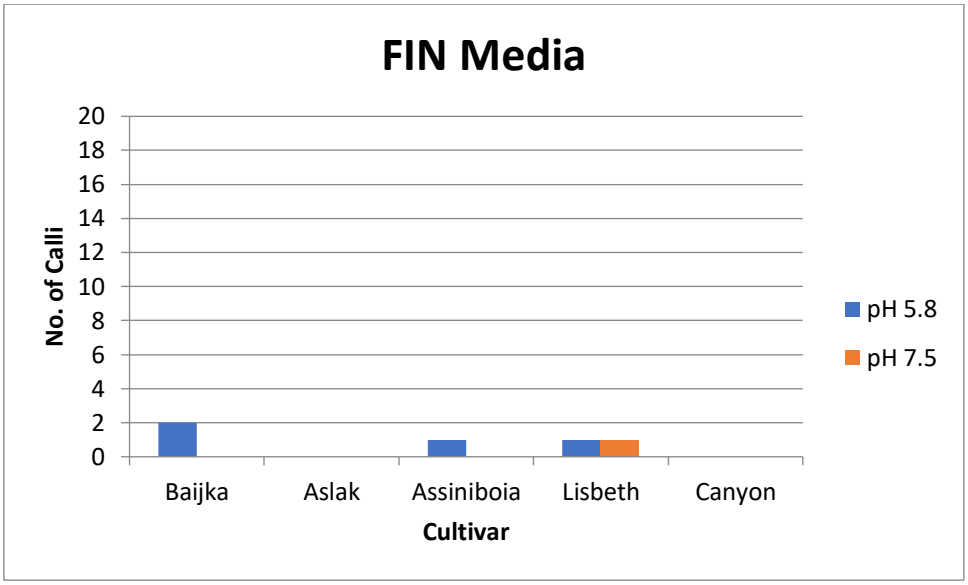


***Figure 2.15**. Total numbers of calli produced on FIN medium with different genotypes and pH.*

The only plant like structure was regenerated from an Assiniboia callus induced on POL media. While the plant was green and therefore photosynthesizing, it did not produce a normal shoot, and failed to root (**figure 2.16.**)

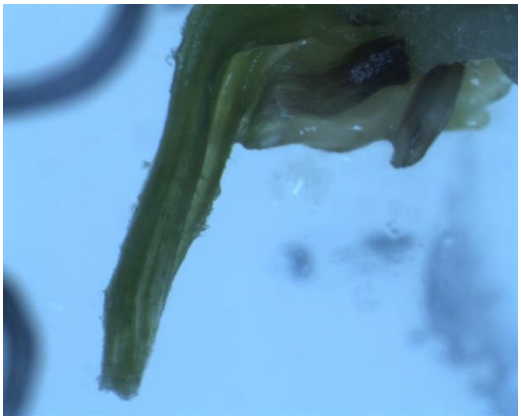


***Figure 2.16.** The green plant like growth, regenerated from Assiniboia on POL media. The plant failed to root and eventually died.*

2.3.1.3. Block two anther culture: Canyon x Firth

For the second block of donor plants, F2 generation plants from a cross of Canyon x Firth were chosen to be screened with the anther culture protocol. Regenerated plants from a population are more valuable than those regenerated from inbred cultivars, because it is possible to screen dihaploid recovered plants for heterozygosity with molecular markers and check whether they are regenerated from somatic (anther wall) tissue rather than the microspores. Both parents are also commercially relevant at the time of experimentation. 23 lines of Canyon x Firth F2 were sown as it is important to have a large number of lines, to increase the chance of seeing segregation in the regeneration rates between lines. Due to the large number of lines to be screened, only three conditions, with 90 anthers per condition (average 30 anthers per anther culture) were tested. These were selected based on the current results at the time with the first block experiment. The three conditions were; MDHM media at pH 5.8 and 7.5, and POL media at pH 7.5.

Canyon x Firth anther cultures responded poorly. Only two calli were induced in Canyon x Firth lines, one in line 10, and one in line 13. In total 204 anther cultures (6120 anthers, not shown) were cultured. Both calli were induced on POL media at pH 7.5.

2.3.1.4. Observations of pH using phenol Red

To see if the high pH media of 7.5 affected the callus induction response of anther cultures, a pH indicator phenol red was added to anther culture induction media petri dishes after different lengths of time. Three replicates of Canyon x Firth anther culture plates were screened on alternate days, from day 0 to day 10. Phenol red staining shows that pH drops below 6 after ten days (**2.17.**) (compared to phenol red chart **figure 2.18.**).

Figure 2.17. *15 anther culture induction plates of Canyon x Firth stained with phenol red. On the furthest left are unused three anther culture induction plates, and at the top and furthest right are petri dishes containing no media. From left to right the stained anther cultures differ by ten days.*

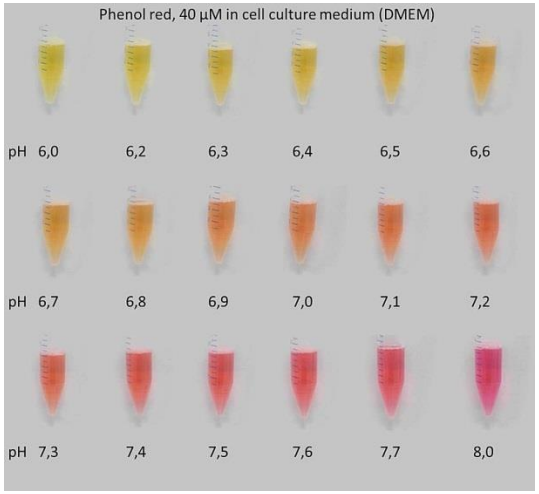


Figure 2.18. *Phenol red chart used to compare with stained anther culture plates. By Max Schwalbe - Own work, CC BY-SA 4.0, https://commons.wikimedia.org By Max Schwalbe - Own work, CC BY-SA 4.0,* https://commons.wikimedia.org/w/index.php?curid=39238574 /w/index.php?curid=39238574

2.3.1.5. Isolated microspore culture

The protocol of Ferrie *et al.* (2013) was used as the basis for isolated microspore culture. Initial experiments were carried out to achieve the optimum microspore concentration of $1x10^6$ /mL without aiming for long-term culture (i.e. without a requirement for aseptic handling). Through these experiments a range of different genotypes were tested, as well as combinations of panicles. The exact moment when panicles were ready, based on microspore stage and pre-treatment, could not be easily predicted, and panicles also had to be used for both the anther culture and isolated microspore culture. At least ten panicles were predicted to be required, so as to have a high enough number of microspores at the correct cell stage, and successfully induced.

Non-sterile attempt one						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
1	5	8.33	83.3	83,333	2	166,666
2	9					
3	3					

Table 2.6. *Concentration of microspores of the first non-sterile IMC.*

The first attempt isolated a very low number of microspores (**table 2.6**.). For the second attempt the centrifugal force was increased from g x 100 to g x 300. Due to the size constraint of the blender cup, ten panicles were split into two runs of five panicles, with the microspores combined at the end.

Non-sterile attempt two						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
Run one						
1.0	9.0	18.0	126.7	126666.0	3.0	379998.0
2.0	11.0					
3.0	18.0					
Run two						
1.0	10.0	8.0	80.0	80000.0	3.0	240000.0
2.0	8.0					
3.0	6.0					

Table 2.7. *Concentration of microspores of the second non-sterile IMC.*

The second attempt with the modification of the centrifugal force was a major improvement on the first first attempt, but still did not attain the required $1x10^6$ /mL (**table 2.7.**). The waste liquid from the filters was checked under a microscope. It was found that many microspores had passed through the 40 µm filter, so this was replaced with a mannitol maltose/gradient centrifugation step using using 0.3 M mannitol and 21 % maltose (Lantos & Páricsi, 2006). The following experiments were conducted with the changes, and were done under sterile conditions so an attempt could be made to culture the isolated microspores. The first run used a mixture of panicles from different genotypes, and the second run contained panicles from only the genotype Aslak (**table 2.8.**). The g was increased from 300 to 400.

Sterile attempt one						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
Run one						
Panicles used: 1x Aslak, 1x Canyon, 3x Assiniboia						
1	17	18.0	180.0	180,000.0	3.0	540,000.0
2	12					
3	25					
Run two						
Panicles used: 5x Aslak						
1	30	28.3	283.3	283,333.0	4.5	1,274,998.5
2	31					
3	24					

Table2.8. *Concentration of microspores of the first sterile IMC experiment*

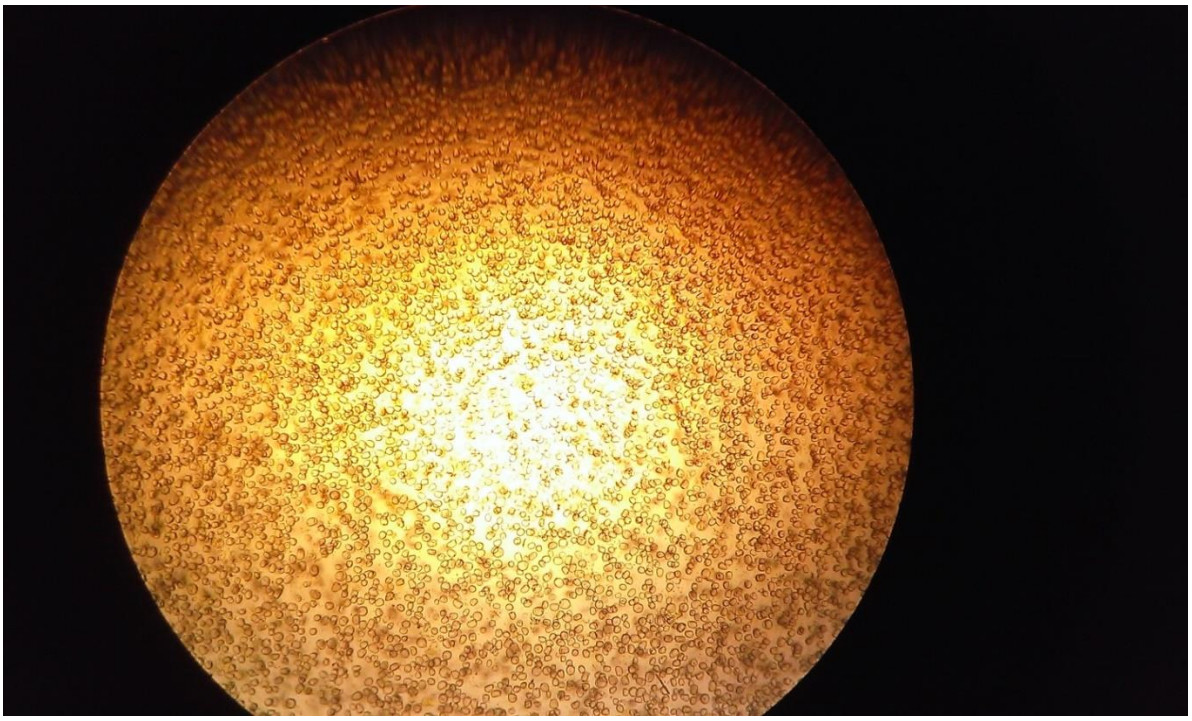


Figure 2.19.*The isolated microspore culture viewed through a 5x microscope.*

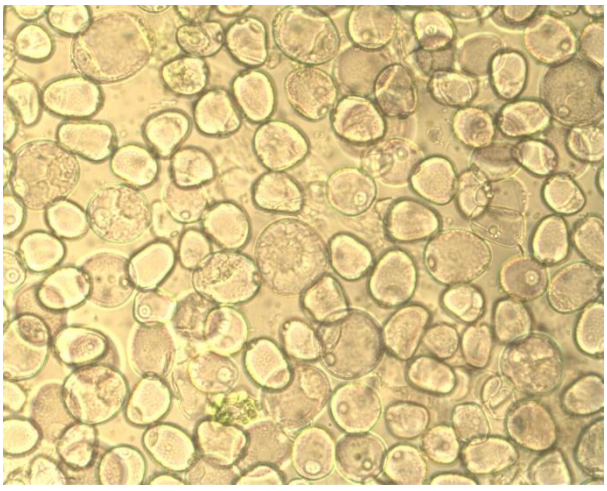
The first and second run for sterile cultures were both more successful than the previous attempts. The second run, made up of only panicles from the genotype Aslak, isolated more than twice as many microspores than the first run made up of mixed panicles (**Table 2.8.**). The mannitol/maltose gradient solution was checked under a microscope and few microspores were observed.

Due to the difficulty of setting the pH of the mannitol solution, 500 mg/L 2-(*N*-morpholino) ethanesulfonic acid **(**MES) buffer was added in future experiments. The centrifugal force was raised again, from 400 to 498 as per Ferrie *et al.,* (2013), and the centrifuge temperature was set to 4 °C as for wheat IMC.

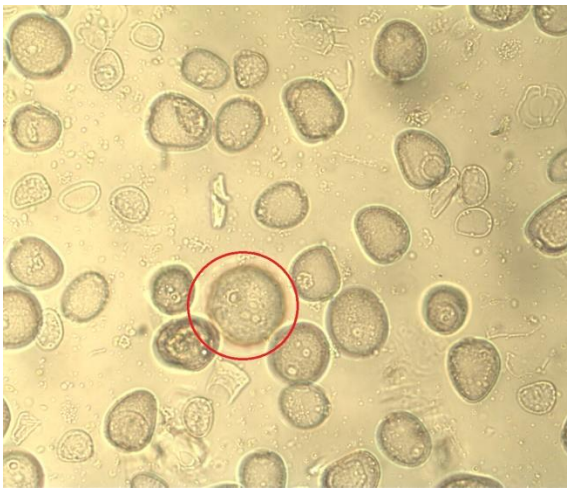
Sterile attempt two						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
Panicles used: Canyon x10						
1	18	14.3	143.3	143,333.0	3.0	429,999.0
2	8					
3	17					

Table 2.9. *Concentration of the second sterile IMC experiment.*

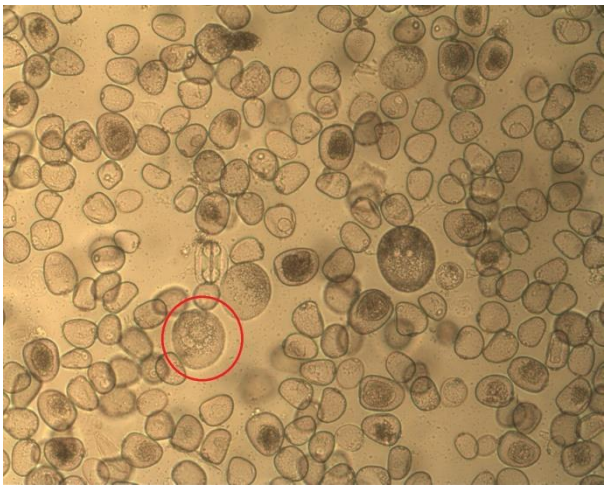
The second sterile attempt combined the microspore containing solution from the two runs of x5 panicles at the first filtering step, to increase the microspore concentration. The total MS concentration did not reach the required $1x10^6$ /mL of microspores (**Table 2.9.**). A camera attached to a microscope was used to photograph the MS to observe how the IMC develop (**figure 2.19.**). On day 0 (**figure 2.20.**) induced microspores were observed along with non-induced microspores at other development stages. By day 9 it was observed that non induced microspores were starting to die and by day 13 the induced microspores did not appear to develop further, and many other microspores had died.



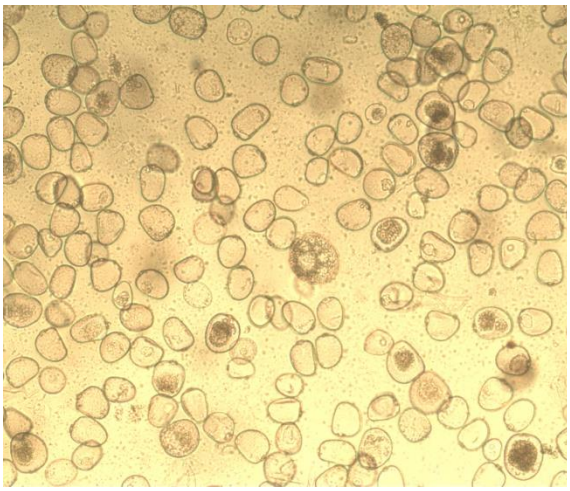
Day 0



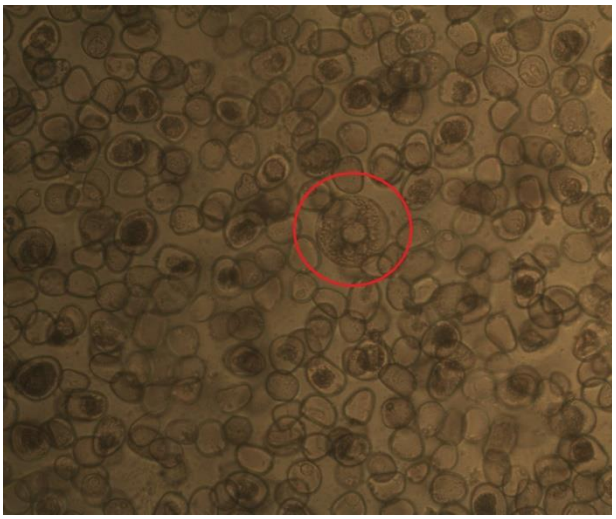
Day 2



Day 9



Day 10

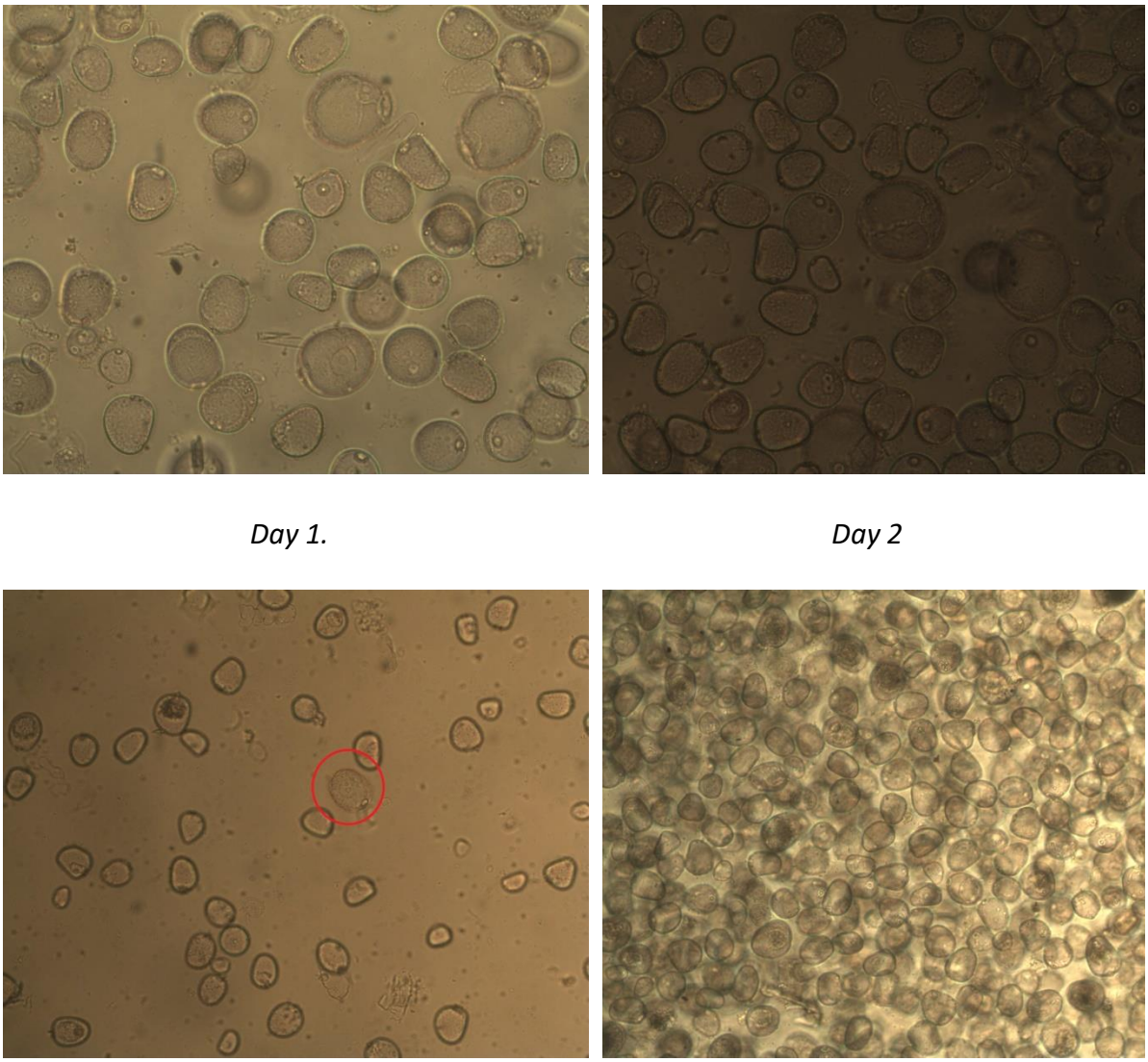


Day 13

Figure 2.20. *Observation of microspores of the second sterile IMC experiment.*

Sterile attempt three						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
Panicles used: Canyon x8						
1	12	6.3	63.3	63,333.0	5.0	316,665.0
2	3					
3	3					

Table 2.10. *Results of the third sterile IMC experiment*



Day 1. *Day 2*

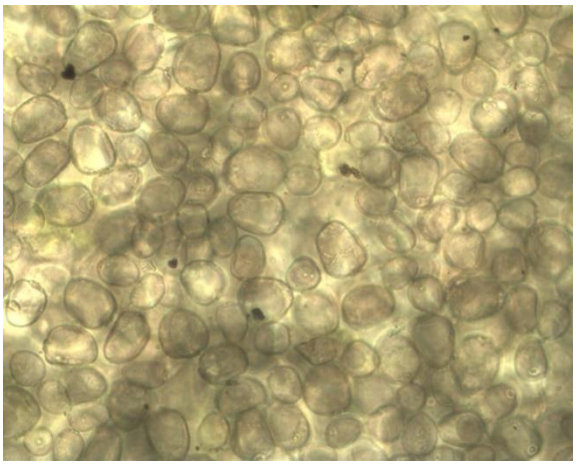
Day 6 *Day 10*

Figure 2.21. *Observation of microspores of the third sterile IMC experiment.*

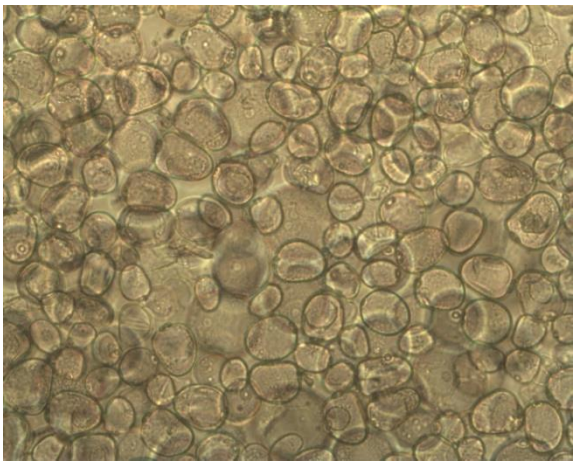
The 3rd sterile attempt used eight panicles instead of ten of the genotype Canyon. Two of the three sample counts were much lower than the third, and much lower than in previous attempts (**Table 2.10.**). The calculated microspore density was lower than previous sterile attempts.

Sterile attempt four						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
Panicles used: Lisbeth x10						
1	102	101.7	1016.7	1,016,666.7	2.0	2,033,333.3
2	98					
3	105					

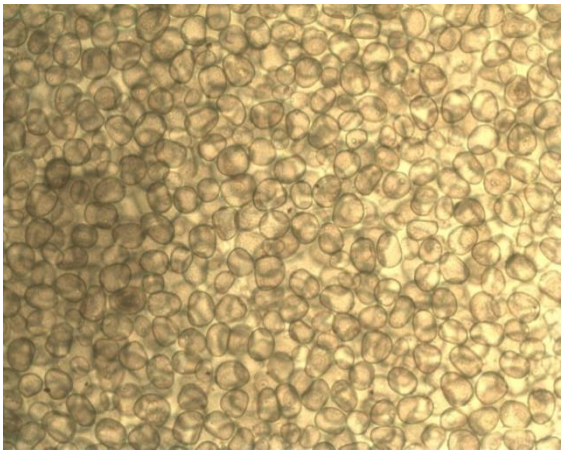
Table 2.11. *Results of the sterile fourth IMC experiment*



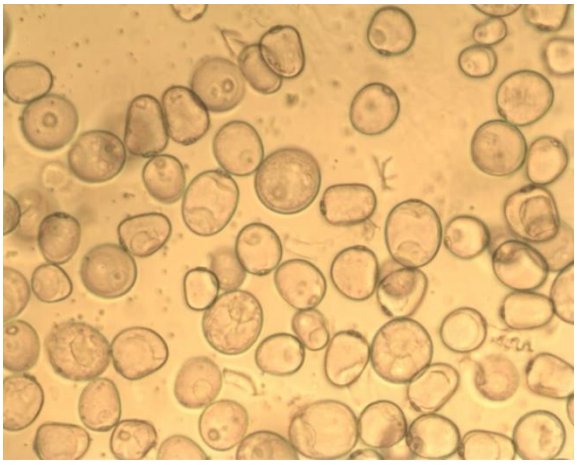
Day 0.



Day 1.



Day 4.



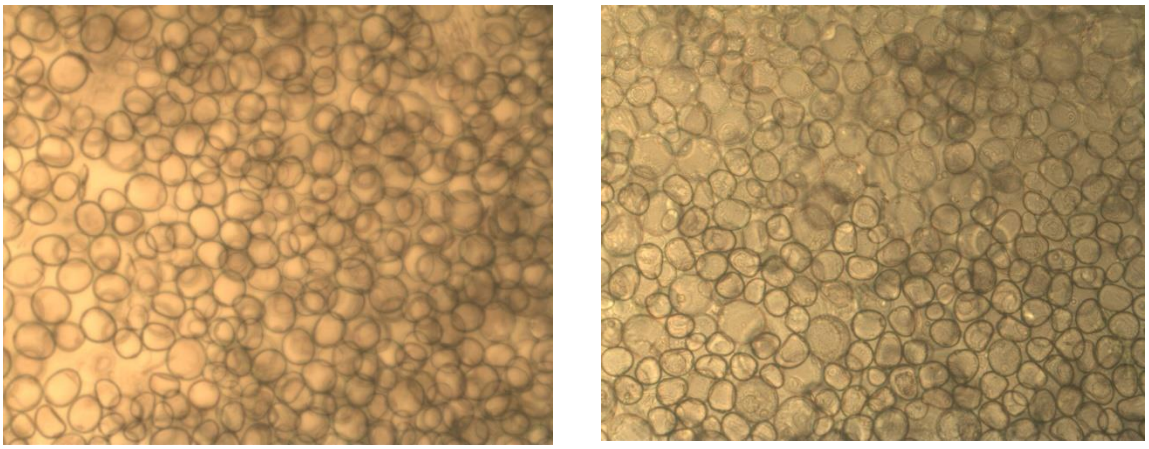
Day 9

Figure 2.22. *Observation of microspores of the fourth sterile IMC experiment.*

The fourth attempt used 10 panicles of Lisbeth and performed far better (**table 2.11.**), reaching the required microspore density of $1x10^6$ /mL of microspores based on three dilution samples, which all scored high microspore counts. The culture appeared viable for nine days before signs of cell death were present. No callus growth was observed.

Sterile attempt five						
Sample no.	MS count	Average	MS /µL	MS /mL	mL	Total MS
Panicles used: Canyon x Firth x10						
1	37	41.0	410.0	410,000.0	4.0	1,640,000.0
2	45					
3	41					

Table 2.12. *Results of the sterile fifth IMC experiment*



Day 0 Day 1

Figure 2.23. *Observation of microspores of the fifth sterile IMC experiment.*

The 5th attempt used 10 panicles from the Canyon x Firth population and did not reach the required microspore density of $1x10^6$ /mL of microspores (**table 2.12.**), and the microspores appeared to die rapidly after isolation (**figure 2.23.**).

2.3.1.6. Preliminary discussion

The primary aim of the preliminary experiments with anther culture and isolated microspore culture was to test the feasibility of each method using published protocols. While isolated microspore culture is preferred over anther culture due to its potentially higher efficiency of explant regeneration, it requires much more highly refined and precise conditions to work successfully. With a recent publication claiming success of isolated microspore culture with oat (Ferrie *et al.,* 2013) it was hoped that it could be the primary method for oat DH production in this project. The initial results from the IMC looked promising, and after two non-sterile attempts it appeared feasible to isolate microspores from the panicles without observing large scale death of microspores. However, with each attempt it was seen that the cultures started to show signs of cell death after several days. This was seen with a variety of cell concentrations and with difference genotypes. This would suggest that the problem lies either in the isolation method or the culture medium and conditions. The method used here followed the method published by Ferrie *et al. (*2013) with one key change. With the observation that microspores were passing through the 40 µm filter in large numbers it was decided that maltose mannitol gradient be used to isolate the microspores more effectively. It is possible that this change to the protocol had a negative impact on the microspores by a shock in osmolarity change; however the images on the days after isolation do not show signs of damage due to adverse tonicity. Cells would have expected to either swell up and even pop in hypotonic conditions or 'shrink' due to hypertonicity caused by the difference in water potential. Another possibility is that the pH may have had a negative impact. The results from the pH anther culture experiment showed that the medium pH dropped rapidly over ten days (**Figure 2.17.**) so it is possible the same drop in pH is occurring in the IMCs. The low pH did not affect the anther cultures, and callus was successfully induced (**Table 2.5.**) so it is possible that the anther wall provides some form of protection for the microspores inside the anthers during the early stages of development. The third possibility is that the concentration of successfully pretreated microspores is too low, in comparison to the non-pretreated microspores. From the images of the various IMCs it is

clear that there are many microspores present which have not undergone successful pretreatment. Day 13 of the second sterile attempt shows a pretreated microspore showing early signs of division, however it is surrounded by microspores with dark patches inside them, which strongly suggests they are in the process of dying. These dying microspores could potentially be releasing compounds or enzymes which create a hostile environment and cause damage to the induced cells. Future work on IMC should focus on several areas for optimization. Either the ratio of successfully pretreated to non-pretreated microspores should be increased to reduce the potential effects of non-pretreated microspores having a negative impact on the IMC, or the isolation method of pretreated microspores should be improved, so that fewer non-pretreated microspores pass through the isolation stage. If the ratio of pretreated to non-pretreated microspores cannot be improved, then using a greater number of panicles with a stricter isolation method may be possible. Optimization of the mannitol gradient could also improve the efficiency of the isolation stage.

Anther culture with oat has a longer history of research, with success in a number of oat species (Kiviharju & Pehu 1998; Kiviharju *et al.,* 1997) and more recently with cultivated oat (Kiviharju & Tauriainen 1999; Kiviharju *et al.,* 2000). The anther culture protocol was further improved and many regenerants were produced (Kiviharju *et al.,* 2005) including Polish cultivars (A Ślusarkiewicz-Jarzina & Ponitka, 2007). Anther culture is a slower, less efficient but more stable method for producing DH plants. The results shown in the preliminary experiments are vastly lower than those in the above published work. This was to be expected, as the preliminary experiments were a learning experience, as well as an initial investigation into current anther culture methods, and responsive genotypes. Despite the very low number of calli induced, with only one plant like structure regenerated, this could be considered a success as it demonstrated that the protocol could be applied in our hands using available genotypes. Callus was induced on all three media types, and the four genotypes (Aslak, Assiniboia, Lisbeth and Bajka) which have been previously proven to work in previously published research have successfully induced callus here, while the untested genotype Canyon failed. The use of a higher pH

did not increase the callus induction efficiency, and may have had a negative effect, with only Assiniboia successfully inducing callus at the pH 7.5 with the media MDHM and POL (**Table 2.5.**). The response of Assiniboia may reflect greater adaptability of this genotype. When measured with phenol red, the pH of the anther culture plates drops significantly, below 5.8, so without the use of a buffer it is likely that setting a higher pH for the medium has little impact in maintaining a stable pH level.

In summary, this preliminary work has been useful as a gauge for how the project should develop, and shows that anther culture is a more reliable method to focus on.

2.3.2. Optimization of the oat anther culture protocol

Following the results from the preliminary experiment, it was decided that future experiments should focus on the optimisation of the anther culture protocol. This procedure was successful, albeit at a much lower level to published work, while the IMC failed to produce any output. It was considered that these low levels were still sufficient to allow detection of improvements in the anther culture protocol that may result from modifications to the culture medium or pre-treatment. Once validated in anther culture, incremental improvements could be applied to IMC at a later date.

In order to accommodate the necessary minimal number of replicates, it was decided to select one base medium for the vast majority of future work to keep the numbers at a manageable level. The medium MDHM with SeaPlaque had been the most consistent in terms of callus induction and so was chosen. The number of replicates for experiments with genotypes was increased to a minimum of ten anther cultures. The genotype Assiniboia showed the most promise in the preliminary experiment, and so was chosen as the positive standard for future experiments.

2.3.2.1. The effect of anther density on callus induction

A higher density of anthers on the induction medium increases the anther response and plant regeneration rate

Early reports showed that the density of anthers in culture was a fundamental factor for successful induction of callus in barley anther cultures (Xu & Sunderland, 1982), particularly when exogenous hormones were not used. In the preliminary anther culture experiments 25-30 anthers were cultured per replicate. High densities of microspores was found to be important for improving the rate of callus and embryo-like structure (ELS), and it is possible that this factor affects anther culture as well. The genotype Assiniboia was sown in a controlled environment room at Aberystwyth University in October 2015. The plants were sampled for anther squashes and cold pre-treated for 14-16 days. Anthers of Assiniboia were cultured on MDHM medium in two different amounts, either 33 or 42 anthers. This equates to 11 and 14 florets per culture respectively. Fifteen replicates for each treatment were produced. Plates were scored by the number of anthers from which calli grew. The calli were moved to regeneration medium once they were approximately 2-3 mm in diameter.

In **Table 2.13.** the results show that the amount of anthers producing callus per 100 anthers at the higher density was more than double that of the lower density.

Genotype	Media	Anther concentration	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers ±SE	No. of regenerants	No. of regenerants /100 anthers	albino	green
Assiniboia	MDHM	low	495	21	4.2 ± 1.10	3	0.61	2	1
Assiniboia	MDHM	high	630	74	11.7 ± 1.34	6	0.95	5	1

(Table 2.13.) *Influence of anther density on anther culture embryogenesis of the genotype Assiniboia*.

2.3.2.2. The effects of co-culturing anthers from different panicles and genotypes

Mixing anthers from different panicles within and between genotypes did not increase anther response or plant regeneration

One of the major factors in carrying out anther culture is the quality of the donor plants from the point of germination through to the end of the panicle harvesting. Even when multiple individuals of the same inbred genotype are grown there will inevitably be differences between individual plant's vigour. This has the potential to influence the results of callus induction and plant regeneration efficiency. Some environmental variation is unavoidable without great effort, as positions of plants in the greenhouse or growth room will affect the amount of light, water and air circulation a plant receives compared to others. Where plants are grown in greenhouse conditions, variation in external light levels are likely to increase variability in plant growth. This is particularly important during the harvest period, which is the crucial stage. The second major factor is the effect harvesting has on the donor plants. The first panicles to be harvested are the primaries, which are larger and stronger than the later secondary panicles. Cutting primary panicles removes significant resources from the plant, and may impact on the fitness of subsequent panicles. It is not feasible to grow sufficient donor plants for only primary panicles to be used, and, indeed, leaf tips are routinely clipped in the early growth stage of donor plants to mimic grazing, which has been shown to encourage tillering so that a minimum of five to six panicles per donor plant can be expected for experimental use. It is therefore important to investigate the effect of panicle identity, to see if there is a marked difference between panicles. If the panicles of the same genotype tested on the same anther culture plate respond at a similar rate, then the effect of variable environmental factors involved during their culture can be discounted. However if the panicles vary markedly in anther response and plant regeneration, then this could be traced back to the relevant environmental factors, or used to optimise harvest time differently between primary and secondary panicles. It is also critical to establish the

consistency of panicle responses to avoid confounding any donor plant variability with the effect of any specific modifications to culture protocols being tested.

An additional consideration is that variable responses by anthers may create different local environments for neighbouring material during culture, as a result of release of compounds or hormones into the medium. Highly responsive anthers, for example from 'ideal' developmental stages or from primary panicles, could potentially aid the response of poorly responsive ones, for example by providing the microspores with hormones which help guide the development of callus, while poorly responding material may in turn not only fail to develop, but may also create adverse conditions such as altered pH or release of stress signals which suppress the growth of potentially responsive anthers. The potential for anthers to influence neighbours in positive or negative ways may most easily be modelled by using mixtures of anthers from responsive and non-responsive genotypes.

To test these potential influences, a number of different experiments were set up. To test the effect of mixing different panicles from the same genotypes, two different anther culture plate layouts were designed. The first is a pair design, where two panicles are split across two anther culture plates to mix the anthers the top, middle and bottom anthers from along the sample area of each panicle (**Figure 2.25.**). The second design is a four plate layout where the anthers from four panicles are split across four plates; bottom, lower mid, upper mid, and top (**Figure 2.24.**). The plate design in **figure 2.26.** was used to test the effect of Assiniboia acting as a 'feeder' genotype for recalcitrant genotypes.

To test the effect of four panicles split across four different anther cultures, three replicates were prepared. For each, four panicles which were deemed ready by anther squash at the same time were used and plated in the same period. The plates were marked to recognize each panicle on each plate. In this experiment, 16 florets for each panicle were used, so that there were an equal number of anthers in each row, for each plate (see **figure 2.24.**).

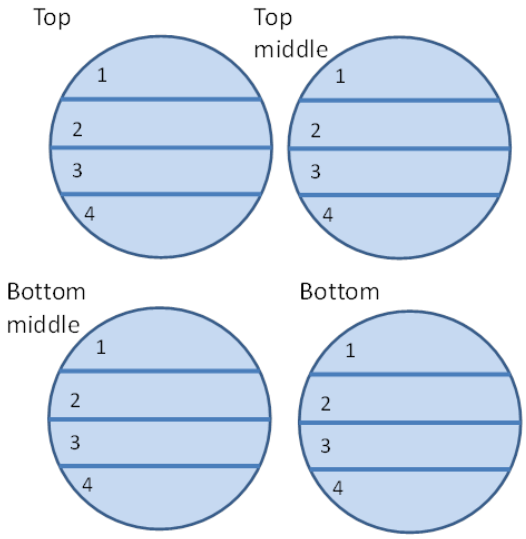


Figure 2.24. *The layout of four panicles across four anther culture plates.*

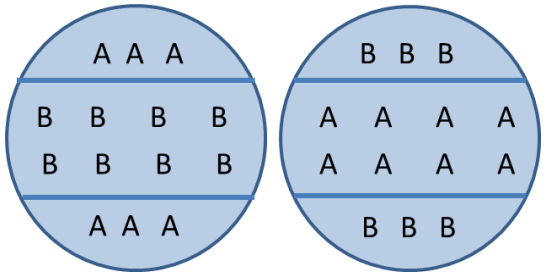


Figure 2.25. *The layout of paired plates for two panicles (A and B)*

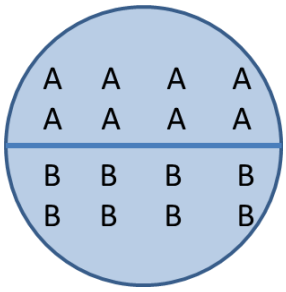


Figure 2.26. *The layout of the anther co-cultures, where Assiniboia (A) acts as a potential feeder to recalcitrant genotypes (B).*

The paired experiment plates were conducted with a range of genotypes. All experiments were conducted with standard pretreatment and plated on MDHM1 media. The first tested 12 pairs of panicles of the genotype Assiniboia split across two plates. Five pairs were plated as per in **Figure 2.25**, and the other seven with four florets of anthers in each row. The second was conducted using 18 pairs of panicles of Assiniboia x Firth, all plated as in **Figure 2.25.** The other paired plate experiments tested mixtures of genotypes. Assiniboia was used as a feeder genotype, with the goal of initiating successful

embryogenesis in currently recalcitrant genotypes. The co-culture plates of Assiniboia with *A. fatua* and Firth were laid out as per in **Figure 2.26**. due to both genotypes producing small panicles with few florets. The co-cultures of Assiniboia with Assiniboia x Firth were laid out as per **Figure 2.25.**

Pair no.	Plant no.	No. anthers	Responding anthers	no. of responding anthers /100 anthers	no. of plants	No. of plants /100 anthers	Green plants	Albino plants
1	18	42	9	21.43	**8**	11.90	5	3
	22	42	7	16.67	**4**	4.76	2	2
2	3	48	12	25.00	4	2.08	1	3
	28	48	13	27.08	6	4.17	2	4
3	1	48	**22**	45.83	5	8.33	4	1
	2	48	**18**	37.50	3	2.08	1	2
4	9	48	11	22.92	4	4.17	2	2
	12	48	12	25.00	3	4.17	2	1
5	7	42	15	35.71	2	0.00	0	2
	15	42	17	40.48	3	4.76	2	1
6	14	42	**15**	35.71	**1**	2.38	1	0
	29	42	**10**	23.81	**5**	7.14	3	2
7	4	48	**10**	20.83	0	0.00	0	0
	6	48	**23**	47.92	3	2.08	1	2
8	15	48	**9**	18.75	**1**	0.00	0	1
	21	48	**20**	41.67	**5**	8.33	4	1
9	2	48	20	41.67	1	2.08	1	0
	10	48	22	45.83	2	2.08	1	1
10	4	42	**6**	14.29	1	2.38	1	0
	20	42	**10**	23.81	0	0.00	0	0
11	9	48	**17**	35.42	3	2.08	1	2
	11	48	**13**	27.08	4	4.17	2	2
12	24	42	10	23.81	6	4.76	2	4
	30	42	9	21.43	3	2.38	1	2
Total	-	1152	330	Avg. 29.99	77	Avg. 3.60	39	38

Table 2.14. *The results from the Assiniboia paired panicle experiment.*

Results from the Assiniboia paired experiments show a mix of results. The overall result was good, with a total of 330 responding anthers from a total of 1152. Across all plates the number of responding anthers per 100 anthers was 28.99. The total number of plants regenerated across all plates was 77, which equates to 3.60 plants per 100 anthers plated. Of the 77 plants, 39 were green, and 38 were albino, an almost 50/50 split. The ratio of green to albino plants for each culture varied. The lowest number of responding anthers recorded was 6, for plant no. 4 in pair no. 10. The highest number of responding anthers was 23 anthers, almost 50% of the total, for plant no. 6 of pair no. 7. Not one plant had zero anthers respond. The number of responding anthers did not appear to influence the number of plants regenerated. Plant six of pair seven only regenerated a total of three plants (one green, two albino) while plant 18 of pair one regenerated a total of eight plants (five green, and three albino) from only nine responding anthers. In bold (**Table 2.14.**) are pairs which have a difference of four between the number of responding anthers, and plants, which equates to roughly 10% difference between the total responses. Of the twelve replicates, six have anther responses which differ by 10%. Replicate eight has a major difference between the two panicles of 9 to 20. This is also reflected in the number of plants which regenerate. Plant number 21 regenerated five plants, of which four were green, while panicle 15 only regenerated one, which was albino. Pair no. 7 also showed a large difference (of 13 responding anthers) between pairs, which was mirrored in the number of plants regenerated.

Table 2.15. shows the results from the four plate experiments. The number of responding anthers ranged from as low as two to as high as 28 (replicate one, plant 28). The average anther response per hundred anthers was 20.83, which is lower than the average anther response per hundred anthers from the paired anther culture plates (**Table 2.14.**) The average regenerants per hundred anthers was also lower at 1.70 plants than 3.60 plants for the Assiniboia paired experiment (**Table 2.14.**). From the total count of responsive anthers, the middle rows had a higher number of responding anthers than the bottom or top rows. Of the total of 12 panicles used, only six regenerated plants, of which only three regenerated green plants. Plant panicles responded differently on the sample plate. For

the first replicate, the difference in responding anthers was 26 between panicles, with the lowest response of plant panicle seven having just two responding anthers, and plant panicle 25 having 28 responding anthers. Smaller differences were also seen in the other two replicates. None of the replicates showed a homogenous response across all plant panicles, which suggests that there is not a clear panicle to panicle interaction.

The results of the Assiniboia x Firth paired panicle experiment (**table 2.16.)** showed similar results to the Assiniboia paired experiment (**table 2.14.**). The overall anther response was low, however several lines stood out. Lines 63, 45 and 58 had an anther response over 14 % (marked with *, **table 2.16.**). Lines 45 and 63 had pair partners which had at least one anther respond, however line 58 did not.

To test the effect of mixing genotypes, Assiniboia was selected as the positive 'feeder' genotype. It had proven to be a positive responding genotype in a number of experiments, and at the time was the choice genotype to be used for media optimization. *A. fatua* failed to respond to anther culture in solo cultures so was a good candidate to screen for an increase in response. The *A. sativa* genotype Firth also was highly recalcitrant to anther culture and so was selected as a second 'recipient' source.

For each replicate, one panicle of each genotype was used, sampling eight florets from the sample area. The anthers were plated as in **figure 2.26.** Both genotypes were plated at the same time. For *A. fatua*, five replicates were made for one treatment on standard MDHM medium without additional additives. For Firth, three different treatments with five replicates each were tested; 1) MDHM with no additives, 2) MDHM with AGP 200 mg/L, and 200 mg/L gum Arabic, and 3) with media pre conditioned with the eight Assiniboia anthers seven days before the Firth was added.

The results in **table 2.17.** show that the co-culture of a recalcitrant genotype with a non-recalcitrant genotype does not improve the response of the recalcitrant genotype. *A. fatua* G1 failed to produce any callus. Firth had just two responding anthers on treatment MDHM1 only. The anther response per hundred anthers of Assiniboia was relative to that

Replicate	Plant no.	No. responding anthers				Total no. anthers	No. responsive anthers	No. responding anthers /100 anthers	No. of regenerants	No. of regenerants /100 anthers	Green plant	Albino plant
		B	BM	TM	T							
1	25	9	3	8	8	48	28	58.33	3	6.25	2	1
	9	1	7	4	5	48	17	35.42	0	0.00	0	0
	23	0	1	1	4	48	6	12.50	0	0.00	0	0
	7	1	0	0	1	48	2	4.17	1	2.08	1	0
2	19	0	2	1	0	48	3	6.25	1	2.08	0	1
	2	4	7	4	3	48	18	37.50	0	0.00	0	0
	13	7	5	1	0	48	13	27.08	0	0.00	0	0
	35	0	0	0	3	48	3	6.25	0	0.00	0	0
3	34	1	2	7	0	48	10	20.83	1	2.08	0	1
	18	0	2	2	0	48	4	8.33	0	0.00	0	0
	36	1	1	0	0	48	2	4.17	2	4.17	0	2
	24	2	5	7	0	48	14	29.17	2	4.17	2	0
Total		26	35	35	24	576	120	avg. 20.83	10	avg. 1.74	5	5

Table 2.15. *Results of the anther culture of Assiniboia four plate experiment. B= bottom row, BM = bottom middle row, TM = top middle row, T= Top row.*

Pair no.	Plant no.	No. responding anthers	No. responding anthers /100 anthers	No. regenerants	No. regenerants /100 anthers	green	albino
1	51	0	0.00	0	0.00	0	0
	63	1	2.38	0	0.00	0	0
3	21	0	0.00	0	0.00	0	0
	58	6	14.29*	0	0.00	0	0
5	30	0	0.00	0	0.00	0	0
	40	0	0.00	0	0.00	0	0
6	30	0	0.00	0	0.00	0	0
	52	2	4.76	2	4.76	2	0
7	22	1	2.38	0	0.00	0	0
	61	0	0.00	0	0.00	0	0
9	27	2	4.76	0	0.00	0	0
	70	0	0.00	0	0.00	0	0
10	9	0	0.00	0	0.00	0	0
	20	0	0.00	0	0.00	0	0
11	19	0	0.00	0	0.00	0	0
	49	0	0.00	0	0.00	0	0
12	34	0	0.00	0	0.00	0	0
	52	2	4.76	0	0.00	0	0
13	45	8	19.05*	2	4.76	1	1
	61	0	0.00	0	0.00	0	0
14	42	1	2.38	0	0.00	0	0
	47	0	0.00	0	0.00	0	0
15	51a	1	2.38	0	0.00	0	0
	51b	3	7.14	0	0.00	0	0
16	14	3	7.14	0	0.00	0	0
	40	0	0.00	0	0.00	0	0
17	34	1	2.38	0	0.00	0	0
	49	0	0.00	0	0.00	0	0
18	60	1	2.38	0	0.00	0	0
	63	6	14.29*	1	2.38	1	0

Table 2.16. *The results from the Assiniboia x Firth paired panicle experiment.*

in other experiments (see **table 2.14., table 2.15.**) and the regeneration per hundred anthers was slightly lower.

Media + Treatment	Genotype	No. anthers	Responding anthers	no. of responding anthers /100 anthers	no. of regenerants	No. of regenerants /100 anthers	Green plant	Albino plant
MDHM1	Firth	120	2	1.67 ± 1.02	0	0.00 ± 0.00	0	0
	Assiniboia	120	48	40.00 ± 7.75	1	0.83 ± 0.83	0	1
MDHM1	*A. fatua* G1	120	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
	Assiniboia	120	38	31.67 ± 1.67	2	1.67 ± 1.02	1	1
MDHM2	Firth	120	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
	Assiniboia	120	25	20.83 ± 3.49	1	0.83 ± 0.83	0	1
MDHM1 +Cond	Firth	120	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
	Assiniboia	120	26	21.67 ± 7.75	1	0.83 ± 0.83	0	1

Table 2.17. *Using the positively responding genotype Assiniboia co-cultured with recalcitrant genotypes. SE = standard error.*

2.3.2.3. The effect of the compounds GAM and putrescine on anther culture

GAM and putrescine did not improve anther response or plant regeneration

Based on the results from the preliminary experiments the two cultivars, Bajka, Assiniboia and Aslak were selected for further experiments. Due to the number of plants, the cultivation was staggered, so Bajka and Aslak were sown first, then Assiniboia later. The medium MDHM was carried forward to be used as a baseline medium, to be compared with a second medium, modified IMI (MIMI) designed by Esteves *et al. (*2014) which is based on the barley medium by Li and Devaux (2003). An important difference between MDHM and MIMI is the substitution of the hormones 2,4-D and kinetin for thidiazuron and dicamba which were found to increase green plant regeneration efficiency. In addition to testing two media, three additional additives were tested. Gum arabic is important for plant cellular activities such as cell division. In wheat, the addition of this compound at 50 mg/L decreased microspore death rates and improved microspore induction. The third additive is putrescine, a polyamine. These compounds have been found to improve induction of microspores. In oats, the addition of putrescine improved somatic embryogenesis rates (Kelley *et al.,* 2002), but it has not been tested for gametic embryogenesis. It was added at 44 mg/L as per Kelley *et al.,* (2002). Two pre-treatments were tested, 9-14 days in autoclaved water at 4 °C, and 2-4 days in 0.3 M mannitol at 4 °C. For each treatment, five replicates of 30 anthers per petri dish were made, so 150 anthers per treatment.

Unfortunately, the anther culture experiments for Bajka and Aslak failed due to experimental error. A problem was noticeable one to two days after anthers had been excised and plated onto induction medium. The anthers split, and spilled microspores over the medium. The effect was noticed across all treatments (about 120 anther culture plants). Initially, it was thought the medium was the problem. The major minerals stocks of both media were re-made and the anther cultures were remade, in case the quantity of SeaPlaque agarose concentration had been added in erroneous amounts. When this

did not solve the problem oat anthers were plated onto anther culture for barley which had been prepared separately by staff at Saaten Union Biotec GmbH. The anthers behaved as before. The solutions used for pre-treatments were remade, and it was then discovered that distilled water had been used instead of tap water. The distilled water had caused a tonicity effect, as the cut panicles effectively have no control over the water potential. After just a few days the plant weight reduces rapidly due to a combination of transpiration and hydraulic resistance (Van Meeteren *et al.,* 2000). Unfortunately by the time the problem had been solved, all panicles of Aslak and Bajka had been affected. However, the Assiniboia donor plants which had been sown later had not yet been harvested and stored in the deionised water. Fewer plants had been sown, so the experiment focused on testing the additives with the MDHM medium. The results from the Assiniboia x Aslak F2 population experiment (**3.3.2.**) had started to be recorded, and the MDHM medium was outperforming MIMI. Four different treatments (MDHM with no additive MDHM with GAM, MDHM with putrescine and MDHM with both) each with ten replicates with 42 anthers per replicate were made.

Table 2.18. shows the results from the experiment. A total of 45 plants were regenerated of which two thirds were green. Of these, almost half were regenerated from the MDHM control. The MDHM control was also just behind MDHM with GAM by a small margin of just ten responding anthers. The lowest anther response was seen in the MDHM medium with both additives, which performed half as well as when the additives were tested individually. Despite this more plants were regenerated (nine) with both additives, than when putrescine was used alone.

Treatment	no. of anthers cultured	No. responding anthers	no. of responding anthers /100 anthers	Regenerants	no. of regenerants /100 anthers	albino	green
None	420	174	41.4 ± 3.94	24	5.7 ± 0.93	10	14
GAM	420	184	43.8 ± 2.01	8	1.9 ± 0.51	1	7
Putrescine	420	148	35.2 ± 3.84	4	1.0 ± 0.31	0	4
GAM + putrescine	420	77	18.3 ± 5.19	9	2.1 ± 0.48	5	4
Total	1680	583	34.7 ± 4.93	45	1.3 ± 0.31	16	29

Table 2.18. *Influence of GAM and putrescine on anther culture and plant regeneration of Assiniboia (SE = standard error).*

Treatment	No. of anthers cultured	No. of responding anthers	No. of responding anthers /100 anthers (±SE)	Regenerants	No. of regenerants /100 anthers (±SE)	Albino	Green
None	420	91	21.67 ± 3.30	4	0.95 ±0.39	2	2
Putrescine	420	80	19.05 ± 3.64	5	1.19 ± 0.53	1	4

Table 2.19. *Influence of putrescine on anther culture and regeneration of Assiniboia. (SE = standard error).*

Putrescine was tested a second time, the following year. Donor plants were grown in the same conditions. All culture media was prepared the same way and the same putrescine concentration of 44 mg/L was used. Ten replicates were produced for each treatment **Table 2.19.** shows the results of the addition of putrescine, compared to a control which was prepared using panicles from the same donor plants. The number of responding anthers was lower than that of the control (difference of eleven) however the putrescine treatment regenerated one plant more, and doubles the number of green plants.

Neither the control nor the putrescine treatment performed as well as the previous year (**Table 2.18**). For both the reduction in the number of responding anthers was almost half. The regeneration efficiency of the control treatment was drastically reduced from a total of 24 regenerants to only four.

2.3.2.4. The effect of the compounds glutamine, casein hydrolysate, ascorbic acid and pyridoxal phosphate

The addition of glutamine and casein hydrolysate increases green plant regeneration efficiency

There are many different compounds which have been added to tissue culture media to increase the successful culture of plants *in vitro*. This section tests more compounds on four different genotypes, to see if they improve the callus induction and plant regeneration efficiency in across a range of genotypes. The four genotypes used were; Assiniboia, the positive standard, Firth, the negative standard, and two new previously untested genotypes from Nordsaat, N15.1407 and N15.1408. Both originated from ongoing breeding programmes so behaviour in anther culture was of great interest. Each genotype was tested on the standard MDHM medium as a control. The first treatment was MDHM with 500 mg /L glutamine, the second treatment with 500 mg/L casein hydrolysate, the third treatment with pyridoxal phosphate. Assiniboia was tested with an additional treatment of 25 mg/L ascorbic acid.

The results are shown in **Table 2.20.** The responses from the different medium additives with Assiniboia are mixed. The addition of glutamine had a minor improvement over the control treatment for the number of responsive anthers inducing callus. The treatments with casein hydrolysate, ascorbic acid and pyridoxal phosphate did not perform much better than the control treatment, and the casein hydrolysate had a decreased response in anther response number compared with the control. The key differences are shown in the regenerant efficiency. The addition of glutamine in the induction media had a major positive impact on the number of plants regenerated, from just under one regenerant per 100 anthers in the control treatment to over six regenerants per 100 anthers. The addition of glutamine greatly improved the number of green plants regenerated, but regenerated a large number of albinos (over 50% of total regenerants). The casein hydrolysate treatment increased the regeneration efficiency as well, with a total of 12 regenerants. It also regenerated three times more green plants than albinos. The pyridoxal phosphate treatment increased the number of regenerants compared to the control, with a significant ratio of green to albino, similar to the casein hydrolysate treatment. The ascorbic acid treatment did not show a marked improvement over the control treatment. The control treatment used here (**Table 2.20.**) is the same as that in **table 2.19.** as the experiments were completed in the same period. The other three genotypes tested all failed to regenerate any plants, and the anther culture response was very low, with either no anthers responding, or just one or two, across ten replicates.

2.3.2.5. The effect of combined glutamine/casein hydrolysate, proline and additional 2,4-D on anther culture

The addition of glutamine and casein hydrolysate combined increases the anther response and plant regeneration efficiency in Assiniboia

This experiment was run at the same time as that in **2.3.2.4.** To study the effect of the addition of proline and a higher concentration of the phytohormone 2,4-D the genotype

Genotype	Treatment	No. of anthers culture	No. of responding anthers	No. of responding anthers /100 anthers (±SE)	Regenerants	No. of regenerants /100 anthers (±SE)	Albino	Green
Assiniboia	None	420	91	21.67 ± 3.30	4	0.95 ± 0.39	2	2
Assiniboia	Glutamine	420	114	27.14 ± 4.89	27	6.43 ± 1.55	15	12
Assiniboia	Casein	420	68	16.19 ± 4.47	12	2.86 ± 1.07	3	9
Assiniboia	Ascorbic Acid	420	82	19.52 ± 3.70	6	1.43 ± 0.63	2	4
Assiniboia	Pyridoxal Acid	420	93	22.14 ± 5.11	15	3.57 ± 2.08	7	8
N15.1407	None	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
N15.1407	Glutamine	420	1	0.24 ± 0.24	0	0.00 ± 0.00	0	0
N15.1407	Ascorbic Acid	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
N15.1407	Pyridoxal Acid	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
N15.1408	None	420	1	0.24 ± 0.24	0	0.00 ± 0.00	0	0
N15.1408	Glutamine	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
N15.1408	Ascorbic Acid	420	1	0.24 ± 0.24	0	0.00 ± 0.00	0	0
N15.1408	Pyridoxal Acid	420	1	0.24 ± 0.24	0	0.00 ± 0.00	0	0
Firth	None	420	2	0.48 ± 0.32	0	0.00 ± 0.00	0	0
Firth	Glutamine	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
Firth	Ascorbic Acid	420	1	0.24 ± 0.24	0	0.00 ± 0.00	0	0
Firth	Pyridoxal Acid	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0

Table 2.20. *The influence of a glutamine, ascorbic acid, pyridoxal phosphate, and casein hydrolysate on the A. sativa genotypes Assiniboia, Firth, N14.1507 and N14.1508.SE = Standard error.*

Genotype	Treatment	Anthers cultured	No. responding anthers	anthers /100 anthers	Regenerants	no. of regenerants /100 anthers	albino	green
Assiniboia	None	420	124	29.52 ± 4.32	40	9.52 ± 1.81	23	17
Assiniboia	Glut/Cas	420	136	32.38 ± 5.58	53	12.62 ± 2.84	34	19
Assiniboia	Proline	420	64	15.24 ± 3.39	16	3.81 ± 1.43	11	5
Assiniboia	3 mg/L 2,4-D	420	31	7.38 ± 2.47	17	4.05 ± 1.63	6	11

Table 2.21. *The effect of glutamine (Glut) (500 mg/L) combined with casein hydrolysate (Cas) (500 mg/L) proline (500 mg/L), 2,4-D (3 mg/L) and on the genotype Assiniboia. SE = Standard error.*

Assiniboia was used and the anther cultures were prepared in the standard way, 14 florets per replicate, and a total of ten replicates per treatment. Proline was added at 500 mg/L and 2,4-D was added at 3 mg/L (the control concentration is 1.5 mg/L 2,4-D).

The addition of proline and additional 2,4-D did not have a positive effect on anther response or plant regeneration (**Table 2.21.**). Additional 2,4-D did not inhibit callus growth, but the number of responding anthers was a quarter of that in the control. The number of plants regenerated was also less than half of the control, however more green plants than albino plants were regenerated. The addition of proline did not have such a negative effect as 2,4-D. The number of responding anthers was half of the control. The number of regenerants was similar to the 2,4-D treatment, however the ratio was the opposite, with more albinos than green plants. The addition of glutamine and casein hydrolysate increased the number of anthers responding and the number of plants regenerating, but regenerating considerably more albino plants.

2.3.2.6. The effect of Copper on anther culture

The addition of copper to the pre-treatment solution and to the media at x100 the normal amount increased anther response

To test the effect of additional copper to anther culture, two different treatments were used. The first added additional copper in the form of CuSO4·5H2O to the MDHM induction medium at 100x the normal amount; 2.5 mg/L. The second treatment applied the same amount of copper to the induction medium but also added copper to the pretreatment water at 2.5 mg/L. The treatment was otherwise the same. The third treatment was a control. All three treatments also included additional glutamine (750 mg/L) and proline (500 mg/L). Three genotypes were selected. Assiniboia, Lisbeth and NORD 16-1421. Assiniboia is a proven positive standard, Lisbeth showed an improve response with additional glutamine and casein hydrolysate (**section 2.3.2.8.**) and NORD 16-1421 is an unknown genotype with Assiniboia pedigree**.**

Genotype	Treatment	Anthers cultured	No. responding anthers	No. anthers /100 anthers +SE	Regenerants	No. of regenerants /100 anthers +SE	Albino	Green
Assiniboia	Control	420	15	3.57 ± 1.19	4	0.95 ± 0.53	1	3
Assiniboia	Cu2+ media	420	17	4.05 ± 1.23	4	0.95 ± 0.63	2	2
Assiniboia	Cu2+media+pretreatment	420	29	6.90 ± 2.05	5	1.19 ± 0.64	1	4
Lisbeth	Control	420	5	1.19 ± 0.81	1	0.24 ± 0.24	0	1
Lisbeth	Cu2+ media	420	7	1.67 ± 0.94	0	0.00 ± 0.00	0	0
Lisbeth	Cu2+ media+pretreatment	-	-	-	-	-	-	-
NORD 16-1421	Control	420	2	0.48 ± 0.32	0	0.00 ± 0.00	0	0
NORD 16-1421	Cu2+ media+pretreatment	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
NORD 16-1421	Cu2+both	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0

Table 2.22. *The effect of copper on the anther culture of different oat genotypes. SE = standard error.*

Due to a lack of good panicles of Lisbeth developing, one treatment could not be completed (**table 2.22.**). The response of the three genotypes varied. Assiniboia was the best performer, with anthers responding and plants regenerating on all treatments. Of the two treatments Lisbeth was tested on, anthers responded on both, and on one a plant regenerated. NORD 16-1421 had anthers respond on only the control treatment. The addition of copper appeared to have a positive impact on the anther response and the plant regeneration for Assiniboia. The addition of copper to the induction medium and pretreatment water showed an increase of almost 100 % in the number of anthers responding. This treatment also had the largest number of green plants regenerate.

The effect of copper did not have such a clear effect with Lisbeth. As one treatment could not be completed only the treatment with copper in the induction medium can be compared. In comparison to the treatment without additional copper, there is only a difference of two in the number of responding anthers, however the response of both is low (5 and 7) so proportionally the difference is potentially significant. The only plant to regenerate from Lisbeth was from the treatment without additional copper. The response of NORD 16-1421 was almost zero for all treatments. The increase in anther culture response seen in Assiniboia was not replicated in NORD 16-1421.

2.3.2.7. The effect of ovary co-culture on anther culture

The addition of ovaries does not increase anther culture efficiency

Ovaries have been used to pre-condition media and also have been co-cultured with IMC to increase the embryogenesis efficiency (Hul & Kasha, 1997). It has been hypothesized that ovaries release compounds into the media which stimulate either the embryogenic response in microspores or provide important compounds to support successful embryogenesis, increasing the overall rate of embryogenesis (Zheng *et al.,* 2002).

To date there have been no published work on the use of ovaries in DH production of oats. This section looked at the use of ovaries in several different experimental formats, to see if oat ovaries can had a positive impact on embryogenesis in anther culture. In all cases mature ovaries were used, taken from florets positioned above those sampled for anthers which had microspores at the uni-nucleate microspore stage as ovaries at this stage were reported to be better in spring wheat (Castillo *et al.,* 2015).

The first experiment looked at the effect of ovary co-culture on the genotype Assiniboia. It has been suggested that ovaries release AGPs and gum arabic (Letarte *et al.,* 2006) so ovary co-culture was tested in combination with both compounds to see if it also effects oat.

The experiment tested four different combinations; 1) MDHM with no additives, 2) MDHM with AGP 200 mg/L, gum arabic 200 mg/L, 3) MDHM with three ovaries, 4) MDHM with AGP 200 mg/L, gum arabic 200 mg/L and three ovaries. The ovaries were sampled from the top end of the panicle sample area, from florets being dissected for anthers. Ovaries were added to the medium at the same time as the anthers. Ten replicates were made for each combination.

The effects of additional GAM did not have a positive effect on anther response or plant regeneration (**Table 2.23.**). The addition of these compounds reduced the anther response by almost 30 % in comparison to the control, and produced far less regenerants. The addition of ovaries alone performed similarly to the control in terms of number of responding anthers, and produced a third of the number of regenerants. The combined treatment also performed similarly to the control for the number of responding anthers, but also regenerated the fewest plants, and did not regenerate a single green plant. The control treatment produced the largest number of regenerants, though only ~20 % were green plants (six). The treatment with ovaries only also produced six green plants, but far few albino plants.

Treatment	Ovaries	Anthers cultured	No. responding anthers	Anthers /100 anthers ± SE	Regenerants	No. of regenerants /100 anthers ± SE	Albino	Green
none	No	420	93	22.14 ± 4.67	32	7.62 ± 1.45	26	6
GAM	No	420	68	16.19 ± 2.72	11	2.62 ± 0.97	8	3
None	Yes	420	95	22.62 ± 4.04	10	2.38 ± 1.06	4	6
GAM	Yes	420	89	21.19 ± 4.58	3	0.71 ± 0.36	3	0

Table 2.23. *The influence of ovary co-culture and GAM at 200 mg/L on the anther culture of the genotype Assiniboia.*

Ovary co-culture was also tested with the oat species *A. sterilis*. This experiment is discussed in **chapter 3.** and the results are shown in **table 3.2.** The experiment was conducted in the same was as here, with three ovaries from the *A. sterilis* taken from the upper section of the anther sample area plated at the same time the anther cultures were prepared.

2.3.2.8. Applying medium improvements to recalcitrant genotypes

The addition of glutamine and casein hydrolysate to the callus induction media causes an increase in anther response and regeneration in a previously recalcitrant genotype

In the **section 2.3.2.5.** the application of glutamine (500 mg/L) and casein hydrolysate (500 mg/L) respectively increased the number of responding anthers compared to the standard medium in the genotype Assiniboia (**Table 2.21**). The additional glutamine did not have an effect on the three other genotypes tested, however all three produced very low, or no results for the four different treatments tested.

In this experiment additional glutamine and casein hydrolysate was tested on three genotypes previously used in the preliminary experiments (**section 2.3.1.2.**). The genotypes Lisbeth, Aslak and Bajka all showed a very low anther response on MDHM medium. With the application of additional glutamine and casein hydrolysate, the response of the genotypes could potentially be improved. Ten replicates for each treatment with each genotype were made.

Genotype	Treatment	Anthers cultured	No. responding anthers	anthers /100 anthers ± SE	Regenerants	no. of regenerants /100 anthers ±SE	albino	green
Assiniboia	None	420	124	29.52 ± 4.32	40	9.52 ± 1.81	23	17
Assiniboia	Glut/Cas	420	136	32.38 ± 5.58	53	12.62 ± 2.84	34	19
Bajka	None	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
Bajka	Glut/Cas	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
Aslak	None	420	3	0.71 ± 0.51	1	0.24 ± 0.24	0	1
Aslak	Glut/Cas	420	14	3.33 ± 2.11	1	0.24 ± 0.24	1	0
Lisbeth	None	420	4	0.95 ± 0.95	1	0.24 ± 0.24	1	0
Lisbeth	Glut/Cas	420	52	12.38 ± 5.72	19	4.52 ± 2.29	16	3

Table 2.24. *The effect of glutamine (500 mg/L) and casein hydrolysate (500 mg/L) on the genotypes Assiniboia, Bajka, Aslak and Lisbeth. SE = Standard error.*

The addition of glutamine and casein hydrolysate promoted anther culture response in two of the three previously recalcitrant genotypes tested (**table 2.24.**). (The results here for Assiniboia are those from **section 2.3.2.5.**, alongside the other genotypes here to aid comparison). The greatest difference between treatments was seen with two genotypes; Aslak and Lisbeth. The addition of glutamine and casein hydrolysate increased the rate of anther response from 0.71 to 3.33 per hundred anthers for Aslak. The difference was even greater for Lisbeth, with an increase in responding anthers from 0.95 to 12.38 per hundred anthers. Lisbeth also showed a large increase in plant regeneration, from 0.24 to 4.52 regenerants per hundred anthers. However, the majority of the plants regenerated were albino.

2.3.2.9. Can phenotypic data predict callus induction efficiency?

Anther response can be correlated with oat panicle phenotypic measurements

In the section **2.1.4.** the roles of donor plant growth conditions and of the quality of donor plants were reviewed. A surplus of donors are grown routinely. It can be expected to obtain four or five good tillers before plant quality declines and noticeable deterioration in panicle size and development is seen (personal observation). Unforeseen problems can occur during the life time of the donor plants. Where possibly, growth chambers are preferred due to their strict climate controls which helps to ensure donor plants develop without environmental stresses affecting normal development. New developments in artificial lighting, such as light emitting diodes (LEDs), have vastly improved growing conditions (Darko *et al.,* 2014). Environmental stresses influence plant development and flowering (Kumar *et al.,* 2012) with photoperiod playing an important role (Riboni *et al.,* 2014). Growth chambers are much more expensive to run per m^2 though, so greenhouses supplemented with heating and lighting are often used. With good weather during the growing period, natural day lengths and light quality may be very beneficial, however periods of poor, cloudy weather may slow plant development

and delay flowering. A general plant development period can be estimated, so that an expected time of harvest can be predicted from the date of sowing, however the actual date could vary by weeks.

Donor plants are staggered in their sowing, so that the intensive work load does not fall within a very small window. As androgenic process rely on a critical cell stage for success, the room for error across the whole protocol is very small, particularly at the point of harvest, where the optimum harvest time can fall on a single day. If the donor plant culture is very successful there may be too much material to process. In all the experiments conducted in this project, there is a general bias to select healthy looking panicles over panicles which are pale/yellow or small and weedy in appearance however no strict phenotypic measurements are used to gauge choosing one panicle over another. The key measurement was always the microspore stage. However when the differences between panicles are less obvious other phenotypic traits might be useful when selecting which panicles would be more likely to give a high response, and which to discard. To see if phenotypic traits of the harvested panicles from donor plants can be used as an accurate guide for panicle selection, phenotypic measurements were taken from panicles being used in anther culture experiments. Four measurements were chosen; length of the panicle inflorescence, number of florets on the inflorescence, length from the flag leaf to the second leaf, and length from the second leaf to the third leaf. The measurements of the inflorescence length and the number of florets on the inflorescence could not be measured until the panicle had been dissected prior to sterilization, so all measurements were made just prior to use for anther culture. Phenotypic data was taken from two experiments, which were investigating the effects of different compounds on anther culture. The experiments are covered in **section 2.3.2.4.** and **section 2.3.2.7.** The experiment in **section 2.3.2.7.** investigated the effects of arabinogalactan, gum Arabic and ovary culture on anther culture of the genotype Assiniboia. The second experiment (**Section 2.3.2.4.**) investigated the effects of glutamine, casein hydrolysate, ascorbic acid and pyridoxal phosphate on anther culture of the genotypes Assiniboia, Firth, N14.1507 and N15.14.08. As the anther culture response was very poor for the genotypes Firth,

N14.1507 and N14.15.08 only data from the genotype Assiniboia was analyzed. The null hypothesis was that there is no correlation between the number of responding anthers and the phenotypic traits measured. As many harvested panicles did not have an intact third leaf this measurement was not analysed. Each trait was compared to the number of responding anthers using the Pearson correlation coefficient, conducted in R Studio with the package 'mix'. The first experiment compiled the results from all treatments together, and the second experiment analysed each treatment individually.

The results from the Pearson correlation coefficient of the first experiment are shown in **table 2.25**. The correlation coefficient is comprised between 1 and -1. -1 indicates a strong negative correlation, 1 indicates a strong positive correlation. A moderate correlation is considered to exist when the r correlation index is over 0.5, and a strong correlation over 0.7. The only strong correlation seen here is between the length of the panicle and the distance from the flag leaf to the second leaf, with an r value over 0.7. The p value of the correlation is 0.000, which is ≤ 0.05 giving strong evidence against the null hypothesis that there is no correlation. There is a weak correlation between the number of flowers and the number of anthers responding, r correlation index of 0.421. The supporting p value of this correlation is 0.007. This is ≤ 0.05 which gives strong support for this weak correlation.

Figures 2.27. and **2.28**. show the scatter plots of the correlations. Both show weak, linear and positive association between the variables**. Figure 2.27.** shows that the data points are generally close to the line of best fit. There are no major outliers. **Figure 2.28.** shows a weaker fit. In the second analysis, each treatment was analysed individually, so that there were only ten replicates for each treatment; however different treatment effects could not then influence the correlations between the traits measured.

r - correlation index					
	No. responding anthers	Flag to second leaf	second to third leaf	number of florets	Panicle length
No. responding anthers	1.000	-0.012	-0.184	0.421	0.224
Flag to second leaf	-0.012	1.000	-0.132	0.098	0.756
second to third leaf	-0.184	-0.132	1.000	0.058	-0.087
no. of florets	0.421	0.098	0.058	1.000	0.397
Panicle length	0.224	0.756	-0.087	0.397	1.000
P - value of correlation					
	No. responding anthers	Flag to second leaf	second to third leaf	number of florets	Panicle length
No. responding anthers	NA	0.941	0.348	0.007	0.165
Flag to second leaf	0.941	NA	0.503	0.546	0.000
second to third leaf	0.348	0.503	NA	0.769	0.661
no. of florets	0.007	0.546	0.769	NA	0.011
Panicle length	0.165	0.000	0.661	0.011	NA

Table 2.25. *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination for a combined anther culture experiment on Assiniboia with a number of medium additives.*

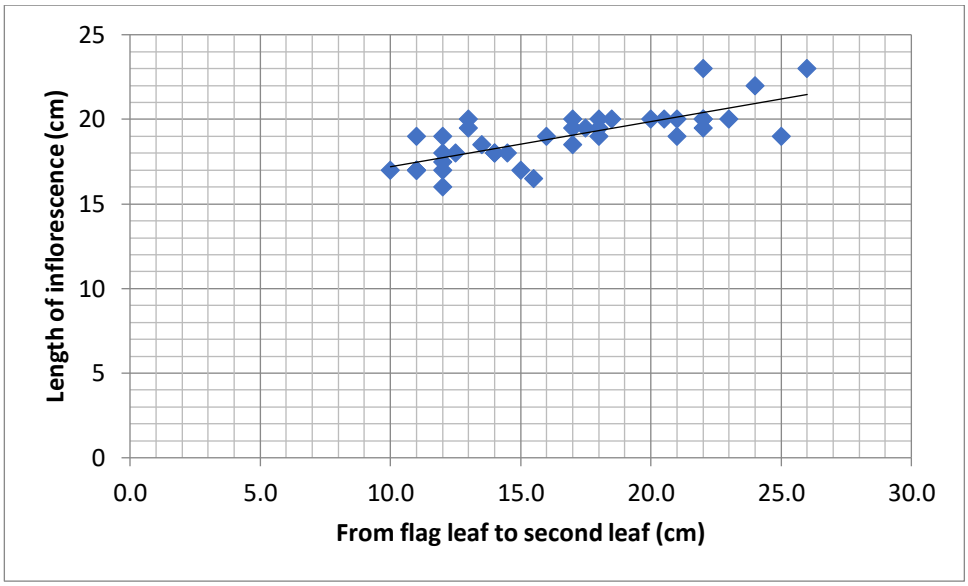


Figure 2.27. *A scatter plot showing a weak, linear and positive association between the variables; length of inflorescence and flag leaf to second leaf (cm).*

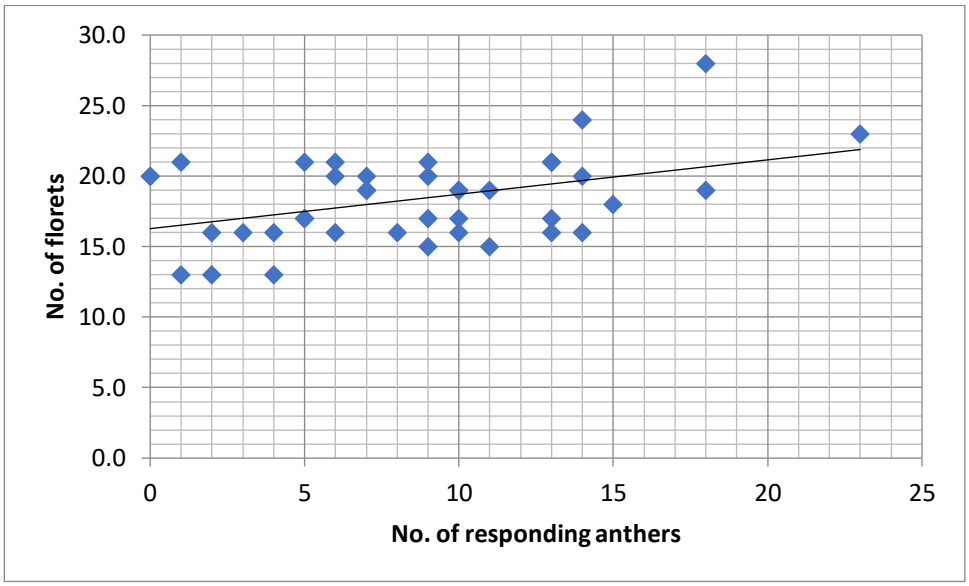


Figure 2.28. *A scatter plot showing a weak, linear and positive association between the variables; no. of florets and no. of responding anthers.*

Treatment one - control				
P - value of correlation				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	NA	0.0494	0.6557	0.0634
Flag leaf to 2nd leaf	0.0494	NA	0.4379	0.2689
Floret no.	0.6557	0.4379	NA	0.0859
Length of panicle	0.0634	0.2689	0.0859	NA
r - correlation index				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	1.0000	-0.6332	0.1616	-0.6057
Flag leaf to 2nd leaf	-0.6332	1.0000	-0.2774	0.3873
Floret no.	0.1616	-0.2774	1.0000	0.5692
Length of panicle	-0.6057	0.3873	0.5692	1.0000

Table 2.26. *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination of treatment one of an anther culture experiment on the genotype Assiniboia.*

Table 2.26. shows the Pearson correlation coefficient results from the first treatment, the control. The only correlation with a p value < 0.05 is the correlation between the flag leaf to second leaf measurement and the total callus response (p value 0.0494). There is a moderate, linear negative correlation between them (r correlation index -0.6332). There were two other correlations which were close to a significant p value; the length of panicle to number of responding anthers, and the length of panicle to number of florets.

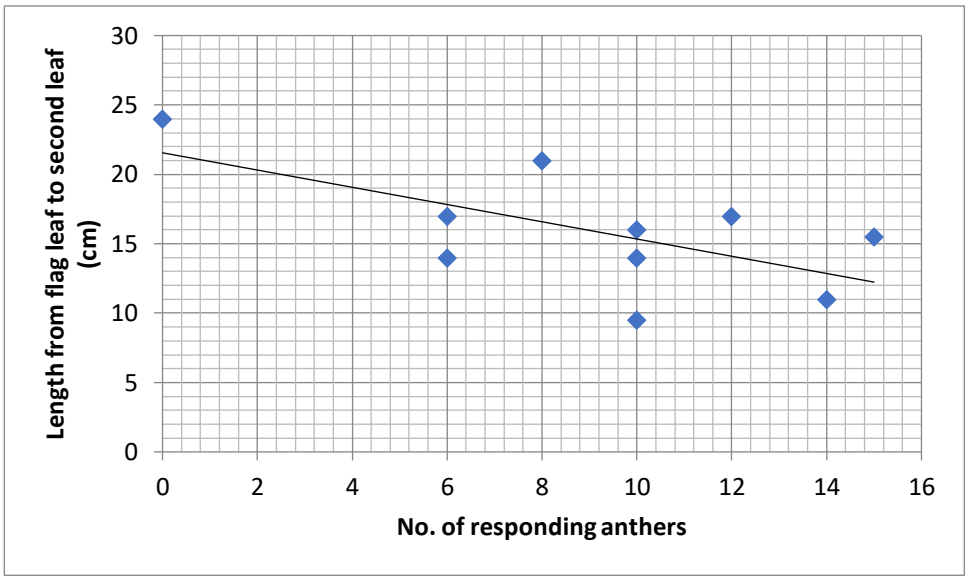


Figure 2.29. *A scatter plot showing a moderate, linear and negative correlation between the length from flag leaf to the second leaf and the no. of responding anthers.*

In **Figure 2.29.** the negative correlation between the length between the flag leaf and the second leaf with the number of responding anthers can be seen. The majority of the data points are not close to the line of best fit. One data point which stands out is the zero anther response, from a panicle which had the longest length between the flag leaf and the second leaf. The panicle which had the shortest distance between the flag leaf and second leaf did not have the highest number of anthers responding.

Treatment two - glutamine				
P - value of correlation				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	NA	0.5302	0.6301	0.0616
Flag leaf to 2nd leaf	0.5302	NA	0.7204	0.5724
Floret no.	0.6301	0.7204	NA	0.7755
Length of panicle	0.0616	0.5724	0.7755	NA
r - correlation index				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	1.0000	-0.2421	0.1869	-0.6432
Flag leaf to 2nd leaf	-0.2421	1.0000	0.1395	0.2184
Floret no.	0.1869	0.1395	1.0000	0.1114
Length of panicle	-0.6432	0.2184	0.1114	1.0000

Table 2.27 . *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination of treatment two of an anther culture experiment on the genotype Assiniboia.*

In **Table 2.27.** the results from the second treatment with additional glutamine do not have any significant correlations. One correlation which is almost significant is that between the length of the panicle and the number of responding anthers (p value 0.0616). The r correlation index is moderate (-0.6432), and is negative.

Treatment three - putrescine				
P - value of correlation				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	NA	0.0313	0.7832	0.1120
Flag leaf to 2nd leaf	0.0313	NA	0.3685	0.3475
Floret no.	0.7832	0.3685	NA	0.1495
Length of panicle	0.1120	0.3475	0.1495	NA
r - correlation index				
	Total callus response	Flag leaf to 2nd leaf	Floret no.	Length of panicle
Total callus response	1.0000	-0.6777	0.1001	0.5338
Flag leaf to 2nd leaf	-0.6777	1.0000	-0.3193	-0.3328
Floret no.	0.1001	-0.3193	1.0000	0.4911
Length of panicle	0.5338	-0.3328	0.4911	1.0000

Table 2.28. *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination of treatment three of an anther culture experiment on the genotype Assiniboia.*

In **Table 2.28.** The results from the third treatment show there is one significant correlation, flag to second leaf correlated with the number of responding anthers. The r correlation index (-0.6777) is moderate and negative. **Figure 2.29.** shows a scatterplot of the correlation. The data point with the longest length between the flag leaf and the second leaf has the lowest number of responding anthers, and the data point with the shortest distance between the flag leaf to second leaf had the highest number of responding anthers.

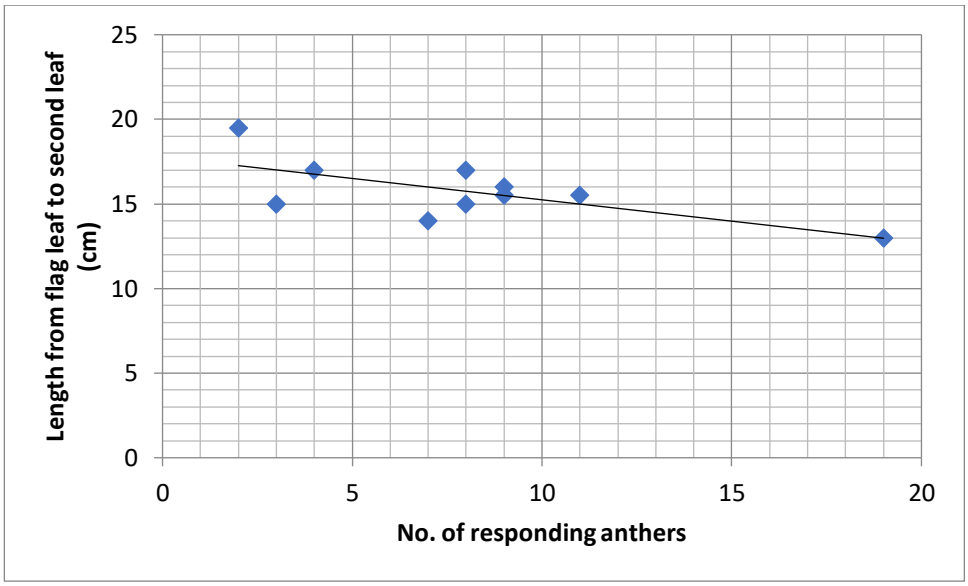


Figure 2.30. *A scatter plot showing a moderate, linear and negative correlation between the length from flag leaf to the second leaf and the no. of responding anthers.*

Treatment four - casein hydrolysate				
P - value of correlation				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	NA	0.0868	0.2852	0.0689
Flag leaf to 2nd leaf	0.0868	NA	0.2184	0.1479
Floret no.	0.2852	0.2184	NA	0.6754
Length of panicle	0.0689	0.1479	0.6754	NA
r - correlation index				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	1.0000	-0.5679	0.3753	-0.5962
Flag leaf to 2nd leaf	-0.5679	1.0000	-0.4270	0.4928
Floret no.	0.3753	-0.4270	1.0000	0.1519
Length of panicle	-0.5962	0.4928	0.1519	1.0000

Table 2.29. *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination of treatment four of an anther culture experiment on the genotype Assiniboia.*

In **Table 2.29.** the results from the Pearson correlation coefficient results shows that there are no significant correlations. There are two correlations which are close to the p value of 0.05; flag leaf to second leaf with number of responding anthers, and length of panicle with number of responding anthers.

Treatment five				
P - value of correlation				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	NA	0.6451	0.7040	0.8858
Flag leaf to 2nd leaf	0.6451	NA	0.9900	0.0023
Floret no.	0.7040	0.9900	NA	0.4021
Length of panicle	0.8858	0.0023	0.4021	NA
r - correlation index				
	No. responding anthers	Flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	1.0000	-0.1668	0.1379	-0.0524
Flag leaf to 2nd leaf	-0.1668	1.0000	0.0046	0.8406
Floret no.	0.1379	0.0046	1.0000	0.2986
Length of panicle	-0.0524	0.8406	0.2986	1.0000

Table 2.30. *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination of treatment five of an anther culture experiment on the genotype Assiniboia.*

In **Table 2.30.** the results from the Pearson correlation coefficient results shows that there is one significant correlation, length of panicle with flag leaf to second leaf distance. However, there are no significant correlations with the number of responding anthers, and none are close to being significant.

Treatment six				
P - value of correlation				
	No. responding anthers	flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	NA	0.4084	0.7684	0.6774
flag leaf to 2nd leaf	0.4084	NA	0.7439	0.7239
Floret no.	0.7684	0.7439	NA	0.2701
Length of panicle	0.6774	0.7239	0.2701	NA
r - correlation index				
	No. responding anthers	flag leaf to 2nd leaf	Floret no.	Length of panicle
No. responding anthers	1.0000	0.2948	-0.1071	-0.1508
flag leaf to 2nd leaf	0.2948	1.0000	0.1187	-0.1283
Floret no.	-0.1071	0.1187	1.0000	0.3864
Length of panicle	-0.1508	-0.1283	0.3864	1.0000

Table 2.31. *Pearson correlation coefficient results showing the r correlation index and corresponding p – value of correlation for each combination of treatment six of an anther culture experiment on the genotype Assiniboia.*

Treatment six (**table 2.31.**) did not have any correlations between number of responding anthers and any phenotypic measurements.

2.3.2.10. Measuring the callus induction and plant regeneration period

The vast majority of regenerants originate from callus induced in week four

When conducting anther culture experiments, all callus from responding anthers is typically moved when the majority of calli are at a suitable size, usually after five to six weeks, rather than moving each callus as it reaches a threshold size. This is to reduce the labour intensity of multiple 'harvests' and the risks of contamination that come with it. To see if the incubation period is important in the callus growth in responding anthers and of successful regeneration, a number of anther culture experiments were conducted. In these experiments once the first calli were visible, they were transferred when ready to regeneration medium on a weekly basis. The week each callus was transferred was recorded and linked to regeneration rates.

The experiments analyzed were anther cultures with the genotypes Assiniboia, Lisbeth and NORD 16 1421. The experiments tested the induction medium additives of copper, glutamine, and proline. It also tested the effect of substituting SeaPlaque agarose with gelrite, and the addition of copper in the pretreatment water. The effects of these treatments are analyzed in other sections. The treatments are shown in **table 34.** NORD 16 1421 produced only two calli, so was not analysed.

Genotype	Treatment	Anthers cultured	No. responding anthers	anthers /100 anthers +SE	Regenerants	no. of regenerants /100 anthers +SE	albino	green
Assiniboia	Control	420	15	3.57 ± 1.19	4	0.95 ± 0.53	1	3
Assiniboia	Glut+pro	420	17	4.05 ± 1.23	8	1.90 ± 0.69	7	1
Assiniboia	gelrite	420	19	4.52 ± 1.61	3	0.71 ± 0.51	2	1
Assiniboia	Cu2+ media	420	17	4.05 ± 1.23	4	0.95 ± 0.63	2	2
Assiniboia	Cu2+both	420	29	6.90 ± 2.05	5	1.19 ± 0.64	1	4
Lisbeth	Control	420	5	1.19 ± 0.81	1	0.24 ± 0.24	0	1
Lisbeth	Glut+pro	420	8	1.90 ± 0.59	0	0.00 ± 0.00	0	0
Lisbeth	gelrite	420	6	1.43 ± 0.81	0	0.00 ± 0.00	0	0
Lisbeth	Cu2+ media	420	7	1.67 ± 0.94	0	0.00 ± 0.00	0	0
Lisbeth	-	-	-	-	-	-	-	-
NORD 16-1421	Control	420	2	0.48 ± 0.32	0	0.00 ± 0.00	0	0
NORD 16-1421	Glut+pro	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
NORD 16-1421	gelrite	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
NORD 16-1421	Cu2+ media	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
NORD 16-1421	Cu2+both	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0

Table 2.32. *The results from anther culture experiments. Control= MDHM + glutamine (750 mg/L), proline (500 mg/L).Glut+pro = MDHM + glutamine (100 mg/L), proline (750 mg/L). Gelrite = MDHM with gelrite (3 g/L). Cu2+ media = MDHM + 2.475 mg/L. Cu2+both = MDHM + 2.475 mg/L, 2.5 mg/L Cu2+ pre-treatment solution.*

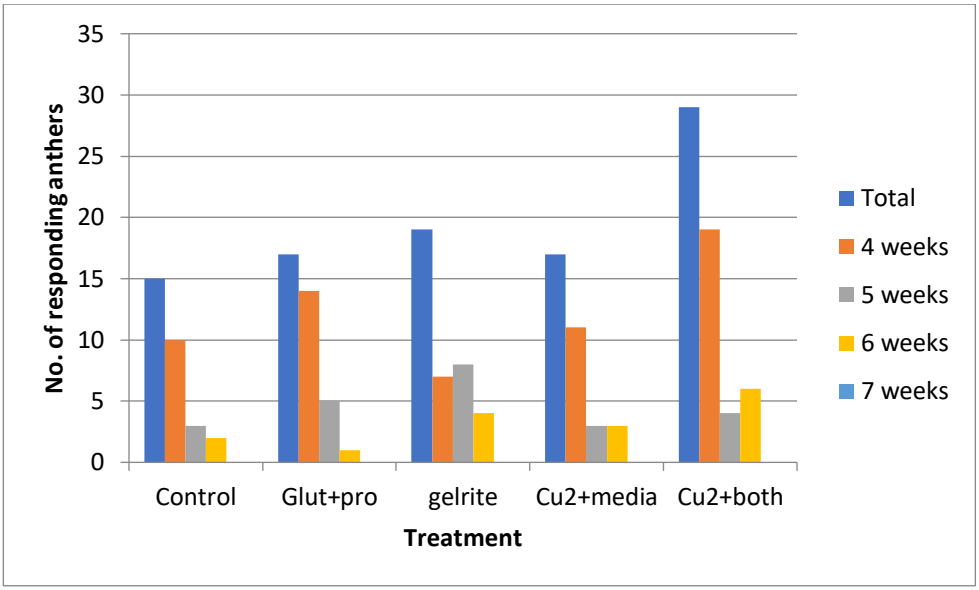


Figure 2.31. *The number of responding anthers of Assiniboia for different treatments and when the calli was ready to be transferred to regeneration medium.*

The first calli started to emerge from the anthers at week four (**Fig 2.31.**). In four of the five treatments, most of anthers responded in week four. The gelrite treatment showed a more even spread across weeks four, five and six. No anthers responded after week six.

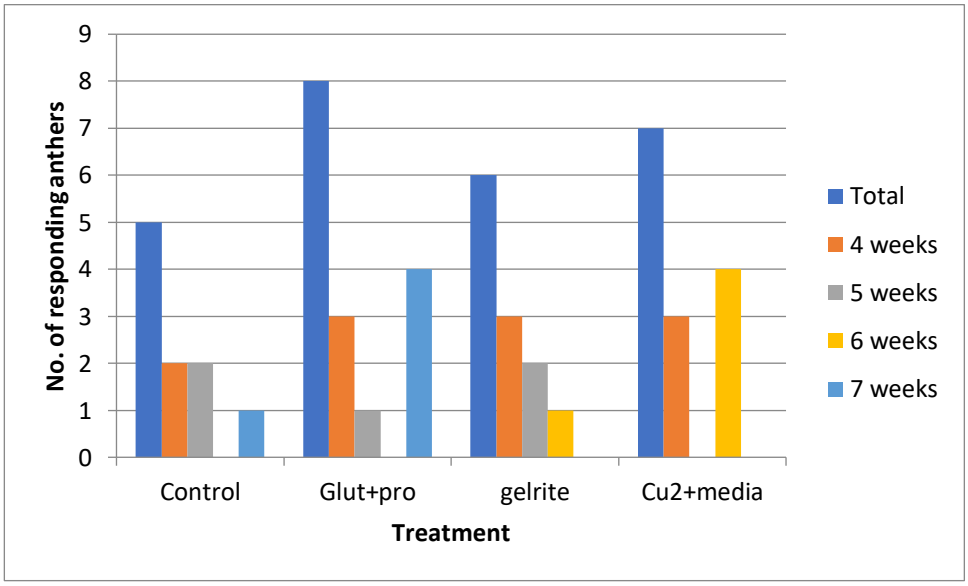


Figure 2.32. *The number of responding anthers of Lisbeth for different treatments and when the calli was ready to be transferred to regeneration medium.*

The results from the anther culture of Lisbeth are more varied and differ significantly from Assiniboia in terms of anther response time (**Figures 2.32, 2.33**). Only one treatment with gelrite shows the pattern seen in four out of five of the treatments with Assiniboia where week four has the highest number of responding anthers, and this number decreases through weeks five and six. Interestingly the gelrite treatment with Assiniboia does not follow this pattern. The other three treatments with Lisbeth also show an inconsistent pattern. For three of the four treatments, a week where no calli were ready to move occurred between weeks where suitable calli were seen. This is particularly apparent with the treatments glut+pro and Cu2+ media, where the final week generated the largest number of responding anthers.

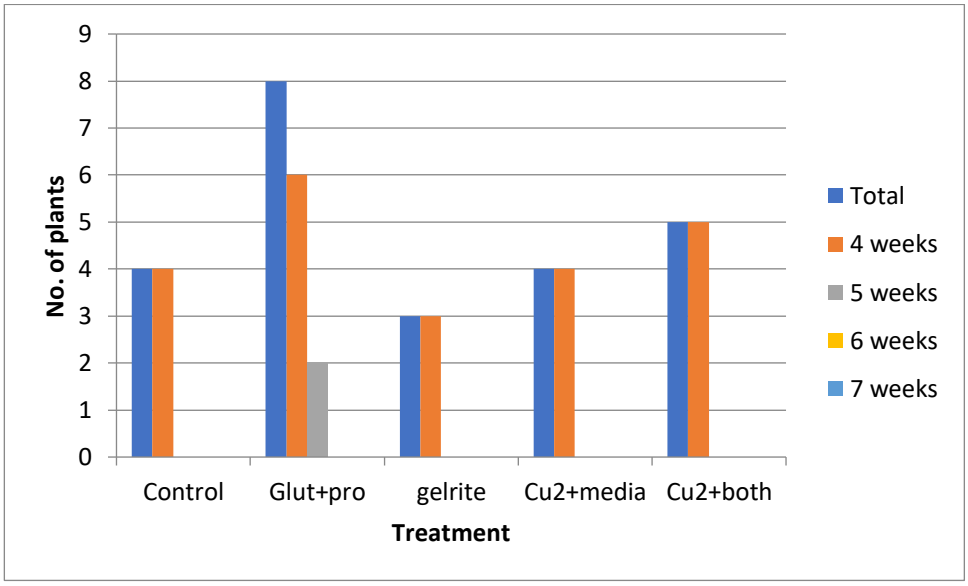


Figure 2.33. *The number of plants regenerating from Assiniboia on different treatments. The week represents the time the callus was moved, that the plant regenerated from.*

Only one plant regenerated from Lisbeth (**table 2.23**). This plant had regenerated from a callus from an anther in week four (not shown). A total of 24 plants were regenerated from Assiniboia. **Fig 2.33.** shows that all but two of the plants regenerated from callus that was moved in the fourth week. The exceptions were two albino plants which regenerated from callus cultured on glut+pro and moved in week five.

2.3.2.11. The effect of panicle development on anther culture

The sample area on the panicle is key to maximising the plant regeneration rate

The microspore stage used is very important for successful androgenesis. When anthers are cultured *in vitro* the switch from gametophytic to sporophytic pathway occurs between the uninucleate and binucleate stages (Smykal & Pechan, 2000).

The oat floral morphology and the sequence of microspore maturity in the oat panicle make it impossible to identify microspores at the correct stage without destructive sampling. Compared to wheat and barley whose spikes develop in a linear order the panicle of oat is more complex. As shown in **figure 2.34.** the microspore stage varies considerably along the panicle so it is important either to sample anthers from a large area along the panicle to ensure at least some of the microspores harvested are at the right stage, or to conduct more sampling and be more selective about which anthers are cultured. While anther squashes to observe microspore stage can be linked to phenotypic traits to aid in harvesting (**see section 2.2.6.2.**) this indirect approach may not be always accurate.

When anther culture plates are set up, the florets are harvested in order from bottom to top of the sample area. The positions of responding anthers were recorded, and in later experiments the same was recorded for the plants regenerated. In this way trends in callus induction rates along the panicle can be observed. It can also be used to see if the sample area covers the part of the panicle with microspores at the correct stage. It would be expected to see a bell curve like shape in the number of responding anthers along a panicle, with the most productive area in the centre of the harvest area.

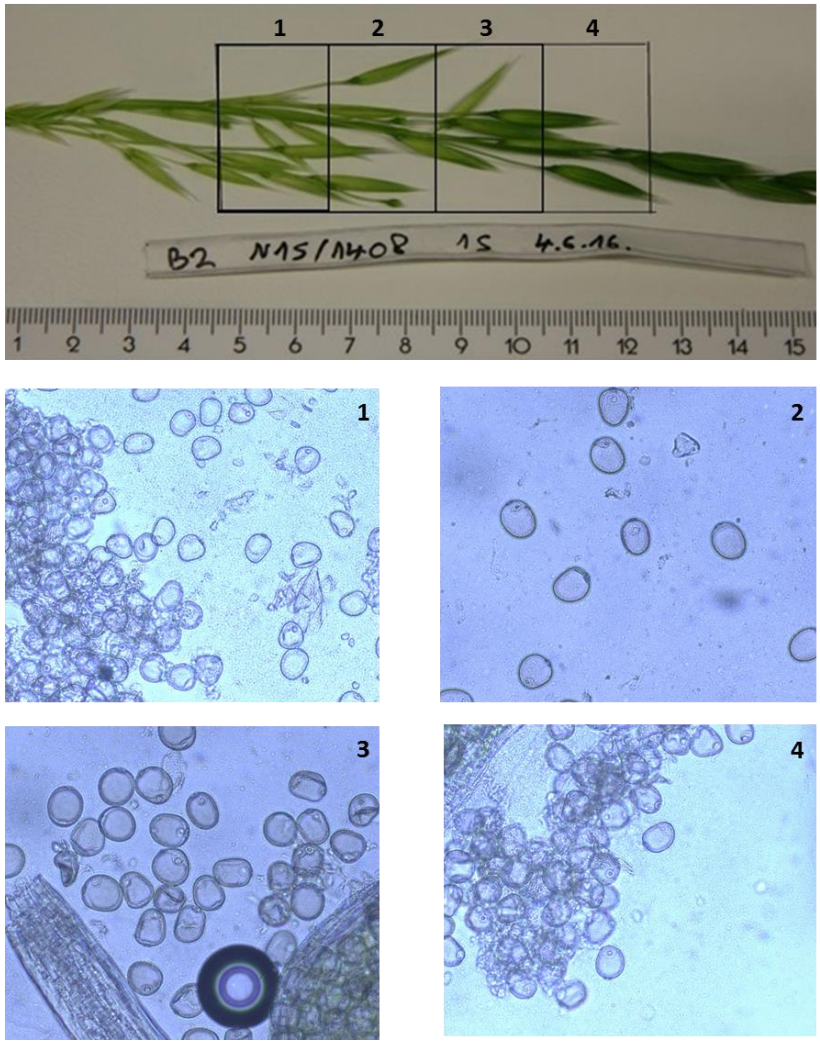


Figure 2.34. *An annotated image of an oat panicle after it has been removed from its sheath. Based on anther squashes of previous panicles, a harvest area is decided on for anther culture use, shown in the black rectangle. The microscope images show the stages of microspores taken from anthers in the areas of the panicle above (one, two, three and four).*

Several experiments were selected for analysis which had been conducted in this project and have been analysed earlier in the chapter. Experiments with Assiniboia were chosen as they had the best anther response and plant regeneration. The first experiment analysed was conducted on Assiniboia looking at the effects of ovary co-culture and the medium additives GUM at 200 mg/L. Four different treatment combinations were tested,

with ten replicates for each. These experiments were conducted in the same period with the same donor plants so can be compared together here.

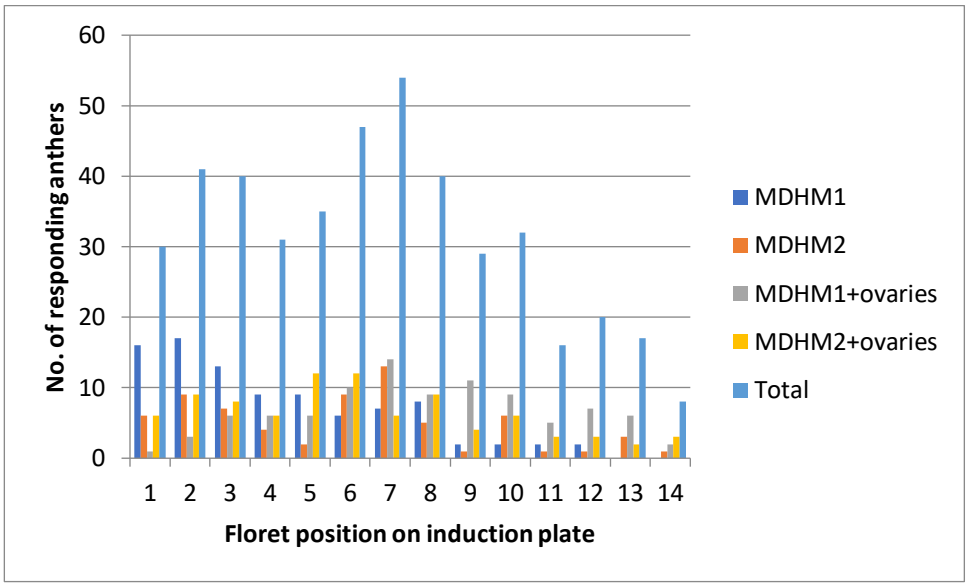


Figure 2.35. *The number of responding anthers at each floret position of four different treatment combinations and a combined total of all treatments.*

The results from the first analysed experiment show a varied response across the induction plate (**figure 2.35.**). The MDHM1 treatment has the most responding anthers at positions one and two. Response is lower for positions three to eight are less, and then low or absent for positions nine to fourteen. MDHM2 shows a different pattern, with a spread of responses that have marked dips at position five and nine. MDHM1+ovaries shows the most bell like shape curve with the highest number of responding anthers at positions six to ten. MDHM2+ovaries show a similar pattern to MHDM+ovaries but the values are generally lower across all positions. The total response for all treatments shows a loose bell shaped response, with the highest values at positions six and seven, and a gradual tail off in number of responding anthers to position 14. No positional data for the regenerated plants was taken so this cannot be analysed.

The second experiment tested the effects of additional compounds to the MDHM medium (**figure 2.36.**). Four treatments were tested with ten replicates each; MDHM with the addition of glutamine and casein hydrolysate (Glut/Cas), MDHM with the addition of proline, and MDHM with a higher concentration of 2,4-D, with a fourth treatment of MDHM only as a control.

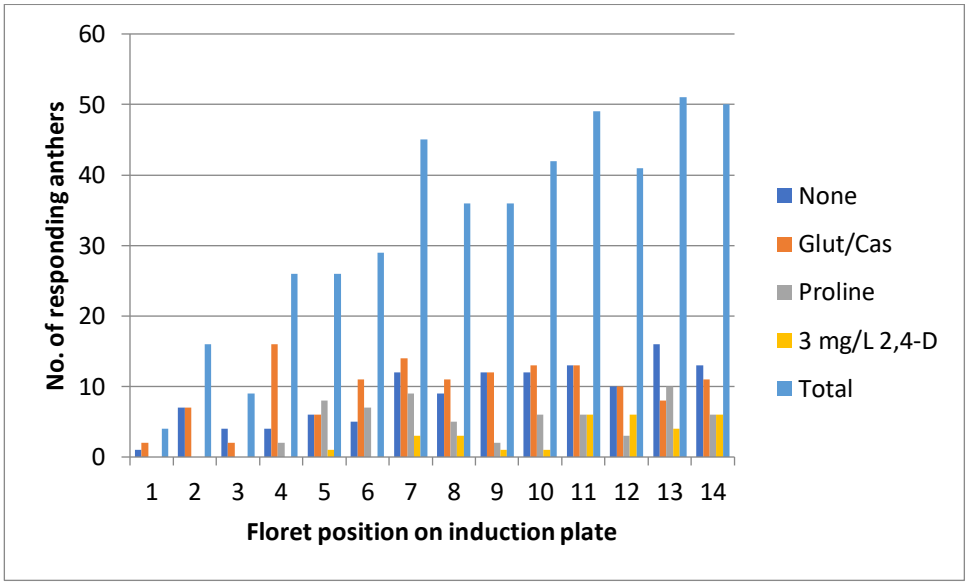


Figure 2.36. *The number. of responding anthers at each floret position of four different treatment combinations and a combined total of all treatments.*

The control treatment (none) shows a larger number of responding anthers in the top half of the plate, with the exception of the higher value at position two. The Glut/Cas treatment also shows this pattern; however the highest responding position for that treatment is position four, in the lower half of the plate. The treatment with proline shows a zero anther response in the first three positions. Positions five through to fourteen show larger number of responses, intermixed with low responses at position nine and twelve. The treatment with 3 mg/L 2,4-D has a very low response across all positions, but particularly from position one through to ten. Positions eleven to fourteen

show an increased response. The combined response as the total shows a trend that the number of responding anthers increases from a low response at position one and two, which then increases to the highest response at position thirteen.

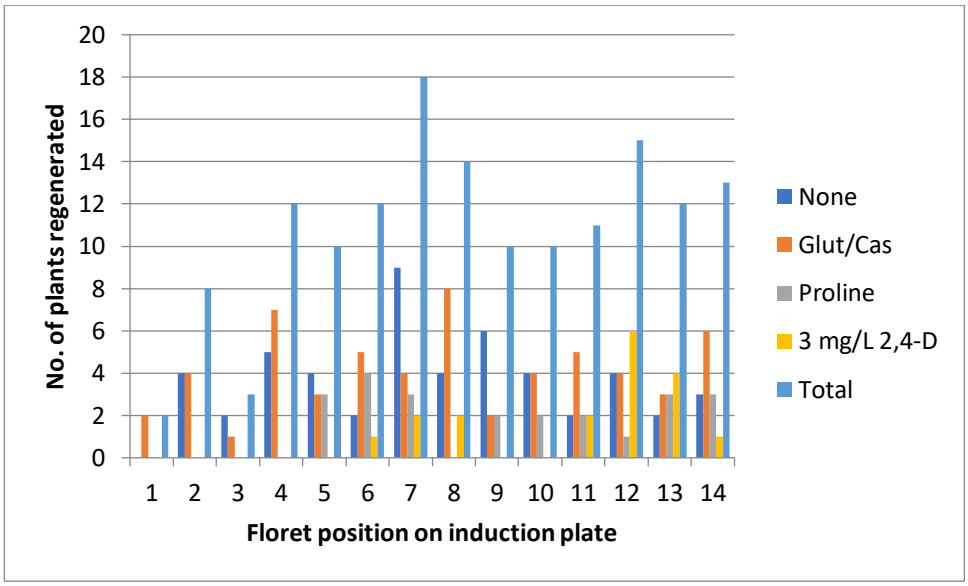


Figure 2.37. *The number of regenerated plants at each floret position of four different treatment combinations and a combined total of all treatments.*

Plant regeneration association with floret position does not correlate exactly with anther response (**figure 2.37.**). The control MDHM treatment with no additions shows a fairly even spread of regeneration across the positions. The outlier positions are one, where no plants regenerated, and seven, where nine plants regenerated. MDHM with Glut/Cas shows a more mixed response, with 'peaks' of high numbers of regenerated scattered infrequently. MDHM with proline and MDHM with 3mg /L 2,4-D showed a similar pattern. Both had zero regeneration in the first positions, as there were no responding anthers with callus present. The other positions show a range of responses, with positions with zero plants regeneration intermixed. When the treatments are combined, the total plant regeneration response is also mixed. More plants are regenerated from the mid and

upper sections, however there is a dip from position seven (the highest response) in positions nine, ten and eleven.

The third experiment looked at the effects of copper in the induction medium and pre-treatment solution (Cu2+media), the use of gelrite as a replacement to SeaPlaque agarose as the gelling agent, and the addition of glutamine and proline on anther culture (Cu2+both). These experiments have been analysed in other sections. As with the previous experiments, ten replicates for each treatment were cultured.

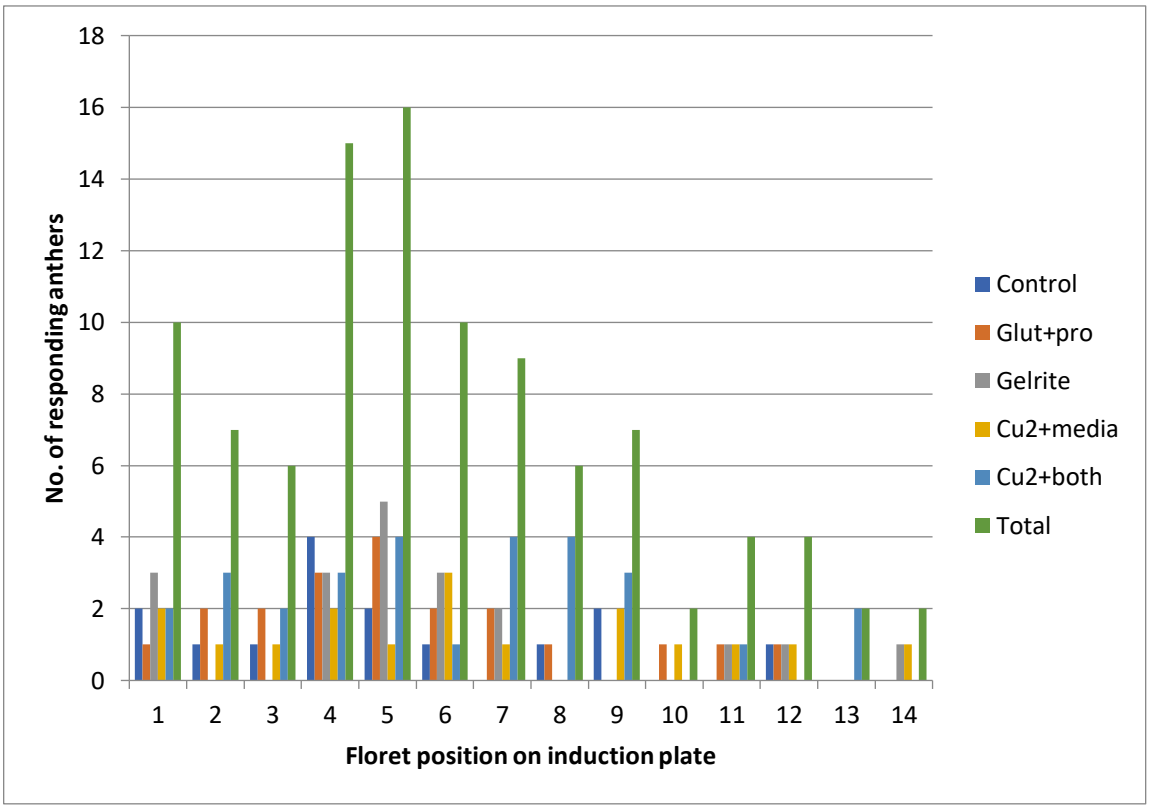


Figure 2.38. *The number of responding anthers at each floret position of four different treatment combinations and a combined total of all treatments.*

Overall the number of responding anthers in this experiment is less than those previously analysed (**figure 2.38.**), however the response is high enough to see some patterns. In the control treatment, the responding anthers were found in the positions from one to nine, with just one anther responding in positions ten to fourteen. The treatment with gelrite

had most responding anthers in the positions four to seven, but position one also stood out with a comparatively high response. Both copper treatments showed most anther responses in the positions one to nine. Across the experiments, responsive anthers are generally found in the lower half of the anther cultures, with the largest number of responsive anthers found in positions four and five. The anther response tails off in the second half of the plate.

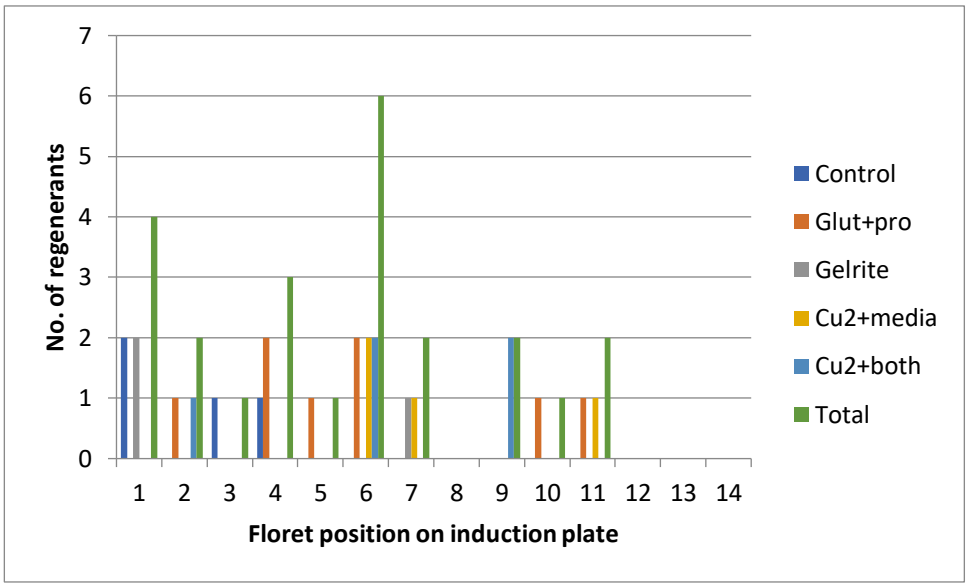


Figure 2.39. *The number of regenerated plants at each floret position of four different treatment combinations and a combined total of all treatments.*

The small number of regenerants were not sufficient to compare patterns in a positional effect for plant regeneration (**figure 2.39.**). Plants regenerated from the control came only from callus formed in positions one, three and four. The glut+pro treatment regenerants were more dispersed, with intervening positions that did not generate plants. The gelrite treatment was similar to the glut+pro. The Cu2+media treatment had regenerants in the middle positions six, seven and eleven, while the treatment Cu2+both had regenerants at two, six and nine.

2.4. Discussion

This section discusses the results of the anther culture experiments conducted highlighting the key findings and how they compare to existing literature. Limitations and problems are also highlighted and discussed as well as perspectives for future research and experimentation.

2.4.1. The effect of anther density on callus induction

The anther density (number of anthers per volume of media) during culture is known to be a key factor for successful anther culture (Xu & Sunderland, 1982). Identifying an optimal and feasible concentration of oat anthers per mL of medium was therefore a priority for this project. This study looked at two densities of anthers, using 15 replicates of 33 anthers and 42 anthers, equivalent to 11 anthers per mL and 14 anthers per mL media respectively. The results showed that a density of 14 anthers per ml increased the number of responding anthers per 100 anthers by seven, and that twice as many plants were regenerated than compared with anthers cultured at a density of 11 per ml.

The optimal density of anthers varies even between genotype (Xu & Sunderland, 1982). In rice, higher densities were initially reported to promote callus induction (Fouletier, 1974) while in more recent studies it was found that anther densities greater than 3 per ml in rice appeared have a detrimental effect on callus induction (Kinoshita *et al.,* 2005). Microspore density is a crucial factor for IMC, including in oat (Ferrie *et al.,* 2013). This may reflect the lack of the anther and its role in supporting pollen embryo development, as anthers have been used to 'nurse' androgenic development of isolated pollen (Sharp *et al.,* 1972). Therefore the need for a high concentration of microspores may be more important for isolating enough microspores at the correct stage which survive the isolation procedure, than there being some co-culture effect between microspores. Studies have shown that the role of the anther wall 'factors' can be substituted with the addition of compounds to the medium such as glutamine, serine and *m*-inositol in IMC

culture of *Datura* while glutamine alone was helpful for the pollen culture of tobacco (Wernicke & Kohlenbach, 1977).

Here, only two different anther densities were tested, as the plating density of anthers was not thought to be a major factor in successful anther culture. However it was important to have a standard set up for all anther culture experiments to ensure repeatability. The approach was to culture panicles individually so that each anther culture was derived from a single panicle, rather than pool anthers together, with the intention of analysing variability within treatments. The total number of florets on a panicle is limited, according to genotype and culture conditions, and the non-uniform development of the panicle may further reduce the number of useable florets. With a fixed volume of media of 3ml, 14 florets per plate gives a density of 14 anthers per ml, which was found to provide good callus response., however if other aspects of the protocol are improved to the point of limitation, it may well be important to return to the aspect of anther density in the future.

2.4.2. The effect of mixing anthers from different panicles and genotypes

Optimisation of anther culture and IMC has largely focused on factors such as genotype, donor plant growth, pre-treatment, and culture medium. Pre-conditioning medium has also been tested, and in barley it was found that pre-conditioning anther medium with anthers and ovaries of cv. Sabarlis increased the yield of anther cultures of cv. Sabarlis (Lane *et al.,* 1981). These authors found that anthers from another genotype of barley or from other species including rye, oats, wheat, maize were less effective. There has been little subsequent research into the use of inter-culture in anther culture, as the compounds secreted by highly responsive genotypes have not been identified and added to the medium directly.

The experiments in this section tested a variety of different combinations mixing panicles from the same genotype, as well as using the responsive genotype Assiniboia, as a 'feeder' for recalcitrant genotypes by inter-culture.

The pair and four plate experiments with Assiniboia were set up to look for patterns in anther culture response. If the secretion of beneficial substances such as AGP into the media by embryogenic microspores occurred, it might be expected to see pairs reacting in the same way, with consistently high or low numbers of calli. **Table 2.14.** shows that about half of the cultures show pairs of panicles responding the same way, and the other half not. This outcome was mirrored in the regeneration of plants. Across pairs of panicles, there was significant variation in the number of anthers responding and in the number of plants regenerating. As all pairs were prepared with the same protocol, using the same pre-treatment and media, the differences in response could be due to different vigour of the panicles from slight variations in harvest time, panicle quality or floret selection during plating. Of all the panicles used, none had a zero anther response, with the lowest frequency being 14 % (**table 2.14.**) so it is unlikely that this was a major issue. The difference in panicle response was much more pronounced in the four plate experiment (**table 2.15.**) with large differences in the number of responding anthers between panicles cultured on the same plates. The regeneration rate was lower for all panicles though, including those with high anther responses. As with the paired experiments, this would suggest that it is the panicle quality that is causing the differences in response within the culture. In the experiments where inter-culture has been tested the medium has been liquid, however in these experiments SeaPlaque agarose had been used as a gelling agent. It is possible that the nature of the medium reduces the ability for the compounds to diffuse from the anther of a positively responding anther to one which does not.

In future work, using a liquid medium might prove more fruitful, as well as testing long pre-conditioning periods, as here the only pre-treatment tested was Assiniboia anthers being plated to the medium seven days prior to plating Firth anthers. Once the IMC protocol for oat has improved it might also be beneficial to pre-condition IMC medium with anthers of Assiniboia to improve the embryogenic response of recalcitrant genotypes.

2.4.3. The effect of different additives to the anther culture of oat

The composition of the induction medium is a very important factor for the induction of microspore embryogenesis. There is no universal induction medium for all plants which makes medium composition an important target for optimisation. Various additives which have positively affected microspore embryogenesis in other species were tested, and this section discusses the results of the experiments presented in **section 2.3.**

Arabinogalactan proteins (AGPs), present in gum arabic (GAM), have been identified as important proteins for plant growth and development, cell division and programmed cell death (Seifert & Roberts, 2007) and are secreted from microspores during culture (Borderies *et al.,* 2004). They are often included in microspore culture medium for wheat (Würschum *et al.,* 2012; Santra *et al.,* 2012) but there are no reports to date of their effect on oat.

Two different experiments were conducted with GAM. The first tested GAM at 50 mg/L against a control without (**section 2.3.2.3.**), which showed no positive improvement. The number of regenerants was in fact far lower, which would suggest that the additives had a negative effect on anther culture. This is a surprising result compared to the many reported cases. The control treatment produced three times as many plants, though many were albino, in comparison to the treatment with GAM, where the ratio of green to albino plants regenerated was far better, in agreement with the results of Letarte (*et al.,* 2006) which also saw an improvement in the rate of green plants regenerated.

In the second experiment the concentration of GAM was increased to 200 mg/L and was also tested with ovaries. This concentration was double the largest concentration used by Letarte (*et al.,* 2006) so an effect should be even more clear. As with the first experiment, the addition of AGP and GAM even at the high concentration did not have a positive effect on callus induction, or on plant regeneration. The presence of ovaries or GAM did not significantly improve the anther response. The difference in culture technique might be the reason why the results here conducted with anther culture differ from Letarte *et al.,* (2006) who used an IMC technique. The anther wall here might be acting as a barrier

to GAM. AGPs are secreted from various parts of the plant, so the GAM in the medium plus those secreted by the ovaries (Letarte *et al.,* 2006) would be expected to be of a high enough concentration to show a positive impact. AGPs are a large and diverse family (Knox, 1995) so one reason for the difference in response might have been due to specific differences in AGP composition. Research has shown that the AGPs present in wheat and oat are both Wattle-blossom type AGPs, (Göllner *et al.,* 2011; Göllner *et al.,* 2010) and differences within that group of AGPs are expected to be subtle. Returning to these proteins could be useful once an IMC protocol for oat is available.

Putrescine was seen as a good candidate additive as it had been beneficial in wheat anther culture (Amina Redha & Suleman, 2011). It was already found to improve somatic embryogenesis response in oat (Kelley *et al.,* 2002). Two separate experiments tested putrescine used at the concentration of Kelley *et al.,* (2002). In both instances the anther response rate underperformed compared to the control treatment although the green/albino regenerant ratio was better which might be related to the positive effect putrescine has on embryogenetic development. This effect does not appear to justify inclusion of putrescine in future medium.

Use of organic forms of nitrogen such as glutamine and casein hydrolysate in preference to inorganic nitrogen, usually provided as ammonium nitrate, has been found to important for DH efficiency. The use of glutamine was a major breakthrough for improving barley DH production (Olsen, 1987) and is routinely used at 500mg/L or higher (Maluszynski *et al.,* 2003; Li & Devaux 2001). It has also been found to be highly effective at promoting callus induction and plant regeneration of rice *in vitro* (Pawar *et al.,* 2015). A positive effect has been reported in oat (Kiviharju *et al.,* 2005). Casein hydrolysate is an amino acid rich protein derived from cow milk (Wang *et al.,* 2013) which has been used previously in oat anther culture (Elina Kiviharju & Pehu, 1998).

Glutamine and casein hydrolysate were first tested separately (**section 2.3.2.4**), both at 500 mg/L based on Li & Devaux, (2003) for casein hydrolysate and Pawar *et al.,* (2015) for glutamine. The results in **table 2.20.** show that the addition of glutamine increased both

anther response and regeneration in Assiniboia. This result agrees with published studies on the use of glutamine suggesting that it is a key component for culture growth and development and for callus regeneration (Pawar *et al.,* 2015; Olsen 1987). The impact of casein hydrolysate was not as large as for glutamine, as it did not improve the anther response rate, however it did contribute to more plants regenerated than the control with a 3:1 green/albino rate. Casein hydrolysate has been used to improve the regeneration *in in vitro* in many different plant species (Pandey & Tamta 2014; Ali *et al.,* 2012) and a similar result can be seen here with the oat genotype Assiniboia. The use of glutamine and casein hydrolysate did not overcome the recalcitrance of the genotypes Firth, N14/1507 and N14/1508 however.

In the second experiment using Assiniboia (**table 2.21.**) glutamine and casein hydrolysate were tested together, both at 500 mg/L. The results showed that the combination had a higher anther response rate than the control treatment for both anther response and plant regeneration, however, in this second experiment the albino plant regeneration number was higher than when the components were tested separately (**table 2.21.**). This difference might be due to the increased overall nitrogen availability resulting from the combination. As the second experiment had to be conducted at a different time of year to the first for logistical reasons, the role of environmental factors should also be considered. The initial experiments were carried out during the optimal growing season months of May-July with donor plants grown in greenhouse conditions at Saaten-Union Biotec GmbH, while the second were carried out in December-February, with plants grown at Aberystwyth University in a growth chamber. Previous work has described seasonal effects on embryogenesis. Conducted over a three year period in a controlled environment, scutella from immature embryos of barley cv. Salome showed an increased frequency of plant regeneration from January to March, peaking in in March and April and then followed by a strong decrease from May to December (Sharma *et al.,* 2005). Seasonal variation in regeneration rate has also been seen in barley IMC (Ritala *et al.,* 2001). It is possible that a similar seasonal effect underlies the differences in albinism rate seen here. Albinism frequency was reported to be reduced when plants originating from

anther cultures were regenerated in low light intensity conditions in the grass *Phleum pratense* (Guo *et al.,* 1999). In oat the opposite has been reported, although with genotype dependent specificity (Kiviharju *et al.,* 2005). When moving callus to regeneration medium in all experiments conducted, the regeneration plates were covered with several layers of tissue paper to reduce the shock of being subjected to light.

Ascorbic acid was another candidate additive for improving oat anther culture based on positive effects for tissue culture in a number of different species. It has been previously tested in oat at higher concentrations (200 mg/L, 400 mg/L) to act as an antioxidant, as oat anthers have a tendency to turn brown during culture. At those concentrations it was not found to have an effect (Kiviharju *et al.,* 2005), however with other species it is used at much lower concentrations, so it was applied here at 25 mg/L. The results from the anther culture with Assiniboia (**table 2.21.**) show that the effect on both anther response and plant regeneration was negligible.

Different vitamin B compounds have found to affect androgenesis in *Capsicum annuum* (Ozsan & Onus, 2017), but little work has been done to test the effects of different forms of specific vitamins. Pyridoxine, pyridoxal, and pyridoxamine are collectively called vitamin B6 (Chen & Xiong, 2005). Vitamin B6 is essential for all living organisms, and is added to tissue culture medium in the form of pyridoxine HCL. It has an important role in nitrogen metabolism (Colinas *et al.,* 2016), is required for root development, and has a role in osmotic and oxidative tolerance (Chen & Xiong, 2005). Pyridoxal phosphate is an active form of vitamin B6, and is an important organic cofactor for many enzymes (Percudani & Peracchi, 2003). The addition of pyridoxal phosphate did not have a significant effect on the number of anthers responding (**table 2.20.**), however there was an increase in the number of plants regenerating. This could be due the oxidative tolerance during callus induction providing better conditions for healthy callus development.

Proline was selected as a candidate for improving oat anther culture due to its positive effect on pollen embryogenesis in wheat (Redha *et al.,* 1998) and maize (Büter *et al.,* 1991) while the result in oat anther culture was not so clear (Kiviharju *et al.,* 2005). The results shown in **table 2.21.** show agreement with Kiviharju, Moisander and Laurila, (2005) that the addition of proline does not improve anther response or plant regeneration.

Copper is an essential micronutrient for plants with roles in photosynthesis, antioxidant activity and cell wall metabolism. It is tightly regulated as at higher concentrations it becomes toxic (Pilon *et al.,* 2006). In barley, adding copper to the pre-treatment solution and induction medium at 100 times the usual concentration increased the rate of responding anthers from 57.3 % to 72.3 % and improved the number of regenerated plants from 2.4 to 11.1 per responding anther, with a positive effect on the proportion of albino plants (Wojnarowiez *et al.,* 2002). Copper was added to the induction medium and to the pre-treatment medium as per Wojnarowiez *et al.,* (2002) and tested with three oat genotypes (**section 2.3.2.6., table 2.23.**). The effect of increased copper on the responsive standard Assiniboia was very positive, and there was a clear improvement when copper was added to both the induction medium and pre-treatment solution, which agrees with the results of Wojnarowiez *et al.,* (2002). The positive effect of copper has been reported by other research, however the effect of increased copper does not always improve the proportion of albino to green plant regeneration even where there is an overall increase in plant regeneration (Makowska *et al.,* 2017). Strong genotype dependency for the effect of copper has been seen (Jacquard *et al.,* 2009). In this work, additional copper had a strong positive effect on the genotype Assiniboia, but did not improve anther response or plant regeneration in the more recalcitrant genotype Lisbeth. Copper has shown to be an important factor in successful regeneration *in vitro* with cereals such as barley, (Dahleen 1995; Nuutila *et al.,* 2000), being used to good effect at levels much higher than the standard 0.1 µM used widely in tissue culture. Copper deficiency is known to affect male sterility (Dell, 1980) by altering tapetum physiology and RNA in the anther (Azouaou & Souvre, 1993), which therefore could be having an important negative effect on

microspore viability in anther culture. The addition of a higher concentration of copper in oat, shown with Assiniboia could therefore explain the increased anther response seen when copper was added to the pre-treatment and callus induction stages, as it is supporting microspore fertility, which could explain the increased rate of anther response. However as the improvement was not seen in the other two genotypes tested, it would suggest that while increased copper is beneficial, it is not a solution to the genotype dependency of DH in oat.

2.4.4. The effect of ovaries on anther culture of oat

The use of ovaries for co-culture and conditioning of medium for IMC in wheat and barley has been widely reported to be beneficial or even crucial for success. In **section 2.3.2.7.** mature Assiniboia ovaries were co-cultured with Assiniboia anthers with and without the addition of GAM. The results did not show any benefit, and the plant regeneration rate was lower than the control treatment. Ovary co-culture with *A. sterilis* B443 was also attempted but showed no positive effect. Due to the poor overall response of *A. sterilis* it is likely that it is recalcitrant to anther culture with the current method, so little can be drawn from the failure shown here. Kohler and Wenzel, (1985), reported that 10 ovaries/ml for 7 days provided the greatest benefit for barley, with important amino acid and sugar changes in medium and a potential phytohormone as likely responsible for the effects. The use of ovary co-culture has been shown to reduce the effect of genotype dependency, but not overcome it completely (Hul & Kasha, 1997). The use of ovaries has mostly been used with IMC. However Broughton, (2008), showed that the addition of five ovaries to anther cultures of different Australian wheat cultivars had a positive effect on embryogenesis and green plants per spike. They also showed that ovary co-culture without pre-conditioning produced the highest number of green plants. In these cases, the ovaries are taken from the same genotypes that are used for anther culture or IMC (Broughton, 2008; Hul & Kasha, 1997). More recently, a study looking at ovary genotype and development stage and found that the genotype of the ovary was significant, and that mature ovaries were better than those excised from flowers with microspores at a mid-late uni-nucleate stage (Castillo *et al.,* 2015). Assiniboia has been a positive standard

for oat anther culture, but its ovaries may not be beneficial for improving anther culture. It was seen in **section 2.3.2.2.** that anthers of Assiniboia did not have a positive impact on the anther response of other species. Mature ovaries of oat were used in co-culture experiments based on the Castillo, Sánchez-Díaz and Vallés, (2015) in wheat. However for oat this development stage may not be optimal for effective ovary co-culture. Testing ovaries from different development stages as well as different genotypes and different densities will be necessary to understand if ovaries are an important factor for successful microspore embryogenesis.

2.4.5. Applying media improvements to recalcitrant genotypes

DH technology has made great strides over the decades, improving efficiency and overcoming recalcitrance in many species. This has been done by focusing on the role of stress factors in androgenic induction (Würschum *et al.,* 2013), sources of organic nitrogen in the media (Olsen, 1987), the form of carbohydrate in the culture media (Hunter, 1987) and the use of various growth regulators (Esteves *et al.,* 2014). Using model genotypes, protocols have developed, and recalcitrant genotypes have improved in efficiency. The genotype effect will likely never be eliminated, but with protocol optimisation, most genotypes can be expected to be induced to respond to DH methods.

The additions of glutamine and casein hydrolysate were found to improve both anther response and plant regeneration in the genotype Assiniboia (**section 2.3.2.5**, **table 2.21.**). The genotypes Lisbeth, Aslak and Bajka were tested in the preliminary experiments in **section 2.3.1** and responded poorly to all treatments. To see if the improvements with glutamine and casein hydrolysate could be applied to these genotypes, three previously recalcitrant genotypes were tested (**section 2.3.2.8., table 2.24.)**. The results showed a dramatic improvement in the genotype Lisbeth, and also a big improvement in the genotype Aslak. Bajka however did not show improvement. The results showed that by altering by modifying the induction medium to include an organic form of nitrogen, the microspores of the genotypes Lisbeth and Aslak were responsive to anther culture. This is an important step forward, showing that optimisation of the culture medium can help to overcome the genotype factor that plagues the DH technique.

2.4.6. Using phenotypic data to select panicles for maximum efficiency

Time and costs are always major factors in plant breeding. While producing homozygous lines through DH technology saves time, the costs involved are high. Setting up greenhouses to grow donor plants and maintaining tissue culture facilities to produce anther cultures is expensive. Donor plant growth can take 6-9 weeks, then there is a two week pre-treatment, followed by a callus induction phase of 4-8 weeks, followed by a regeneration stage of regeneration phase of 2-4 weeks and then finally the maturation of regenerants in the greenhouse to set seed. Preparing anthers for culture is also time consuming. It takes 15 minutes to prepare each culture and the exact timing of this is strictly tied to the microspore stage. Therefore maximising efficiency at all stages is crucial to minimise wasting time, effort and ultimately money. The cheaper the service is, the easier it is to market to plant breeders. During the project it was clear that the panicles of the donor plants would vary slightly. Environmental factors such as whether they were grown in a glasshouse or a controlled environment, and in which season, had effects on the size of panicles, the number produced per plant, and the speed at which they were ready to use. More donor plants than were expected to be needed were grown to compensate for potential losses during germination, early growth stages, and the timing of panicles being ready for use. This meant that in most experiments more material was available than necessary. Due to the complexity of the oat panicle architecture it is difficult to accurately gauge when microspores are at the correct stage without direct observation by anther squashes. Attempts have been made to use phenotypic traits like anther size to predict which anthers were best to use, variation was observed between and within genotypes (Cesaro *et al.,* 2009). An anther culture experiment was set up to test medium additives, and four phenotypic measurements were taken from the panicles used for each replicate. In the correlation analysis (**table 2.25.**) few significant correlations are visible. Panicles used were selected based on perceived quality and microspore stage, so panicles which would have been small, had a low number of florets etc. would not have been used. This might have skewed the results, as it would be expected that small panicles with few florets or panicles with few florets on

a long panicle are abnormal and would perform poorly. The other possibility is that these phenotypic traits are not important, as the general variation in the panicle development and structure is not significant, or does not affect the quality or viability of the microspores.

The analysis of individual treatments in the second experiment revealed slightly more. Treatment one and three showed a correlation between the distance between the flag leaf and second leaf with the number of responding anthers. This is a useful measurement as it can be made without destructive sampling, and can be used as a proxy for optimal harvest based on recent anther squashes. When this measurement is between 7-9 cm for example, might be when the central area of the panicle has microspores at the correct stage. This of course highlights that if panicles were harvested when this measurement is less or more, that the number of anthers responding would decrease. The correlation, taking these extremes into account would not be a linear correlation; the results would presumably look more like a bell shape if presented on a histogram. The length of the panicle and number of florets also correlated with the number of responding anthers in some treatments. This confirms the presumption that large panicles are healthy and more capable of enduring the pre-treatment stage. Having more florets available also means it's possible to be more selective of anthers at the correct stage for culture. The different treatments did not show similar correlations, which would suggest that the treatment used is having a greater effect. This measurement could also be strictly genotype dependent; however, it might also be linked to plant fitness or a certain stage in their development where the plant is focussing on inflorescence development. Panicle use for each treatment was random, however always with a bias for using the best panicles available at the moment in time. It might be possible to link this measurement to a particular metabolite in the plant, which could then be used as a future screening method. For instance by studying sugar distribution in the plant, on the expectation that healthy vigorous plants near the correct stage of harvest for DH are investing heavily in inflorescence development, which would predictively mean healthier, better microspores.

In summary, this analysis shows that the assumptions made for panicle selection was good, but extreme examples could help to make the correlations clearer. This could form the basis of a guide for harvesting panicles for anther culture in future experiments, would need to be repeated and tested with different genotypes, as well as with more microspore squash analysis analysed with a more stringent statistical analysis to draw further conclusions, but screenings which avoid destructive sampling would be very beneficial.

2.4.7. Measuring the callus induction and plant regeneration period

As mentioned in **2.3.8**., time is an important factor, which translates into cost. The anther cultures are incubated at 28°C for 4-8 weeks, which is expensive to maintain. Visual observations while conducting anther culture experiments revealed that callus does not visually emerge from anthers at the same time, and can occur over several weeks. In experimental conditions, every callus is important, but in business terms there will be a cost benefit analysis for how long the callus induction period should last, if callus inducing in the 7-8 week period has a low regeneration rate for example. Results from **figure 2.31.** and **figure 2.32.**, show that with both genotypes, the majority of callus was induced and ready to move in week four, particularly with Assiniboia. The callus induction of Lisbeth was more erratic however. Due to Lisbeth only regenerating one plant, a pattern could not be analysed, but Assiniboia regenerated many plants, and the callus they regenerated from are shown in **figure 2.33.** show that all but two plants regenerated from callus which was induced in week four. This is particularly interesting with the gelrite treatment with Assiniboia, which had more calli induced in week five, yet only regenerated plants from callus induced in week four. These calli which induce earlier might be due to them being more fit or vigorous than calli which are slower to appear. This is potentially useful for anther culture on a commercial level, as these results suggest that culturing anthers for longer than four weeks does not lead to more regenerants and so is an unnecessary cost. It would need to be shown to be repeatable, and it may be specific to Assiniboia, as Lisbeth did show the same pattern so the result may be genotype dependent.

2.4.8. The effect of panicle development on anther culture of oat

The oat panicle has a complex structure and a non-linear inflorescence development pattern. A few efforts have been made to try to understand how microspore stage can be linked to the development stage, however variation between and within genotypes have made this difficult (Cesaro *et al.,* 2009). To ensure microspores are used at the correct stage, regular anther squashes are required.

The results of several experiments looking at anther position were scored and are shown in **section 2.3.2.11.** The first experiment shows that the distribution of responding anthers fits a bell curve shape (**figure 2.35)**, presumably reflecting the content of microspores at the mid to late uni-nucleate stage amenable to the pre-treatment and change pathway. **Figure 2.36** shows another anther culture experiment testing different additives, but with the same genotype Assiniboia. The anther response is similar. In this experiment it looks as though sampling was started a little too low, as the top end of the sample area is higher than the lower end, and has not peaked. This also counters the argument that anthers in the centre of culture plate are in more favourable conditions hence why more of them are responsive. **Figure 2.37** shows the number of plants regenerated from anthers at those positions. Compared to **figure 2.36** they do not strictly correlate, but it can be seen that more plants regenerate from the higher end of the panicle than the lower, which agrees with the anther response in **figure 2.36**. A similar pattern is seen in the third experiment, where the number of responsive anthers is in the central area of the plate (**figure 2.38.**) and the same for the plant regeneration **(figure 2.38.**).

These results show that the area of the panicle to be harvested is important for efficient plant regeneration, and that the target sample area is relatively small, though microspores at the uni-nucleate stage may also be present in anthers outside of the optimum stage. Anthers at the extremes of this sample area vary in size and colour, which is likely why anther morphology has not been a successful phenotype to use as a proxy for microspore stage (Cesaro *et al.,* 2009). As this data only becomes available weeks after the panicles have been harvested it is not useful as a guide for initial panicle selection,

but with a more in depth study of the oat panicle and microspores, it could be used to create a more precise model of the oat panicle, which could be used for future anther culture experiments as a prediction tool. Oat panicles are not only complex in structure, but their floret morphology means that extracting the anthers from the primary florets is difficult and time consuming, as any damage to the anther compromises the microspores. A prediction tool to identify anthers on the panicle most likely to be useful for regenerating plants would speed up the anther culture set up, by reducing the number of anthers needing to be excised, and could also reduce the number of cultures to be set up, saving time and materials.

2.5. Discussion summary

The aim of this section was to build on current knowledge of the oat DH methodology and take it forward by drawing on the work conducted from successes in other species, and by testing new approaches. **Table 2.33.** highlights the key outcomes from the many experiments conducted during the project. The early identification of Assiniboia as a positive genotype was a very important step, which has allowed for several breakthroughs in the anther culture protocol. The use of glutamine and casein hydrolysate have been shown to increase anther response and plant regeneration in Assiniboia, and make callus induction and plant regeneration a possibility in the previously recalcitrant genotype Lisbeth. The use of high levels of copper in the pre-treatment solution and callus induction media also looks very promising.

The immediate next step would be to test other crosses involving Assiniboia. Assiniboia x Lisbeth using an anther culture media containing glutamine and copper would be an obvious first choice. This would hopefully lead to the creation of a much larger DH population by the increased efficiency expected. A population of 100 + green plants could allow for experimental studies into linkage maps and even marker assisted selection (MAS). An efficient isolated microspore culture would be a major step forward. So far only two has been reported (Ferrie *et al.,* 2013; Sidhu & Davies 2009) and attempts to repeat it earlier in this chapter were unsuccessful. However, with a positive genotype and

a culture media that has been optimized with anther culture, it could now be worthwhile to return to the method. Even with a low efficiency, the reduction in time and labour of anther extraction would be great, and would allow for direction observation of microspore development and callus growth which could offer further insight into protocol optimization.

No.	Experiment	Key outcome	Section
1	The effect of anther density on callus induction	A higher density of anthers on the induction medium increases the anther response and plant regeneration rate	2.3.2.1.
2	The effects of mixing anthers from different panicles and genotypes on anther culture	Mixing anthers from different panicles within and between genotypes did not increase anther response or plant regeneration	2.3.2.2.
4	The effect of the compounds glutamine, casein hydrolysate, ascorbic acid and pyridoxal phosphate	The addition of glutamine and casein hydrolysate in the induction media increases green plant regeneration efficiency	2.3.2.4.
5	The effect of Copper on anther culture	The addition of copper to the pre-treatment solution and to the media at x100 the normal amount increased anther response	2.3.2.6.
6	Applying medium improvements to recalcitrant genotypes	The addition of glutamine and casein hydrolysate to the callus induction media causes an increase in anther response and regeneration in a previously recalcitrant genotype	2.3.2.8.
8	Measuring the callus induction and plant regeneration period	The vast majority of regenerants originate from callus induced in week four	2.3.2.10.
9	The effect of panicle development on anther culture	The sample area on the panicle is key to maximising the plant regeneration rate	2.3.2.11.

Table 2.33. *A summary of the key findings made from the experiments conducted in this chapter.*

Chapter 3: Testing of hybrid and non-cultivated genotypes in double haploid experiments

3.1. Introduction

This section focuses on the use of F2 donor plants to confirm the origin of regenerated plantlets from embryogenic microspores, and to make progress towards creation of DH populations. Flow cytometry and SSR marker methods were developed to characterize regenerants. Non-domesticated accessions were also used to test whether the poor responsiveness of oat cultivars stems from genetic changes that occurred or were selected during domestication.

3.1.1. Generating DH regenerants from F2 populations

Use of responsive genotypes such as Assiniboia are essential in the initial development of reliable DH protocols when induction and regeneration efficiencies are low. A critical check of these initial protocols is the confirmation that regenerants originate from microspores, and not from somatic tissues of the anther. Flow cytometry can be used to identify the ploidy of regenerants, with the expectation that haploid regenerants will have originated from microspores as somatic tissue will be diploid. However it is not possible to discriminate between spontaneous dihaploid regenerants and regenerants with a somatic origin when using inbred donor lines, as little or no heterozygosity is present. By using donor plants from an F2 population, it will be possible to distinguish homozygous regenerants from heterozygous dihaploid regenerants by using markers which are polymorphic for the two parents. Some donor plant progeny will be heterozygous for these markers, but haploid-derived regenerants from these will segregate, having one or the other allele but not both, allowing them to be distinguished from the donor. Using donor plants from crosses with at least one positively responding parent might also be beneficial in terms of exploiting heterosis (hybrid vigor). Heterosis in DH populations has been observed, where progeny performed better in anther culture than their parental genotypes (El-Hennawy, Abdalla, Shafey, & Al-Ashkar, 2011).

3.1.2. Anther culture of wild oat species

Domestication frequently involves loss of traits or responses that are of value only in wild conditions, while millennia of inbreeding may lead to further loss of pathways not under

active selection. It is therefore plausible that the genetic 'tools' for efficient embryogenesis have been lost in modern oat cultivars. This could explain why cultivars of *A. sativa* are so recalcitrant to the process. Rines and McCoy found that *A. fatua* accessions were consistently responsive to tissue culture, although the *A. sterilis* accessions they tested were not (Rines & Mccoy, 1981). Anther culture of the oat species *A. nuda*, *A. sterilis* and *A. byzantina* have been published by Kiviharju *et al.,* (1997), who successfully regenerated the first plants from *A. sterilis*. If responsive genotypes of other oat species can be identified they would be useful for investigating the genetic properties behind embryogenesis or for interspecies crosses, and may serve as 'feeder' stocks for co-culture or conditioning of media.

3.1.3. Analysis of regenerants using flow cytometry

It is important to confirm the ploidy of the regenerated plants. Some spontaneous doubling of chromosomes may be expected so diploid regenerant plants do not necessarily indicate that somatic tissue has been cultured, but the presence of haploid regenerated plants would provide strong evidence for a microspore origin. There are direct and indirect methods to screen ploidy levels. Indirect approaches include studying phenotypic traits in comparison to the donor plants, such as comparing the regenerated plants to their donor plants in terms of physical morphology such as plant height, leaf dimensions and flower morphology. Measurements of stomata and chloroplast number within the stomatal guard cells have been used to identify ploidy levels (Shrestha & Kang, 2015; Beck *et al.,* 2005). Characterization of DH plants based on homogeneity of phenotypic traits has been done (Kernan & Ferrie, 2006). However while these methods do not require expensive or specialized equipment these methods requires all candidate plants to be grown through to maturity and would require an increased amount of space and labour, as well as needing an additional generation of field evaluation (Chen *et al.,* 1998). These methods are unreliable, as they are subject to environmental conditions.

Direct and more accurate methods for screening ploidy level are cytological techniques, such as chromosome counts from root tips (Maluszynska, 2003), and measurement of DNA quantity using flow cytometry (Bohanec, 2003). These methods are not just more accurate, but can also be used much earlier, as the plants do not need to mature to be sampled. The plants can be sampled for leaf tissue (for flow cytometry) or root tips (for chromosome counts) as soon as the plants are big enough to be moved into soil. It is important to identify haploid plants early, as the single set of chromosomes precludes meiosis and leads to infertility. The inflorescence will form, but with phenotypic irregularities. If identified while the plants are still vigorous, the haploids may be rescued by the use of a chromosome doubling agent such as colchicine.

Spontaneous doubling rate varies depending on the DH method used. In barley and wheat, doubling frequencies of 50 %+ of regenerated plants have been observed, resulting in fertile doubled haploids (Slama Ayed *et al.,* 2010; Hoekstra *et al.,* 1993). Triticale also shows similar rates of spontaneous doubling (57.5 %) (Aurelia Ślusarkiewicz-Jarzina & Ponitka, 2003) and in maize it is 40 %+ (Antoine-Michard & Beckert, 1997). In durum wheat spontaneous doubling has been reported as high as 67 % (Cistué *et al.,* 2009). The rate of spontaneous doubling appears to vary based on the species or genotype, but can also be affected by the culture conditions. In barley, genotype was found to be significantly affecting the rate of spontaneous doubling (61.20 – 78.93) between seven different genotypes (Kahrizi, 2009). The use of mannitol in the pre-treatment solution may increase the rate of nuclear fusion following the first mitotic division (Shim & Kasha, 2003) The exact mechanism of spontaneous duplication of chromosomes is not known, but it is has been hypothesised to occur by endoduplication or nuclear fusion (Sunderland, 1974). Sunderland, (1974) described nuclear fusion as the occurrence of two nuclei synchronously entering into division, forming a common metaphase plate and spindle which results in two nuclei, each with more than one set of chromosomes. Physiological methods are also known to improve the rate of spontaneous doubling, for example by growth in the presence of mannitol (Kasha *et al.,* 2001).

The frequency of spontaneous doubling may too low to be useful for plant breeders, and may need to be enhanced. The method needs to be swift, suitable for large numbers of plants and have a low rate of mortality. Haploid plants can be induced to double their chromosomes by using a variety of methods including caffeine (Thomas *et al.,* 1997) oryzaline (Bouvier *et al.,* 1994) and colchicine (Morgan, 1976). Colchicine is a naturally occurring metabolite originally discovered in the plant genus *Colchicum*. It is an anti-microtubule agent, which function by binding to tubulin in the nucleus which disrupts the segregation of sister chromatids which inhibits normal spindle formation (Hervás *et al.,* 1974). Colchicine is normally applied to green regenerants just before they are ready to be moved to soil, but colchicine has also been applied to the anther culture medium (Islam, 2010). Due to the toxicity and cost of the agent, in most cases it is applied *in vivo* to keep the use of the agent to a minimum. The concentration and duration period of the colchicine treatment varies between species. A low concentration or short duration may lead to a low efficiency of doubling in a population of individuals, while a higher concentration or longer duration may lead to a high rate of mortality or tetraploidy.

The efficiency of colchicine on chromosome doubling varies depending on the species. In maize it is 40 % (Eder & Chalyk, 2002), in rice 30 – 60 % (Alemanno & Guiderdoni, 1994) and in wheat, it can range from 20-100 % depending on the genotype and colchicine treatment (Zamani *et al.,* 2000).

3.1.4. Analysis of regenerants using SSRs

In both anther and microspore culture, there is a risk that plants regenerate from somatic tissue, rather than the microspores. In anther culture this can occur in the anther cell wall, filament or from ovaries used for co-culture. In microspore culture somatic tissue from the panicles which passes through the filters in the filtrate can potentially regenerate into plants. Regenerants with a somatic origin will inevitably have a diploid karyotype, however, regenerants derived from microspores may also show high spontaneous doubling rates so that ploidy alone cannot distinguish origin. The most

direct screen, which requires only small samples to carry out, is to genotype regenerants from heterozygous donor plants, where heterozygosity will be maintained in somatic embryogenesis but lost in genuine double haploid regenerants. A range of genotyping assays are available for oat, with microsatellite screening being the method of choice here. Microsatellites, also known as simple sequence repeats (SSRs) are subcategories of tandem repeats (TRs) that make up genomic repetitive regions. SSRs tend to be unstable with changes in repeat copy number occurring through 'stuttering' during DNA replication. The consequent high mutation rates, which lie between 10^3 and 10^6 per cell generation, are far greater than with point mutations (Gemayel *et al.,* 2012), leading to high rates of polymorphism. Fluorescently labelled primers can be used to amplify SSR containing regions by PCR, with subsequent analysis on micro capillary systems capable of detecting single base size differences. The assay is fast, relatively inexpensive, able to be automated and generally applicable to a large number of genotypes once appropriate targets and conditions have been established.

3.2. Materials and methods

This section details the methods of flow cytometry and SSR marker screening used to characterise regenerants.

3.2.1. Determination of ploidy level of regenerated plants

The ploidy level of the regenerated plants was determined by flow cytometry of the DNA content of the leaves stained with 4'-6-diamidino-2-phenylindole (DAPI) using a Partec CyFlow® Ploidy Analyzer. Freshly germinated Assiniboia leaf material was used as a 2N control. Control samples were run before each session of samples to ensure the machine was running correctly. Approximately 1 cm^2 of leaf material was sampled from each plant and stored in 5 by 5 deep well plates with 0.5 mL of tap water. Samples were kept on wet ice during sampling and at 4 °C if the samples were not to be used immediately. Leaf samples were chopped with a razor blade in 400 µL ice cold nuclei extraction buffer (Cystain UV Precise, Partec) for 1 min or until the leaf material was finely chopped. The

suspension was then filtered through a 30 µm screen to remove excess plant material leaving only plant cell nuclei for analysis. The extraction suspension was incubated for a further 5 min on ice, then 1.5 mL of nuclei staining buffer (Cystain UV Precise, Partec) was added to the suspension and the samples were incubated on ice for a further 10 min. The samples were then measured with on the Partec using UV excitation. Results were analysed and compiled with Flowing Software (Terho, 2009).

3.2.3. Colchicine treatment of regenerated plants

The colchicine treatment followed the protocol of Morgan, (1976). The plants were removed from the soil and the roots were washed in water to clean roots. The roots were cut back to 2-3 cm long, and plants put into glass tubes. 10 mL of the colchicine solution was added to each glass tube, so that the roots and meristem were submerged. The tubes were covered in foil and incubated at room temperature in a fume hood for five hours. The solution was then removed, and the plants were washed five times with water, and then incubated in water for one hour. The plants were then potted into soil and covered with clear plastic to provide humidity.

3.2.4. Moving regenerated plants to the greenhouse

Green regenerated plants were moved to the greenhouse once roots of 1-2 cm had developed, and the leaves were reaching the top of their container. The plants were rooted in seedling trays. The plants were removed from their containers and the agar was carefully broken up to ensure the roots were not damaged. Once the plant was removed a little water was used to remove the agar from the base and roots of the plants before being planted into trays. The tray was covered with clear plastic to maintain humidity. The plants were checked daily for watering and to observe progress.

3.2.5. DNA extraction

DNA was extracted from approximately 3 cm^2 of leaf material, using a DNeasy plant mini kit (QIAGEN, Germany) according to the manufacturer´s protocol. DNA concentration of the samples was determined by Epoch™ Spectrophotometer System (Biotek instruments Inc. USA).

3.2.6. SSR marker analysis

SSR markers were adopted to assess homozygosity and to determine the origin (gametic or somatic) of the regenerated plants. Nineteen SSR markers polymorphic for the parents cv. Firth and cv. Assiniboia were selected from panels of primer sets that were developed at IBERS (**table 3.1.** Howarth, Langdon, unpublished). The polymerase chain reaction (PCR) was carried out with two primers: the specific forward and reverse primer for each microsatellite. The forward primer for each microsatellite was labelled with a fluorescent dye for genotyping applications (Applied Biosystems). The dyes for each microsatellite are summarised in **table 3.1.** PCRs were performed in a Veriti Thermal Cycler (Applied Biosystems, USA) in a final volume of 10 µl containing 2 µl of gDNA, and 8.0 µl of QIAGEN Multiplex PCR Master Mix, SDW (sterile deionised water) with both primer pairs. The PCR thermal profile was as follows: an initial denaturation step at 95 °C for 15 min, followed by 40 cycles of (a denaturation step of 94 °C for 30 s, an annealing step at 57 °C for 90 s, followed by a final extension step at 72 °C for 90 s). 2 µl PCR product was diluted with 98 µl SDW, and the 1 µl of this was mixed with 10 µl formamide, 0.06 µl of GeneScan 500 LIZ (-250) (Applied Biosystems, USA). DNA fragment analysis was performed on a 3730 DNA analyser (Applied Biosystems, USA). Allele lengths were determined using the GeneMapper software, version 3.7 (Applied Biosystems, USA).

Name	Forward primer	Reverse Primer	Dye	Repeat motif	Size bp	Source
AM01	5'GGA TCC TCC ACG CTG TTG A	5'CTC ATC CGT ATG GGC TTT A	PET	(AG)21(CA GAG)6	204	Rossnagel & Schole. 2000
AM3	5'CTG GTC ATC CTC GCC GTT CA	5'CAT TTA GCC AGG TTG CCA GGT C	6-FAM	(AG)35	280	Rossnagel & Schole. 2000
AM14	5'GTG GTG GGC ACG GTA TCA	5'TGG GTG GCG AAG CGA ATC	HEX	(AC)21	133	Rossnagel & Schole. 2000
AM30	5'TGA AGA TAG CCA TGA GGA AC	5'GTG CAA ATT GAG TTT CAC G	HEX	(GAA)14	203	Rossnagel & Schole. 2000
AM41	5'CCA AAG GAA ACA AGT CAA TAG	5'TTC CCG CAA AGT CAT CAT	PET	(GAA)10	205	Rossnagel & Schole. 2000
AM42	5'GCT TCC CGC AAA TCA TCA T	5'GAG TAA GCA AAG GCC AAA AAG T	6-FAM	(GAA)16	193	Rossnagel & Schole. 2000
Barc126	CCATTGAAACCGGA TTTGAGTCG	CGTTCCATCCG AAATCAGCAC	6-FAM			Somers *et al.*, 2004
OL0868	CTGAACGGATC TGCTGCTATC	AGCAGGGTGGT GTAGGTCAC	HEX	GAG	263	Oat Link
OL0102	AACACCCAGTC CTACCAGACC	GTTGTCATCGA TCACCTCCTC	NED	TCCAGC	300	Oat Link
OL0875	CTTCGTTGCT CCTCCTTCC	GAACCTCTTGTCG GTGAACTG	PET	TTCCCC	244	Oat Link
OL2133	GCTGTTCCTCC TCTCGAATCT	TTCCATATGATC CCAAGCAAG	NED	CTG	246	Oat Link
OL0256	GTGTGCATCCC TCATCAGTC	ACCTCCATCTCG ACCTCAACT	NED	CAGAGA	112	Oat Link
OL0410	TGCCCTAGTGTA GCACTGACC	AGGATCAGCAG GTCCGTTATC	6-FAM	CGC	151	Oat Link
MAMA3	TTCCCACTCCG TGTTCTCTC	GATGGACGCAC AAGAATCG	PET	TCTA(n)		White *et al* 2010
MAMA7	ATAAATACGCG CCACCACTC	TCCGGTGTGAG TAGGGTAGG	HEX	TC(n)AC(n)TC(n)		White *et al* 2010
MAMA11	TGTATGCACC GATGCAATTT	GACTACCGCCCA GATGAGAC	PET	TCTA(n)		White *et al* 2010
LAM91	CGCAACTCTT CCTACTTTTTGTT	TGGCAAACTCCC TCGATTTA	HEX			Pal *et al* 2002
HvARHGN	TCCATGGAT GCAGAAGTAC	CTCTTTCTAGTG TTCGTGGTC	NED			Ramsay *et al.*, 2000
ASTA	ACCCCAGCGA ACAATATCAG	GTCTGAGGCT GAACGAGACC	PET			Jannink *et al* 2005

Table 3.1. *Polymorphic SSR markers for the parents Firth and Assiniboia.*

3.3. Results

3.3.1. Anther culture of the oat species *A. sterilis* and *A. fatua*

Wild oat species are recalcitrant to the current callus induction medium

In this experiment donor plants of A*. sterilis* accession B443 were grown at Saaten Union for anther culture. *A. sterilis* is a common wild species in most warm-temperate places and an occasional weed of cereal crops. The first panicles were sampled and screened by anther squashes to ensure the panicles were harvested at the optimum time and the standard 14-16 day cold pre-treatment was applied. The B443 accession was tested on the oat medium MDHM with and without the addition of the additives GAM (50 mg/L) and putrescine (44 mg/L). Three ovaries of the donor plants were also added to see if they had a positive effect. Ten replicates of 42 anthers per petri dish were plated for each treatment combination.

In **table 3.2.** the results from the anther culture of *A. sterilis* B443 show that the genotype responded very poorly to all treatments, with zero response for the majority of treatments. Callus and plant regenerants were only obtained from the control treatment without ovaries, and the treatment with added putrescine and without ovaries. The numbers were too small to conduct a statistical analysis.

The second species *A. fatua* was sown at Saaten-Union Biotec GmbH in the spring of 2016. Two different genotypes were sown. The plants were grown as per the materials and methods and as with *A. sterilis* more care was taken to monitor the growth progression. It was not known how many tillers to expect per plant so ten plants for each genotype was sown with the aim to make ten replicates for each on standard MDHM medium. Any surplus panicles could also be tested on MDHM medium with other additive compounds.

Ovary co culture +/-	Treatment	Anthers cultured (no.)	Anther responding (no.)	No. of responding anthers /100 anthers ± SE	Total Regen	No. of regenerants /100 anthers ±SE	Albino	Green
-	None	420	1	0.20 ± 0.10	1	0.20 ± 0.10	0	1
-	GAM	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
-	Putrescine	420	5	1.20 ± 0.27	3	0.70 ± 0.15	1	2
-	Both additives	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
+	None	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
+	GAM	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
+	Putrescine	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
+	Both additives	420	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0

Table 3.2*. Influence of GAM (50 mg/L), putrescine (44 mg/L) and ovary co culture on anther culture embryogenesis of the oat A. sterilis B443. (SE = standard error).*

Genotype	Media	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers ±SE	No. of regenerants	No. of regenerants /100 anthers ± SE	albino	green
A. fatua G1	MDHM	168	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
A. fatua G2	MDHM	210	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0

Table 3.3. *Anther culture of two different A. fatua genotypes.*

The results in **table 3.3.** show that neither *A. fatua* genotypes produced any callus. Only four anther cultures for genotype one and five anther cultures for genotype two were made due to the lack of good panicles. Both genotypes also took 2-3 weeks longer than expected to be at the required development stage for harvest. The plants grew very elongated stems which required stakes and tape to keep erect.

The poor conditions of the donor plants prevents any strong conclusions to be drawn about the responsiveness of these accessions, and growth conditions should be optimized before repeating these tests.

3.3.2. Anther culture of F2 oat populations

The addition of glutamine to the callus induction medium increases the anther response and plant regeneration in the Assiniboia x Firth F2 population

To date only one DH linkage map for oat has been developed (Tanhuanpää *et al.,* 2008) which has since been updated (Tanhuanpää *et al.,* 2010; Tanhuanpää *et al.,* 2012). With the new markers, most of the link age groups could be anchored to the 'Kanota' x 'Ogle' oat reference map. DH mapping has great potential for application by oat breeders.

Following the failure of Canyon x Firth progeny to regenerate green plants (section **2.3.1.3**), populations containing at least one parent which had shown embryogenic ability through anther culture were selected for further experiments. It is essential to confirm that the callus generated by current protocols is derived from haploid cells rather than from diploid tissue (e.g. anther walls). It is also highly desirable to obtain a DH population as soon as possible to allow screening for any bias for or against particular lines. At Saaten-Union Biotec GmbH in 2015 ten donor plants each of seven F2 progeny of Assiniboia x Aslak were sown for anther culture. Both parents have shown embryogenic ability in the preliminary experiments and Aslak has already been used to create a double haploid population in published work (Tanhuanpää *et al.,* 2008). Each line was tested on two different media; MDHM and MIMI, each with and without the addition of

arabinogalactan (50 mg/L), gum Arabic (50 mg/L) and putrescine (44 mg/L). Five replicates were made for each genotype for each treatment combination, with 42 anthers per replicate.

Line	Media	Additive	Anthers (no.)	Responsive anthers (no.)	No. of anther resp. /100 anthers	No. of plants	albino	green
1	1	1	210	3	1.43 ± 0.95	0	0	0
1	1	4	210	1	0.48 ± 0.20	1	1	0
1	2	1	210	0	0.00 ± 0.00	0	0	0
1	2	4	210	0	0.00 ± 0.00	0	0	0
2	1	1	210	22	10.48 ± 4.10	0	0	0
2	1	4	210	7	3.33 ± 2.33	0	0	0
2	2	1	210	0	0.00 ± 0.00	0	0	0
2	2	4	210	0	0.00 ± 0.00	0	0	0
3	1	1	210	0	0.00 ± 0.00	0	0	0
3	1	4	210	8	3.81 ± 1.61	2	1	1
3	2	1	210	0	0.00 ± 0.00	0	0	0
3	2	4	210	0	0.00 ± 0.00	0	0	0
4	1	1	210	5	2.38 ± 1.84	0	0	0
4	1	4	210	2	0.95 ± 0.95	0	0	0
4	2	1	210	0	0.00 ± 0.00	0	0	0
4	2	4	210	0	0.00 ± 0.00	0	0	0
5	1	1	210	16	7.62 ± 2.43	2	0	2
5	1	4	210	5	2.38 ± 1.06	0	0	0
5	2	1	210	3	1.43 ± 1.43	0	0	0
5	2	4	210	0	0.00 ± .0.00	0	0	0
6	1	1	210	0	0.00 ± 0.00	0	0	0
6	1	4	210	19	9.05 ± 3.05	2	2	0
6	2	1	210	0	0.00 ± 0.00	0	0	0
6	2	4	210	0	0.00 ± 0.00	0	0	0
7	1	1	210	7	3.33 ± 2.20	0	0	0
7	1	4	210	7	3.33 ± 1.78	0	0	0
7	2	1	210	0	0.00 ± 0.00	0	0	0
7	2	4	210	0	0.00 ± 0.00	0	0	0

Table 3.4. *Anther response and plant regeneration efficiency of seven progeny of the F2 Assiniboia x Aslak cross. 1 = MDHM media, 2 = MIMI media, 1 = no additive, 4 = all additives. SE = Standard error.*

The results from the Assiniboia x Aslak were more promising than the Canyon x Firth experiment. In **Table 3.4.** MDHM media outperformed MIMI media strongly. Callus induction response in the Assiniboia x Aslak F2 population varied across the progeny. Genotype two had the highest anther response on MDHM media, but genotype five produced the most plants. MIMI media produced an anther response in genotype five only, and none regenerated. Overall the number of callus induced were still very low across all genotypes. Out of a total of 5,880 anthers cultured from seven different F2 Assiniboia x Aslak progeny, only 105 callus producing anthers (1.79 responsive anthers per hundred anthers) and seven plants (0.12 plants per hundred anthers) were produced across all treatments. From the response of Assiniboia x Aslak genotype two it can be seen that a higher number of induced callus does not necessarily result in a higher number of regenerated plants.

After the poor performance with anther culture of the Assiniboia x Aslak population, it was decided to use a different population. Assiniboia x Firth was selected as the parents showed very different responses. Firth is highly recalcitrant based on previous experiments in **section 2.3.1.2.** but has been used extensively for genetic and genomic research at Aberystwyth, potentially aiding future analysis of genetic factors affecting tissue culturability and response. Assiniboia has proven to be a reliable genotype for protocol optimization (**Section 2.3.2.**). F2 seed was already available, and had a good rate of germination. A total of six different experiments were conducted with Assiniboia x Firth lines (**Table 3.5.**), totaling 399 individual anther cultures. All of them except MDHM5c were conducted at Saaten-Union. The first experiment (MDHM1a) with Assiniboia x Firth F2 plants used 20 lines, one donor plant per line, cultured on MDHM medium with no additional additives, three replicates for each. The second and third experiments MDHM1b and MDHM5b were conducted at the same time. MDHM1b consisted of 26 lines, one donor plant per line, three replicates for each. MDHM5b consisted of ten lines, one donor plant per line cultured on MDHM medium

supplemented with glutamine (500 mg/L). The pairs and co-culture experiments are covered in **section 2.3.2.2.**

The final experiment involved 51 lines of Assiniboia x Firth, grown at Aberystwyth University. One donor plant per line. Based on the success of glutamine, it was decided to use MDHM medium supplemented with 1 mg/L glutamine and discontinue the use of casein hydrolysate. To regenerate as many plants as possible for further analysis, a minimum of three replicates was set with the aim to produce as many replicates as possible. This experiment was called MDHM6a.

Experiment	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers	No. of regenerants	No. of regenerants /100 anthers	albino	green
MDHM1a	2520	31	1.23	1	0.04	1	0
MDHM1b	2646	52	1.97	7	0.26	4	3
MDHM5b	1176	14	1.19	5	0.44	3	2
Pairs	1512	38	2.51	5	0.33	4	1
Co-Culture	420	7	1.67	0	0.00	0	0
MDHM6a	11004	291	2.64	70	0.64	40	30
Total	16758	402	2.40	87	0.52	51	36

Table 3.5. *Summary table of efficiencies of anther response and plant regeneration of Assiniboia x Firth under different conditions. These experiments were not conducted at the same time.*

The results from all Assiniboia x Firth anther culture experiments are summarized in **table 3.5.** A total of 16,758 anthers were successfully cultured (not including anther cultures which were contaminated and destroyed), which produced a total of 402 responding anthers. From this 87 plants were regenerated, the majority of which were albino. As the experiments were performed at different locations and times of year direct comparisons between experiments cannot be precise. MDHM1a and MDHM1b cultured a similar number of anthers, and yet show differing levels of response for both the number of responding anthers and plant regeneration. The highest rates of anther response were

recorded in the pair experiment and in the MDHM6a experiment, where both reach over an anther response rate of 2.5 %. Most calli and plants were produced in the final experiment MDHM6c, where 80 % of all plants were regenerated from, despite only containing 65 % of all anthers cultured, suggesting that the additional glutamine increased the response rate of both anthers and regeneration.

Line	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers +SE	No. of regenerants	No. of regenerants /100 anthers +SE	albino	green
1	126	1	0.79 ± 0.79	0	0.00 ± 0.00	0	0
2	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
3	126	2	1.59 ± 1.59	0	0.00 ± 0.00	0	0
4	126	2	1.59 ± 1.59	0	0.00 ± 0.00	0	0
5	126	3	2.38 ± 0.00	1	0.79 ± 0.79	1	0
6	126	2	1.59 ± 1.59	0	0.00 ± 0.00	0	0
7	126	1	0.79 ± 0.79	0	0.00 ± 0.00	0	0
8	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
9	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
10	126	5	3.97 ± 1.59	0	0.00 ± 0.00	0	0
11	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
12	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
13	126	13	10.32 ± 1.59	0	0.00 ± 0.00	0	0
14	126	1	0.79 ± 0.79	0	0.00 ± 0.00	0	0
15	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
16	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
17	126	1	0.79 ± 0.79	0	0.00 ± 0.00	0	0
18	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
19	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
20	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0

Table 3.6. *Results of the MDHM1a experiment with Assiniboia x Firth F2 lines. SE = Standard error.*

The results from the MDHM1a experiment (**table 3.6.**) show a poor rate of callus induction and plant regeneration. Ten of the 20 lines did not induce any callus and reported zero responding anthers. Only one line (no. 13) had a response similar to that seen with the genotype Assiniboia, however no plants regenerated. The only line to

regenerate a plant was line 5, which regenerated a single albino plant.

Line	No. of anthers cultured	No. of responding anthers +SE	responding anthers /100 anthers	No. of regenerants	No. of regenerants /100 anthers +SE	albino	green
7	126	0	0.00 ± 0.00	1	0.79 ± 0.79	0	1
9	84	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
13	84	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
17	84	1	1.19 ± 1.19	1	1.19 ± 1.19	1	0
18	42	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
24	42	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
26	126	20	15.87 ± 10.13	2	1.59 ± 1.59	0	2
33	126	1	0.79 ± 0.79	0	0.00 ± 0.00	0	0
35	126	3	2.38 ± 2.38	0	0.00 ± 0.00	0	0
36	126	6	4.76 ± 2.75	0	0.00 ± 0.00	0	0
39	84	1	1.19 ± 1.19	0	0.00 ± 0.00	0	0
43	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
44	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
45	84	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
47	126	3	2.38 ± 0.95	0	0.00 ± 0.00	0	0
50	126	3	2.38 ± 2.38	0	0.00 ± 0.00	0	0
51	84	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
53	84	3	3.57 ± 3.57	1	1.19 ± 1.19	1	0
56	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
58	126	9	7.14 ± 7.14	1	0.79 ± 0.79	1	0
62	84	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
65	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
66	126	1	0.79 ± 0.79	1	0.79 ± 0.79	0	1
68	84	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
69	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
70	42	1	2.38 ± 0.00	0	0.00 ± 0.00	0	0

Table 3.7. *Results of the MDHM1b experiment with Assiniboia x Firth F2 lines. SE = Standard error.*

The response of Assiniboia x Firth lines on MDHM1b medium was slightly better than on MDHM1a. A total of 52 anthers responded (**Table 3.7.**), and seven plants were regenerated of which four were albino and three were green. The best response was

seen in line 20, which had 20 anthers respond, and two green plants regenerated. 14 of the 26 lines did not induce any callus and only six lines regenerated at least one plant.

Line	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers +SE	No. of regenerants	No. of regenerants /100 anthers +SE	albino	green
1	126	0	0.00 ± 0.00	1	0.79 ± 0.79	1	0
8	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
11	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
16	126	3	2.38 ± 1.37	1	0.79 ± 0.79	0	1
25	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
31	126	1	0.79 ± 0.79	0	0.00 ±0.00	0	0
36	42	1	2.38 ± 0.00	0	0.00 ± 0.00	0	0
41	126	3	2.38 ± 0.00	1	0.79 ± 0.79	1	0
54	126	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
64	126	6	4.76 ± 1.37	2	1.59 ± 1.59	0	2

Table 3.8. *Results of the MDHM5b experiment with Assiniboia x Firth F2 lines. SE = Standard error.*

The additional glutamine and casein hydrolysate did not appear to have a clear positive effect on anther response or regeneration (**3.8**). Of the ten lines, five had at least one responding anther. Of those, four regenerated at least one plant.

The final experiment with Assiniboia x Firth was the most successful. A total of 70 plants were regenerated, of which 30 were green, and 40 were albino; a regeneration efficiency of 0.62 plants per 100 anthers, the highest recorded (**table 3.9.**). The anther response was also the highest recorded, with 2.64 anthers per 100 anthers responding. The addition of 1 g/L of glutamine appears to have a positive effect. The lines have been listed by highest to lowest number of anther response per 100 anthers as the difference in replicates is large across the lines. This makes it difficult to compare lines against each other. Of the 51 lines, only six failed to have any responsive anthers. The number of responsive anthers did not appear to be closely correlated with the number of regenerants. This can be seen in the lines, both of which regenerated many plants from a

small number of responsive anthers. A number of anther cultures showed this response, where multiple plants regenerated from a single callus structure, making it difficult to identify if the plants are from different microspores or not.

Line	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers ± SE	No. of regenerants	No. of regenerants /100 anthers ± SE	albino	green
42	84	8	9.52 ± 4.76	1	1.19 ± 1.19	0	1
12	168	15	8.93 ± 2.81	7	4.17 ± 1.79	6	1
32	336	28	8.33 ± 3.85	4	1.19 ±0.71	3	1
10	84	6	7.14 ± 7.14	4	3.57 ± 4.76	3	1
39	336	24	7.14 ± 1.91	2	0.60 ± 0.39	0	2
24	84	5	5.95 ± 3.57	0	0.00 ± 0.00	0	0
40	294	17	5.78 ± 1.93	8	2.72 ± 2.35	3	5
37	294	15	5.10 ± 1.60	3	1.02 ± 0.48	3	0
23	126	6	4.76 ± 2.75	1	0.79 ± 0.79	1	0
17	42	2	4.76 ± 0.00	0	0.00 ± 0.00	0	0
38	42	2	4.76 ± 0.00	0	0.00 ± 0.00	0	0
13	294	13	4.42 ± 1.51	2	0.68 ± 0.44	1	1
22	252	11	4.37 ± 2.71	3	1.19 ± 1.19	0	3
9	336	14	4.17 ± 1.61	1	0.30 ± 0.30	0	1
8	210	8	3.81 ± 1.93	10	4.76 ± 3.01	10	0
30	294	11	3.74 ± 1.36	10	3.06 ± 2.31	3	7
50	294	10	3.4 ± 1.26	0	0.00 ± 0.00	0	0
2	210	6	2.86 ± 0.89	1	0.48 ± 0.48	1	0
43	294	8	2.72 ± 1.68	2	0.68 ± 0.68	2	0
6	84	2	2.38 ± 2.38	0	0.00 ± 0.00	0	0
51	126	3	2.38 ± 1.37	0	0.00 ± 0.00	0	0
29	294	7	2.38 ± 0.90	0	0.00 ± 0.00	0	0
26	378	8	2.12 ± 0.48	1	0.26 ± 0.26	1	0
25	336	7	2.08 ± 0.83	0	0.00 ± 0.00	0	0
16	336	7	2.08 ± 0.70	1	0.30 ± 0.30	1	0
48	252	5	1.98 ± 1.29	0	0.00 ± 0.00	0	0
4	252	5	1.98 ± 0.73	1	0.40 ± 0.40	0	1
1	210	4	1.90 ± 1.39	0	0.00 ± 0.00	0	0
33	210	4	1.9 ± 1.39	1	0.48 ± 0.48	0	1

Line	No. of anthers cultured	No. of responding anthers	responding anthers /100 anthers ± SE	No. of regenerants	No. of regenerants /100 anthers ± SE	albino	green
41	294	5	1.70 ± 0.68	1	0.34 ± 0.34	0	1
49	126	2	1.59 ± 1.59	0	0.00 ± 0.00	0	0
27	252	4	1.59 ± 1.00	0	0.00 ± 0.00	0	0
7	252	3	1.19 ± 1.19	0	0.00 ± 0.00	0	0
46	294	3	1.02 ± 0.71	0	0.00 ± 0.00	0	0
19	210	2	0.95 ± 0.58	2	0.95 ± 0.58	0	2
20	126	1	0.79 ± 0.79	0	0.00 ± 0.00	0	0
44	126	1	0.79 ± 0.79	1	0.79 ± 0.79	0	1
18	252	2	0.79 ± 0.50	0	0.00 ± 0.00	0	0
31	168	1	0.60 ± 0.60	0	0.00 ± 0.00	0	0
3	210	1	0.48 ± 0.48	0	0.00 ± 0.00	0	0
45	210	1	0.48 ± 0.48	0	0.00 ± 0.00	0	0
11	462	2	0.43 ± 0.29	0	0.00 ± 0.00	0	0
28	294	1	0.34 ± 0.34	2	0.68 ± 0.68	2	0
35	336	1	0.30 ± 0.30	1	0.30 ± 0.30	0	1
5	42	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
14	210	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
21	168	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
34	42	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
36	42	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
47	336	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0
Total	11004	291	2.64 ± 0.25	70	0.62 ± 0.14	40	30

Table 3.9. *Results of the MDHM6a experiment with Assiniboia x Firth F2 lines. SE = Standard error.*

3.3.2. Flow cytometry

Leaf samples (1cm^3) were harvested from Assiniboia x Firth regenerants from the experiment MDHM6a (**table. 3.9.**) and from regenerants of Assiniboia and Lisbeth from the copper experiment (**table 2.3.2.6.**) when the rooted plants were moved from *in vitro* culture to soil in the greenhouse. Plants were not sampled and transferred until they were at least 5 cm tall. Plants which needed to be resampled after a failed flow cytometry measurement were sampled from the greenhouse. As green plants were of more value than the albino plants, efforts were focused on green plants.

No.	Regenerant	Albino/green	Ploidy
1	axf42,p14,12.6.17.	green	2n
2	axf39,p1,12.6.17.	green	2n
3	axf39,p6,18.6.17.	green	2n
4	.axf12,p5,15.6.17.	green	2n
5	axf40,p3,15.6.17.	green	2n
6	axf9,p6,4.7.17.	green	2n
7	axf10,p13,15.6.17.,p2	green	2n
8	axf12,p12,8.6.17.	albino	2n
9	AxF35,p2,5.7.17.	green	2n
10	axf40,p9,15.6.17.	green	2n
11	axf32,p10,21.6.17.	green	1n
12	axf10,p3,15.6.17.	green	1n
13	axf41,p3,26.6.17.	green	1n
14	axf19,p2,20.6.17.	green	1n
15	axf22,p5,20.6.17.	green	1n
16	axf44,p2,12.6.17.	green	1n
17	axf32,p10,21.6.17.	green	1n
18	axf40,p5,3.7.17.	green	1n
19	axf22,p9,20.6.17.	green	1n
	Summary		
	Green	2n	9
	Albino	2n	1
	Green	1n	9
	Albino	1n	0

Table 3.10. *Results of regenerants of Assiniboia x Firth analysed with flow cytometry.*

Results of the flow cytometry are shown in **table 3.10.** A total of 19 plants were successfully measured of which 18 were green and one was albino. Of the 19 plants ten were dihaploid and nine were haploid. The only albino plant was dihaploid.

no.	Regenerant	Albino/green	Ploidy
1	Lisbeth.3.2.4.	green	1n
2	Assiniboia.4.1.4.	green	2n
3	Assiniboia.5.6.4.	green	2n
4	Assiniboia.1.2.4.	green	2n
5	Assiniboia5.6.4.	green	2n
6	Assiniboia.6.9.4.	green	2n
	Summary		
	Green	2n	9
	Albino	2n	1
	Green	1n	9
	Albino	1n	0

Table3.11. *Results of regenerants of Lisbeth and Assiniboia successfully analysed with flow cytometry.*

Table 3.11. shows the results of the second batch of successful flow cytometry analysis of plants regenerated from the copper experiment

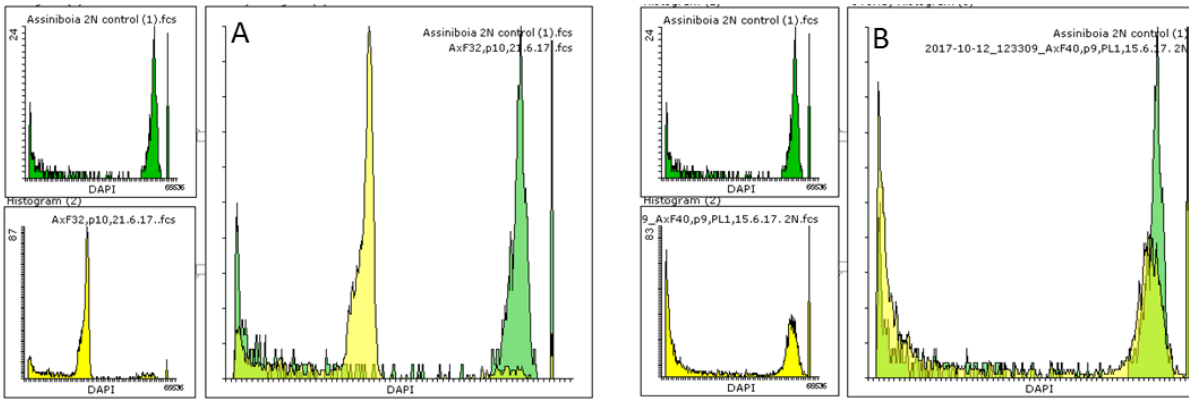


Figure 3.1.*Examples histograms of haploid (A) and dihaploid (B) regenerants from Assiniboia x Firth (yellow) compared to a control diploid Assiniboia plant (green).*

In **figure 3.1.** examples of haploid and dihaploid regenerants of AxF are shown. Peak height and width varied between samples. Many samples were poor, and could not be scored as being either 1n or 2n due to the low count of fluorescing cells. The availability of leaf material prevented repeat of some screens.

3.3.3. Analysis of regenerants using SSRs

Leaf samples were taken from Assiniboia x Firth donor plants and from DH regenerants for DNA extraction and then for SSR marker analysis. The donor parents of DH regenerants which were successfully analysed with flow cytometry were selected. They were screened against the SSR markers to identify Assiniboia x Firth donor plants which were heterozygous. In **figure 3.2.** a regenerant of Assiniboa x Firth 40 which was successfully screened with flow cytometry is compared to its donor plant parent, and the parents of the cross Assiniboia and Firth. The results show a haploid, segregating regenerant (4) from a heterozygous donor plant A x F 40 (3) of the segregating parents Assiniboia and Firth (1 & 2).

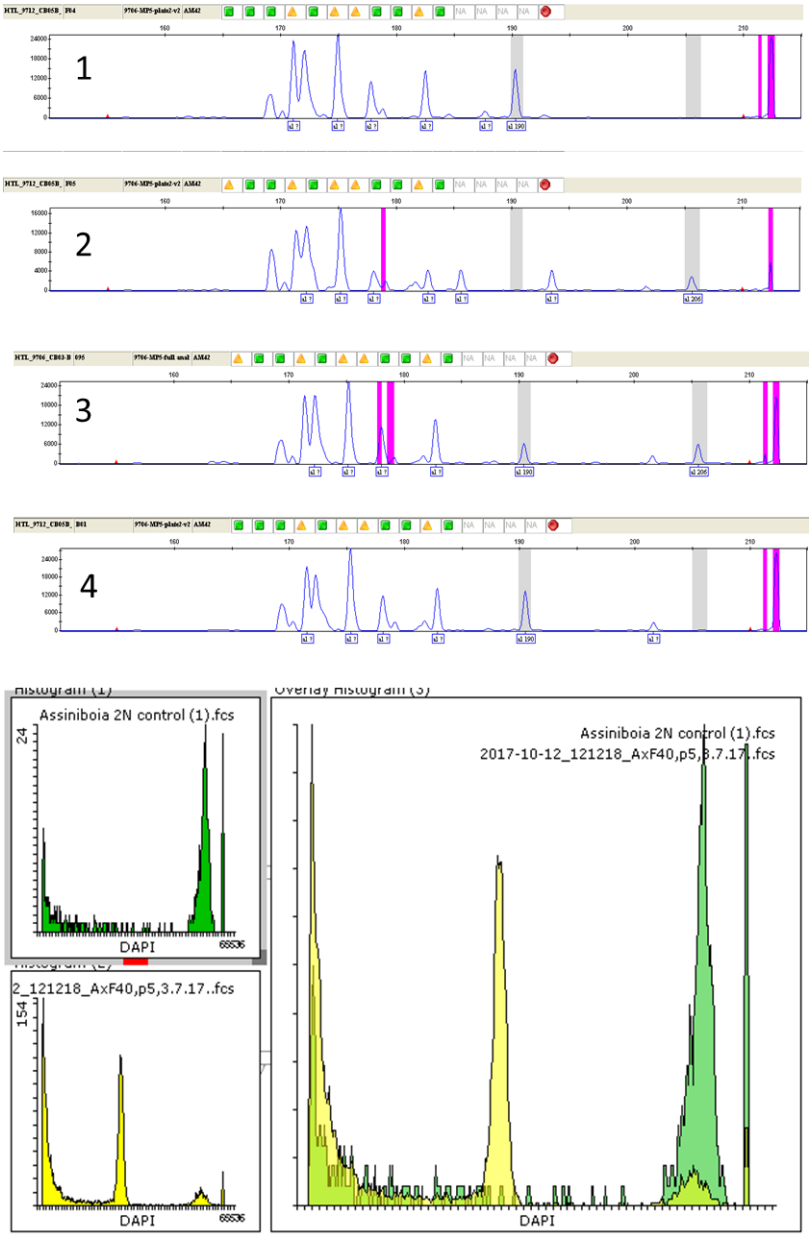


Figure. 3.2. *Results of the SSR marker AM42 segregating in the offspring of Assiniboia x Firth 40. 1:* Assiniboia., 2: Firth., 3: AxF 40 donor parent., 4: AxF40,p5.3.7.17. regenerant. The flow cytometry histogram shows the number of events (Y axis) against the fluorescence (x axis). The 2n Assiniboia in green represents the dihaploid, and in yellow, a haploid regenerant showing a strong haploid peak at half the size of the dihaploid.

3.3.4. Colchicine treatment

Colchicine treatment was not attempted on the haploid regenerants of the due to the high risk of mortality. Regenerants from the Assiniboia and Lisbeth genotypes were selected due to their low value after flow cytometry screening. The development stage was also important (Morgan, 1976). The single haploid Lisbeth regenerant (Lisbeth.3.2.4.) and two diploid Assiniboia regenerants (Assiniboia 5.6.4. and Assiniboia 6.9.4.) were selected for treatment as per the methods described in **3.2.3.** The treatment was not successful and all the plants died.

3.4. Discussion

3.4.1. Anther culture of the *Avena* species *A. sterilis* and *A. fatua*

Avena sativa is one of the more recalcitrant species of cereal crops for double haploid production. One hypothesis for this is that the 'genetic tools' required for efficient embryogenesis have been lost during domestication. The wild oat species *Avena sterilis* L. and *Avena fatua* L. both contain a wealth of genes that would be highly beneficial for the cultivated oat *Avena sativa*. Both have been crossed with *A. sativa* (Lawrence & Frey, 1975; Stevens & Brinkman, 1986) followed by backcrossing to *A. sativa* in an attempt to introgress candidate genes. *A. sterilis* for example contains potential powdery mildew resistance that would be highly beneficial for cultivated oat (Okoń *et al.,* 2016). Both species may have retained greater developmental plasticity than the cultivar, which could enable DH response.

Three genotypes of wild species were tested, *A. sterilis* B443 and two *A. fatua* accessions. *A. sterilis* was tested with ovary co-culture and with the addition of GAM (**table 3.2.**) while *A. fatua* was tested on standard MDHM medium. The poor anther response and low number of regenerated plants (two *A. sterilis* only) do not support the idea that the wild relatives shows significantly greater DH potential than *A. sativa*. Only 19 anther cultures were tested for *A. fatua,* however, as the donor plants were difficult to grow,

and produced poor quality panicles. Larger quantities of better quality donor material would be required to confirm this poor response. To date, there are no reported attempts to conduct DH production of *A. fatua* although anther culture of *A. sterilis* has been conducted previously and was successful regenerating some plants (Kiviharju *et al.,* 1997; Kiviharju & Pehu 1998; Kiviharju & Tauriainen 1999; Kiviharju *et al.,* 2004). The concentration of 2,4-D used at 5-6 mg/L did have a positive effect on *A. sterilis* (E. M. Kiviharju & Tauriainen, 1999), which is higher than the 1.5 mg/L used here. They also reported that the use of kinetin at 0.5 mg/L to be beneficial, which is the same as used here. Further experimental work, testing higher levels of 2,4-D as per (E. M. Kiviharju & Tauriainen, 1999) could be key. It is important however to highlight that the genotype of *A. sterilis* is different, and the differences in phytohormone concentration might be due to this factor.

3.4.2. Anther culture of F2 oat populations

The use of F2 hybrids is a valuable extension to the use of inbred varieties for confirming that DH protocols are behaving as expected, as only plants regenerated from heterozygous lines can be screened for homozygosity, a key test of whether the regenerants originate from microspores. Segregating lines also raise the possibility of identifying relevant genetic factors at a later date, and indicate the likely efficiency of the protocols when applied to commercial breeding material. The first population used was Canyon x Firth. Both are current, agronomically important cultivars and so generating a DH population from lines of this cross would be highly beneficial, particularly if they showed high efficiency to current protocol. The results in **2.3.1.3.,** however, showed that the lines tested were recalcitrant to the DH protocol.

Following this, populations using genotypes which had been proven to show some response to anther culture were tested in **section 3.3.2.** Seven lines of Assiniboia x Aslak progeny were tested (**Table 3.4.**) and showed a clear preference for the MDHM media over MIMI. A difference in response appeared to be segregating, with several lines having comparatively many responding anthers. The number of different treatments being

tested restricted the number of donor lines that could be used so that firm conclusions about the segregation pattern could not be made.

Progeny of an Assiniboia x Firth population was used for the next experiments. The experiments conducted with Assiniboia are summarised in **table. 3.5.** The key finding from these experiments is the effect glutamine had on the anther response and plant regenerant number. Glutamine was tested with Firth (**table 2.18.**) and had no effect, suggesting that Firth's recalcitrance cannot be overcome with the use of organic nitrogen. Assiniboia is a responsive genotype and its efficiency was greatly improved with additional glutamine. This parental difference in response to glutamine is apparent in the results of the final experiment, where 1 g/L of glutamine is used in the medium (**table 3.9.**). Not only is the mean efficiency rate the highest, but more significantly, more lines have anthers responding. In the experiments conducted without glutamine, about half of the lines did not have a single anther respond (**table 3.6., 3.9.**). Firth was not tested with 1 g/L glutamine, which might be key to inducing a response in the genotype.

Differential response of parents to DH protocols have been identified in barley and shown to cause segregation distortion in progeny by analysis with single sequence repeat (SSR) markers (Sayed *et al.,* 2002). It was shown that the proportion of loci deviating from the expected monogenic segregation ratios in the DH population was significantly higher than in the F2 population, with the deviation biased to the parent which had been found to regenerate more plants in IMC (Sayed *et al.,* 2002). There is an additional selection pressure applied through the use of the IMC protocol. Segregation distortion has been reported often in barley regenerated from anther culture (Graner *et al.,* 1991; Zivy *et al.,* 1992) which usually favours the parent which less recalcitrant to the DH method.

The highest efficiency with Assiniboia x Firth was recorded with the MDHM medium containing 1 g/L glutamine, yielding 0.3 green plants per hundred anthers. This far below the best results of Assiniboia, which reached around 5 green plants per hundred anthers (**table 2.15.**) however it is a big improvement on the previously tested populations of Assiniboia x Aslak and Canyon x Firth. These variations have been recorded in other oat

populations used for anther culture. Ponitka and Slusarkiewicz-Jarzina, (2009) tested nine different cross combinations, of which five did not regenerate any green plants. The highest performing cross was Flämingsprofi x Rajtar which regenerated 19 green plants, 0.6 green plants per hundred anthers. The cross used for the first double haploid linkage map of oat created by Tanhuanpää *et al.,.,* (2008) was between Aslak and Matilda, which created a total 186 green regenerants from 26,070 anthers, meaning 0.7 green regenerants per hundred anthers. With some further improvements to the protocol, such as applying the copper treatment which was successful with Assiniboia (**section 2.3.2.6.**) the rate of green plant regeneration could potentially be improved further. Applying medium improvements from **chapter 2.** could also be applied to Assiniboia x Aslak.

3.4.3 Flow cytometry

It is important to know the proportion of haploid to diploid plants regenerated from DH culture. Haploid plants are infertile, and have no little value for the researcher or the breeder so must be treated with a chromosome doubling agent to restore fertility. While expensive and time consuming on a large scale when many hundreds of regenerants need to be screened, flow cytometry offers precise and reliable results when conducted with an optimised protocol. In total 19 plants were successfully measured, of which ten were diploid and nine haploid. The reported ratios of haploid to diploid for oat vary. One haploid and two diploid plants were produced from cv. Stout. (Sun *et al.,* 1991), while in another study of 13 green regenerants, one was dihaploid (Kiviharju *et al.,* 2000). Of 137 DH individuals regenerated from an Aslak x Matilda population, 82 were haploids and required induced doubling of chromosomes (60%) and 55 individuals were spontaneously doubled haploids (Tanhuanpää *et al.,* 2008). *A. sterilis* cultures produced 5 haploid and 3 diploid green plants while progeny of crosses between *A. sativa* × *A. sterilis* produced 29 haploid and 6 diploid green plants (Kiviharju & Pehu, 1998). This suggests that genotype and species affect ploidy level in regenerants, and that the ability to spontaneously double is controlled genetically. This can be investigated in future work as more regenerants from newly responding genotypes such as Lisbeth and Lisbeth x Assiniboia regenerants can be analysed. Genotypes with high levels of spontaneous doubling could

be valuable, as the time and cost of induced doubling increases steeply when large numbers of regenerants must be used.

Overall, the flow cytometry protocol followed did not work well for oat. Many attempts were made on several different machines to get a reliable result. The results presented here are the first which showed consistent peaks for the control plants, as well as the haploid and dihaploid regenerants. Leaf samples taken from older leaves rarely showed florescence of cells in the expected range. Assiniboia used for the control was at first taken from mature donor plants that had been used for anther culture, but leaf material from newly germinated seedlings proved necessary to get a reliable result. This was also a problem with the regenerants as the required sampling significantly reduced the plant's vigour, particularly if plants needed to be sampled multiple times in an attempt to get a clear histogram result. The problem was much worse with albino plants possibly due to their particularly weak, thin leaves. In most cases the entire plant was harvested for flow cytometry analysis as their value was low in the long term, however this meant it was impossible to repeat if the sample failed with flow cytometry. Leaf material also had to be shared with the SSR marker work, to ensure DNA was available.

3.4.4. SSR marker analysis

The use of SSR markers to validate the assumption that plants regenerating from anther culture were originating from microspores was a key step. This is especially important when new methods are developed, or methods are transferred between labs. Combined with the flow cytometry results, there is now strong direct evidence that regenerants originate from the microspore. It was found that many of the markers developed for diversity studies of cultivars were not segregating within the Assiniboia x Firth donor lines, which reduced the number of regenerants that could be screened. A greater number of markers would allow a more universal application of this approach, but the associated labour and costs would then rise.

3.4.5. Colchicine treatment

Efforts focussed on setting up a reliable flow cytometry method to confirm that plants were regenerated from haploid cells, and to identify the proportion of haploid regenerants. Consequently, there were less opportunities to use and optimise the colchicine protocol for oat. It is expected that colchicine treatment will become routine use for treating haploids by using regenerants of positively responding genotypes such as Assiniboia after optimisation of the protocol. With reduced mortality rates, haploid regenerants of important DH cross populations can be doubled, increasing the size of the usable population for use with QTL or MAS breeding techniques.

3.4.6. Summary

It had been hoped that the key to successful DH production might be found in wild oat species, which might have escaped a loss of developmental plasticity through the domestication process. Unfortunately there was no evidence for a greater responsiveness in the accessions used here, and poor response to anther culture may be a general feature of *Avena* species.

The production of DH oat populations is key for successful application of the DH process in oat, and also as a validation system for the process itself. DH plants regenerated from inbred cultivars are virtually indistinguishable from their parent, so regenerating homozygous plants from a heterozygous population was key. By applying DH production optimisation steps conducted with inbred lines it was possible to produce a small DH population of Assiniboia x Firth. Analysis of the population with flow cytometry showed that haploid and dihaploid plants were regenerated while SSR marker analysis confirmed that homozygous plants were regenerated. This was a key proof that the protocol was leading to culture and regeneration of plants from gametophytic material, and is an important step towards the practical implementation of the DH system in oat.

Chapter 4: Genetic transformation of oat

4.1. Introduction

Genetic modification (GM) of crops is commonly associated with the introduction of genes from alien sources such as bacteria or fish. The widespread hostility to such exotic transfers has led to resistance to GM use from consumers even when genes are transferred from one crop to anther, such as Golden Rice 2 (Key *et al.,* 2008). Public opinion may change, but it is unlikely that a GM oat crop would find a market in the UK or Europe in the foreseeable future (Costa-Font *et al.,* 2008) particularly as oat is marketed as a healthy food (Fitzsimmons, 2012).

Despite this, there is increasing pressure to introduce new genes into crops for agronomic performance (disease and stress resistance) and commercial advantage (nutrient content and yield). Introducing novel genes by conventional means from unadapted sources of diversity is a difficult process as linkage drag of undesirable traits requires multiple rounds of backcrossing to be eliminated, and wide crosses can even require the use of intermediates to overcome incompatibilities. GM has the ability to generate lines resembling the intended novel varieties to test phenotype suitability, and validate genes in advance of the laborious conventional procedures.

Efficient tissue culture procedures are key to successful GM and the improvements in DH culture methods described in the previous chapter suggested that it be of interest to test GM protocols too. This chapter investigates two methods of transformation; biolistics and *agrobacterium*-mediated transformation with the aim of creating a reliable genetic transformation protocol for oats.

4.1.1. Genetic improvement

Domestication of the first agricultural plants is estimated to have started around 9,000 to 11,000 years ago. Since this time humans were growing and selecting plants for desirable traits. Over thousands of years this man made selection along with natural evolutionary changes has produced plants which are often unrecognisable from their wild ancestors. This combination of man-made and natural selection has led to increased and more consistent yields, better abiotic stress resistance, disease resistance and improved

nutritional value. However, selection for immediate gains may have inadvertently led to the loss of genetic diversity which has potential value in the long term (He & Bjørnstad, 2012). Oat is particularly prone to genetic bottlenecks due to the nature of its domestication. Oat was domesticated later than wheat and barley, and arose as a result of secondary domestication. The initial adaption of oats' wild progenitors as weeds of barley and einkorn resulted in indirection selection of particular agronomic traits (Preece *et al.,* 2017) with subsequent cultivation primarily as an animal feed and forage crop. It has only been more recently that cultivation has been directed towards milling and human use (Moore-Colyer, 1995). Valuable diversity may have been lost during domestication as a consequence, compared to other crops that have had a much longer history of cultivation for food. Recovering diversity could require returning to landraces or wild relatives that are significantly different from current elite cultivars, particular in traits such as agronomic yield under field conditions or amenability to efficient milling. Crosses between cultivated oat (*Avena sativa*) and wild species such as *Avena sterilis* and *Avena fatua* which contain a multitude of useful genes are known to create a large proportion of undesirable offspring (Lawrence & Frey, 1975; Stevens & Brinkman, 1986). There is a major challenge for conventional breeding to meet predicted cultivar improvements required to meet a population expected to reach 10 billion by 2050 (Evans, 1998), when combined with a predicted loss of arable land due to drought and increased salinity (Wang *et al.,* 2003).

4.1.2. Using genetic transformation to understand gene function

The publication of the genome of the model plant species *Arabidopsis thaliana* (Initiative, 2000) was a monumental achievement. Since then the number of plant genomes being sequenced has grown dramatically. As of January 2018, 472 plant genomes representing 289 different species have deposited in the NCBI database. As expected the focus has been on agronomically important species such as rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*). Important model crops such as *Brachypodium distachyon*, an important model grass with a much smaller genome than its important crop close

relatives like wheat and barley, and Chinese cabbage (*Brassica rapa*), a close relative of the very important vegetable oil crop oilseed rape (*Brassica napus*) which also has a complex genome, have also been sequenced.

However, sequencing the genome is only one step in understanding the growth and development of plants, and in particular in identifying candidate genes responsible for agronomic traits targeted by breeders. The size of many plant genomes presents huge bioinformatics challenges for annotation and prediction of gene function, and this may be further complicated by the presence of multiple homeologues in polyploid species. Many important crops are polyploid such as the tetraploid crops durum wheat, maize, cotton, potato and tobacco, and hexaploid crops like bread wheat, triticale, and oat. Having multiple genomes has advantages in breeding (Sattler *et al.,* 2016) but alters the phenotypic impact of changes in individual genes. For example, domestication frequently proceeds by loss of functions required for survival and propagation in the wild which hinder harvest, storage or use in crops (such as seed shattering, awns). In diploid species, recessive loss of function at a single gene may occur relatively easily, while a hexaploid may require much rarer gain-of-function (dominant) or multiple loss-of-function changes to achieve the same phenotype. In addition, the development of pan-genome resources for increasing numbers of species has demonstrated that gene content is not constant within a species, with as many as ten thousand genes showing potential presence-absence variation in rice (Zhao *et al.,* 2018). Consequently, there is a need to validate candidate genes identified by sequence alone before investing effort in allele discovery or introgression programmes.

In conventional (forward) genetics, experimental populations or diversity panels are used to map the trait of interest and then to attempt to identify candidate genes based on associated sequence variation. Reverse genetics takes the opposite approach by attempting to create a mutant phenotype with a defined sequence. While the most obvious reverse genetics approach is to look at the effect of expressing the artificial sequence in its transgenic hose, a valuable alternative emerged with the discovery of gene silencing mediated by RNA interference (RNAi) (reviewed by Harwood, 2012) has

been one of the biggest discoveries of the last few decades. The interfering RNAs are generated as double strand RNA (dsRNA), which is then cleaved into small strands called small interference RNA (siRNA), which in turn binds to mRNA and direct its degradation. As the gene is not silenced entirely, this method can be used on genes which are crucial for living without killing the organism, something not possible with permanent gene knockout where gene expression is reduced by 100 % permanently (Hood, 2004). This was developed in animal research, but is now applied widely in plants, including for crop improvements (reviewed by Saurabh, Vidyarthi and Prasad, 2014). RNAi may be induced in plants by transient or stable methods with particle bombardment or *Agrobacterium* inoculation, such as in wheat and *Brachypodium* (Lv *et al.,* 2014; Wiley *et al.,* 2007). A powerful extension of RNAi, virus induced gene silencing can also be used for transient expression studies of genes, where stable transformation is lethal to the host (Burch-Smith *et al.,* 2004). However, containment procedures required for VIGS limit availability, while RNAi can be carried out using standard transformation methods and facilities. RNAi vectors may be designed to silence all homeologues in a genome, making it particularly convenient for testing gene function and validating candidates in polyploids such as oat.

A striking example of the use of reverse genetic approaches to directing breeding strategies is given by the development of wheat with very high resistant starch content (Regina *et al.,* 2015). Wheat is a very important food staple around the world, and producing more nutritionally rich cultivars would have a global impact. Resistant starch is an important dietary fibre but its presence in starchy food is generally low. It was found that there is a positive relationship between resistant starch and amylose, and that by supressing the genes behind starch branching enzymes activity by RNAi increased amylose content. Knowing this, traditional breeding approaches were used to generate mutations or deletions in these genes to generate loss of function alleles, thereby creating amylose rich lines without the direct use of GM.

4.1.3. Genetic transformation

Genetic transformation is a technology that introduces genetic material into the genome of a cell, which cannot always be achieved through conventional breeding. Genetic transformation is a highly desirable technology as it has the potential to insert novel, desirable exogenous genes from virtually any source into another organism's genome without disrupting the competence of the host organism (Newell, 2000) or requiring time consuming crossing and backcrossing. Using this form of genetic manipulation crop productivity can be improved by inserting genes which grant disease and pest resistance (Pink & Puddephat, 1999) or increased tolerance to environmental stress factors (Gubiša *et al.,* 2007) to increase yields.

4.1.4. Target cells for genetic transformation

Before genetic transformation can be attempted, a reliable tissue culture and plant regeneration system is necessary. Plants are capable of producing callus which is a mass of undifferentiated plant cells which form on plant wounds. With the right addition of plant hormones, callus can be induced from plant tissue and cultured in vitro on a media (which can be solid or liquid) which contains nutrients for the callus to grow. Under optimal conditions, embryogenic callus with the addition of exogenous hormones can regenerate into whole, fertile plants. While dicots have been successful in *in vitro* culture, regenerating from most tissues, monocots have been more recalcitrant (Morel & Wetmore, 1951) due to the poor regenerative ability of differentiated cells (Aloni & Plotkin, 1985). Protoplasts (Altman *et al.,.,* 1977), root sections (Ron *et al.,* 2014), stem segments (Moore *et al.,* 1992) floral tissues (Clough & Bent, 1998) hypocotyls and other plant cell tissues have also been used for plant transformation, as long as whole plants can be regenerated. Without reliable plant regeneration the genetic transformation technology is of little to no value.

4.1.5. Methods for genetic transformation

There are many methods for inserting exogenous genes into plant genomes. Delivery of the genes may be indirect, where the exogenous DNA is inserted using a biological vector,

and direct, where a physical or chemical process is used to insert the DNA. Of the numerous methods, only three are regularly utilised due the lack of efficiency or repeatability of the others. The two most widely used methods are *Agrobacterium*-mediated transformation and biolistic bombardment transformation. This review will focus on these two methods, and their strengths and weaknesses.

4.1.6. *Agrobacterium*-mediated transformation

4.1.6.1 Introduction

In nature *Agrobacterium* spp. infect the wound sites of dicotyledonous plants. Infection can be recognised by the symptoms of crown gall disease; abnormal swollen growths on the roots, rhizomes and stems of plants. Crown gall disease is a significant problem for crops such as fruit and nut trees. Crown gall was known to be caused by *A. tumefaciens* since the early 1900s (Smith & Townsend, 1907) however it was not until later that research showed that gene transfer was responsible for the 'cancer' like growths. The first evidence for how *Agrobacterium* produced tumours in plants came with the discovery and identification of plant hormones. *Agrobacterium* induced tumours were found to be a source of auxin (Link & Eggers, 1941). These tumours were capable of growing in *in vitro* culture without the presence of the bacteria or the usual plant growth regulators which are normally necessary to elicit growth of callus (White & Braun, 1941). Braun (1947) hypothesised that *Agrobacterium* was the cause of the tumour induced effect, but it was not until molecular techniques were available that the DNA of the gall tumours could be analysed, and shown to contain DNA of bacterial origin (Schilperoort *et al.,* 1967). The tumour inducing (Ti) plasmid was later identified as the facilitator (Van Larebeke *et al.,* 1975). This eventually led to the discovery of T-DNA (Chilton *et al.,* 1978)

4.1.6.2. Taxonomy

Agrobacterium tumefaciens is a gram-negative, motile bacterium found in the soil, and belongs to the class of Alphaproteobacteria, which contains plant parasites and symbionts as well as human pathogens and non-pathogens (Williams *et al.,* 2007). The *Agrobacterium* genus is very closely related to the genus *Rhizobium*, bacteria which infect

the roots of legumes and fix nitrogen. The *Rhizobium* genus was first described by Frank (1889) as nitrogen-fixing bacteria that live in the root nodules of plants. The *Agrobacterium* genus was described by Conn (1942) to encompass plant pathogenic bacteria that caused galls and root disease. The two genera have been considered to be congeneric (Conn, 1942; Graham, 1964) but were still seen as two separate genera, until DNA sequencing technology showed that they could not be clearly separated (Willems & Collins, 1993). Young *et al.,* (2001), reclassified *Agrobacterium* into the genus *Rhizobium*, and changed the taxonomic structure of the family *Rhizobiaceae* based on 16S rDNA which found both genera rather than on the type of plant-microbe relationship. The new classification found the genera to be paraphyletic. However, some authors disagreed based on molecular and phenotypic data that *Agrobacterium* should be kept as a separate genus (Farrand *et al.,* 2003) which created uncertainty in the scientific community on how to call these bacteria. More recently it was found that the type of plant-microbe relationship was controlled by the mobile DNA elements, like Ti Plasmids, and that natural strains of *Rhizobium rhizogenes* can carry legume endosymbionts *nod* and *nif* genes responsible for nodule formation and nitrogen fixation, as well as the pathogenic *vir* genes responsible for the formation of tumours or hairy roots (Velázquez *et al.,* 2005). Based on this evidence it was argued that *Agrobacterium* should be a synonym of *Rhizobium* as *Rhizobium* was the first described name. The situation was finally settled at the International Committee on Systematics of Prokaryotes in 2010 to retain the *Agrobacterium* genus by transferring the more distant species of the genus to *Rhizobium* (Lindstrom & Young, 2011). Bacterial taxonomists and systematists still prefer to use *Rhizobium* but for many molecular scientists *Agrobacterium* is the preferred name.

4.1.6.3. Agrobacterium-mediated transfer

A. tumefaciens has the ability to transfer a piece of DNA referred to as T-DNA into the genome of the infected plant cell. The T-DNA is integrated into the host genome, and transcribed causing crown gall symptoms in the infected plant. The T-DNA is transferred to the plant cell in a tumour inducing (Ti) plasmid (Gelvin, 2017).

All Ti plasmids code for functions associated with virulence, opine utilization, plasmid replication and general maintenance, conjugative transfer, and sensory perception of exogenous signals released by the plant host as well as neighbouring agrobacterial cells at the site of infection (**figure 4.1.**). Genes encoding each of these functions are generally clustered on the Ti plasmid, with the exception of two spatially distinct regions, the virulence or *vir* region and the transfer-DNA or T-DNA required for infection of plants, and the *tra* and *trb* regions required for conjugative plasmid transfer. Two T-DNA fragments in the plasmid are integrated into the plant nuclear genome. The first contains oncogenic genes also referred to as phytohormones, which encode for enzymes involved in the synthesis of auxins and cytokinins which are responsible for the stimulation of uncontrolled plant cell division which create the gall tumours. The second carries genes encoding the synthesis of opines.

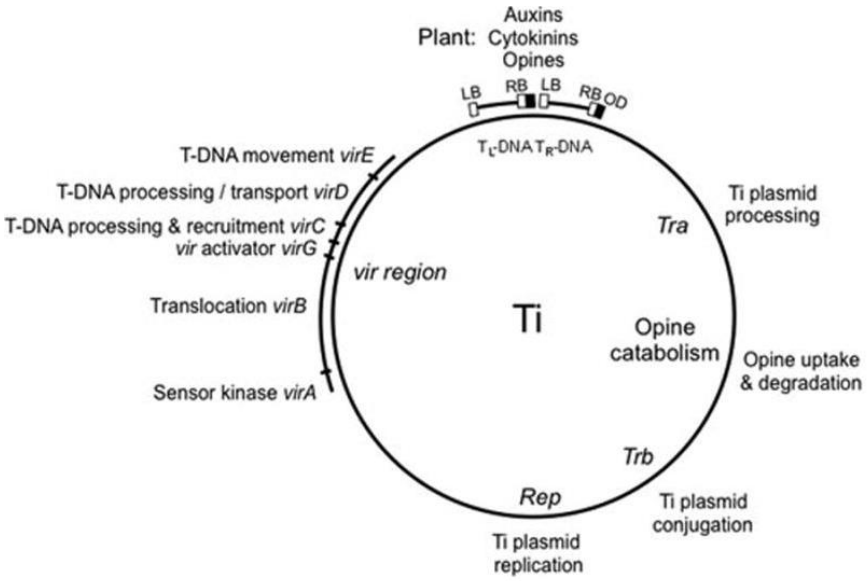


Figure 4.1*. Diagram taken from (Christie and Gordon, 2014) Schematic of octopine-type Ti plasmid pTiA6 showing locations of genes coding for plasmid maintenance (rep), infection of plant cells (vir region, T-DNA), cell survival in the tumour environment (opine catabolism), and conjugative transfer of the Ti (tumour inducing) plasmid to recipient*

agrobacteria (tra and trb).

Opines are produced by condensation between amino acids with ketoacids or sugars, and are synthesized and excreted by the crown gall cells so they can be catabolized and used by *A. tumefaciens* as a source of carbon and nitrogen. Plants are unable to catabolize opines, so opines are released into the extracellular space. Ti plasmids traditionally have been classified by the opine type. Opines are a diverse family of compounds (Moore *et al.,* 1997) produced only by *Agrobacterium*, however the opines produced by the crown gall cells are entirely dependent on the virulent strain of *Agrobacterium*. An *Agrobacterium* which contains genes for the synthesis and metabolism of the opine nopaline does not have the ability to metabolize octopine (Petit *et al.,* 1983). This opine specificity could possibly be a competitive factor, as other *Agrobacterium* strains in the vicinity cannot utilise the specific opine being produced. The majority of the functions of the plasmid are only initialised when extracellular signals are detected. These include those released from plant wound sites or other *Agrobacterium* cells. Upon sensing these extracellular signals the *Agrobacterium* starts the infection process, 1) T-DNA transfer, 2) inducing tumour growth, 3) production and catabolism of opines, 4) multiplication and spread of Ti Plasmids to infect more plant cells. The precise molecular events involved in *Agrobacterium*-mediated genetic transformation are not fully understood. Lacroix *et al.,* (2006) has proposed a possible method for how transferred DNA is integrated from the tumour-inducing (Ti) plasmid of the *Agrobacterium* into the nuclei of the receiving plant cells. *Agrobacterium* virulence is triggered by many extracellular plant derived signals which are present at plant wound sites, including phenolic compounds and monosaccharides and an acidic pH.

4.1.6.4. Manipulating Agrobacterium for genetic engineering

With the discovery that *Agrobacterium*-mediated transfer was able to insert DNA into a plant and that it be stably integrated into the plant genome and be expressed (Chilton *et al.,* 1977) it was immediately proposed that these Ti plasmids could be used as a vector to

insert genes of interest into plants. The problem however is that Ti plasmids are large and the T-DNA regions do not contain unique restriction endonuclease sites, so they are difficult to target for inserting genes of interest. Initial efforts began by trying to introduce the gene of interest into the T-DNA by recombining them into the T-DNA region of the Ti plasmid (Fraley *et al.,* 1985). The problem with this method is that the Ti plasmids are very big, and exist in low copy numbers within the *Agrobacterium* making them difficult to isolate and manipulate *in vitro*. This is further hampered by the fact they cannot be multiplied in *Escherichia coli*, which is commonly used for gene cloning (Cohen, 2013). There was also the need to remove the oncogenes from T-DNA so gall tumour free plants are regenerated. A major breakthrough was made by two groups simultaneously (de Framond *et al.,* 1983; Hoekema *et al.,* 1983). Both groups found that the *vir* and the T-DNA regions of the Ti plasmids could be split into two separate replicons. As long as both were present in the *Agrobacterium* cell then they could function normally. This system was named the T-DNA binary vector system. The T-DNA is located on the binary vector, and the non T-DNA region of the vector contained the origin of replications that could function in both *E. coli* and *Agrobacterium*, so the plasmid could be multiplied in *E. coli* before being introduced into the *Agrobacterium,* as well as antibiotic genes, so both organisms could be selected for to maintain the plasmid within them. This system revolutionised *Agrobacterium*-mediated transformation, as it was now not necessary to introduce into the T-DNA region of the Ti plasmid, but instead clone the gene of interest into small T-DNA regions within the binary vectors which were more suited for this purpose. This could be verified by culturing in *E. coli*, and then the plasmid could be introduced into an *Agrobacterium* strain by conjugation or transformation, which already contained the *vir* helper region.

4.1.7. Biolistics: transformation by particle bombardment

4.1.7.1. Introduction

Biolistics (short for "biological ballistics") is the method of transformation by accelerating high density micro-carrier particles, approximately 1-2 × 10^{-6} m in diameter coated with genes that pass through the cells and leave the DNA inside them where it is hopefully

incorporated into the organism's genome. The method was first designed at Cornell University in 1987 for the genetic transformation of cereals (Sandford *et al.,* 1987). The method was developed with the aim of transforming monocotyledons, which were until recently recalcitrant to transformation by *Agrobacterium*. Biolistics can be used on a wide range of animal, fungal and plant species. In plant research the main uses of biolistics are for transient gene expression studies, production of transgenic plants and inoculation of plants with plant pathogens.

4.1.7.2. Biolistics method

The biolistic particle delivery system or "gene gun" fires DNA coated micro carrier particles at high speed using gas acceleration to penetrate the tissue. The micro carrier particles are normally made out of gold, tungsten or platinum because they are non-toxic to cells and are high in density (Frame *et al.,* 2000). Gold particles are use more frequently because they are biologically inert and can be produced in smaller sizes (0.7 to 1.0 µm) (Altpeter *et al.,* 2004). For the micro-carriers to be more effective, and travel faster, a vacuum is created in the bombardment compartment. Current biolistics devices work on a very similar model, but vary in small ways. The Biolistic® PDS-1000/He particle delivery system, (Bio-Rad Laboratories, Hercules, CA.) uses a rupture disk system. Once the vacuum is created in the bombardment compartment, the pressure in the above chamber is increased using helium. When the pressure reaches the sufficient level, the rupture disk containing the macro-carriers breaks and the helium launches the plastic macro-carrier (which is covered in the DNA coated micro-carriers) down until it hits the stopping screen. The macro-carrier is halted but the micro-carriers continue through, collide with and penetrate the target tissue cells. Another method is the Particle Inflow Gun (PIG) (Finer *et al.,* 1992), and use 13 mm Swinney Filter Holders (Pall, UK). The DNA coated micro-carriers in an ethanol solution are placed onto the Swinney filter which acts as a replacement to the rupture disk. Instead of relying on the rupture disk to break at the required pressure, a "shot" of helium at the required pressure is fired down the gas acceleration tube at the Swinney filter, launching the micro-carriers at the target tissue cells. A baffle, in the form of a steel mesh placed above the target tissue, helps to

spread the micro-carriers (in case they are in a clump) across the target tissue, and also prevent the tissue from flying around when hit with the shot of gas. The velocity of the micro-carriers is dependent on the helium pressure in the gas acceleration tube, the distance from the rupture disk or Swinney filter to the stopping screen, and the distance from the stopping screen to the target tissue cells, and the amount of vacuum in the bombardment chamber. Helium is used over nitrogen or other compressed gases because it is very light, inert, has a high diffusivity, and expands faster, which means the carriers travel faster (Klein & Fitzpatrick-McElligott, 1993). It is important to optimise these conditions; if the velocity is too low the micro-carriers will not penetrate into the target tissue, and if the velocity is too high the target tissue can be destroyed.

4.1.8. *Agrobacterium*-mediated transformation vs Biolistics

Agrobacterium-mediated transformation is widely thought to be more precise, controllable and efficient than biolistics. However there are still many reasons why biolistics is not yet obsolete, with the choice between methods now being based more on what the aim of the research is. This section looks at the advantages and disadvantages of each method.

Genetic transformation is made up of two stages, DNA transfer into the cell, followed by DNA integration into the genome. The integration step is far less efficient than the transfer stage, so only a small number of cells will be stably transformed. DNA which enters the cells but does not integrate into the genome can be expressed for a short time (transient expression) until it is degraded by nucleases. Transient expression occurs almost immediately after gene transfer, occurs at a much higher rate than transformation and does not require the regeneration of whole plants (Ueki *et al.,* 2008; Kuriakose *et al.,* 2012). Transient expression after particle bombardment with a reporter gene such as *gusA* (Basu *et al.,* 2003) or *gfp* (Shah *et al.,* 2013) is used routinely to compare different expression constructs and identify those with the highest or most appropriate activity. Transient expression is also possible using *Agrobacterium*, (Bhaskar *et al.,* 2009) by the

method of agro-infiltration (Yang *et al.,* 2000) which allows for rapid analysis of plant promoters and transcription factors *in vivo*. Biolistics-mediated transformation does have the ability to transform plastids (Ruhlman, 2014), which is not possible with *Agrobacterium* as the T-DNA is targeted to the nucleus only. Biolistics can also be used to transform mitochondria (Kemble *et al.,* 1988). Biolistics does have the advantage of not relying on the biological constraints of a particular organism. Monocots were until the late 1990s recalcitrant to *Agrobacterium*-mediated transformation, and even now the transformation of cereals and legumes are limited to a small number of genotypes. The difficulties are reviewed by Sood, Bhattacharya and Sood, (2011). Biolistics-mediated transformation was devised in part due to this issue and is not limited in this way as it uses physical properties to force the DNA into organisms, and then relies on DNA repair systems to incorporate the DNA into the organism's genome. By optimising the physical factors of biolistics, the limitation of successful transformation is limited to the target tissues ability to regenerate after transformation and has allowed the successful transformation of even the most recalcitrant species. Examples of this are reviewed here (Christou, 1995). Due to the nonspecific targeting of biolistics any type of cell has the potential to be transformed if the DNA can be introduced without killing the target cell. The biological limitation is of the target cell's totipotency to successfully regenerate after transformation. This means the transformation of particular cells or tissues that cannot or are much more difficult to target such as apical meristematic cells (Rech *et al.,* 2008) are possible. Other cells include leaf discs (Ueki *et al.,* 2013), microspores (Yao *et al.,* 1997) and non-embryogenic tissues (Romano *et al.,* 2005). Another advantage of biolistics that a vector is not required. The exogenous DNA that is used in transformation experiments is usually made up of a plant expression cassette which is inserted into a vector that is based on a bacterial cloning plasmid. Successful DNA transfer does not rely on either, and only the expression cassette is required for the transgene expression in the target tissue. After vector cloning and purification of the plasmid, the rest of the vector is not required for successful transformation. This makes biolistics still a very useful tool for transformation. *Agrobacterium*-mediated transformation however is more efficient at co-

transformation, and is less prone to inserting multiple gene copies, which cannot be easily controlled in biolistics-mediated transformation (Komari *et al.,* 2004) Both techniques still have their merits, and have both been used in oats.

4.1.9. Genetic transformation of oat

The first published research of successful oat transformation was by Somers *et al.,* (1992) using the particle bombardment method. Friable calli derived from immature embryos were transformed with a plasmid carrying the *gus* and *bar* genes. A total of 111 tissues were recovered, from which 38 plants were regenerated. Phosphinothricin (PPT) was used as the selective agent. GUS activity was identified in the seed of fertile regenerated plants and PPT resistance in the progeny of these plants co-segregated with *bar* and *uidA* sequences demonstrating stable inheritance of the transgenes. As PPT is a herbicide, the risk of transformed oats cross pollinating with wild species led to a search for an alternative antibiotic resistance selective marker (Somers *et al.,* 1992). Oat callus was successfully transformed by particle bombardment with two different plasmids containing the *nptII* gene using the antibiotic paromomycin sulphate (Torbert *et al.,* 1995). 17 fertile plants were successfully regenerated from 88 transgenic tissue cultures, an efficiency similar to that using PPT. The use of biolistic transformation has been successful with a range of different oat tissue sources including callus from mature embryos (Cho *et al.,* 1999), leaf base segments (Gless *et al.,* 1998) and shoot meristems from germinated seed (Zhang *et al.,* 1999).

Using immature embryos requires the growth of mature oat plants over a wide time period to provide a stable supply of embryos at the desired development stage. This process is both labour intensive and requires a lead time of several months. Using leaf base segments from germinated seedlings *in vitro* removes the requirement for mature donor plants, and reduces the time period of culture from up to a year down to three – four months. (Pasternak *et al.,* 1999) A reliable protocol for regenerating plants from leaf base segments in oat have been published by Chen, Zhuge and Sundqvist, (1995) and Gless, Lörz and Jähne-Gärtner, (1998). Successful transformation of oat leaf base segments by bombardment was published by Gless *et al.,* (1998). The tissue was

cultured for two weeks on embryogenesis inductive medium for two weeks, and then bombarded with a plasmid carrying the genes *uidA* and *pat*. The calli were selected for on medium containing 2 mg/L PPT. The transformation frequency was 5%.

An efficient protocol for reliable regeneration of plants from shoot meristematic cultures (SMC) was developed by Zhang *et al.,* (1996), and later developed for successful transformation with bombardment (Zhang *et al.,* 1999). Bialaphos at 2 mg/L was used to select for transformed plants. Of the 61 oat plants transformed, 70% were fertile. This is higher than in oat transformation methods which use embryogenic callus and leaf base segments, possibly due to this method not requiring a callus stage that can reduce fertility by somaclonal variation (Zhang *et al.,* 1999).

More recently, attempts have been made to transform oat SMC with an osmotic stress resistant gene *hva1* (Xu *et al.,* 1996) as oat is not suited to dry environments (Maqbool *et al.,* 2002). The gene was successfully inserted into three of the four cultivars using biolistics, with the *bar* gene used for selection. The progeny showed stable expression of the gene, and their osmotic tolerance was increased. Kuai *et al.,* (2001) were able to regenerate plants transformed by biolistics-mediated transformation with the *bar* gene, and stable transmission and expression in the T_1 progenies occurred in a Mendelian manner.

Other reporter genes have also been tested in oat. The use of green fluorescent proteins (GFP) isolated from cnidarians is a universal reporter gene in bacteria (Prasher *et al.,* 1992). It has been successfully utilised as a marker gene in plant transformation (Chiu *et al.,* 1996). It is superior to *uidA* and *gus* because it can be observed without destructive sampling. In oat, plasmids containing the serine green fluorescent protein (*sGFP)* (S65T) gene and one of three selectable marker genes, phosphinothricin acetyltransferase (*bar*), hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase (*nptII*) were transformed by bombardment. The cultures were selected for with bialaphos, hygromycin and geneticin. Out of 289 transformed plants

regenerated, 23 independent transformants were obtained, of which 64% produced fertile plants.

To date even less work has gone into *Agrobacterium*-mediated transformation in oat. The successful *Agrobacterium*-mediated transformation of oat, and some of the factors involved, has been reported by Gasparis *et al.,* (2008). Three different cultivars, two forms of explant and three combinations of strain/vectors (which had been successfully used with other cereals) were tested. Transgenic plants were obtained from the immature embryos of all three cultivars Bajka, Slawko and Akt, and from the leaf base explants of the cultivar Bajka after transformation with the *A. tumefaciens* strain LBA44404(pTOK233) (Gasparis *et al.,* 2008). The highest transformation rate was obtained with immature embryos of the cultivar Bajka, of which 79% of the selected plants were transgenic. However, only 14.3% of the T_0 and 27.5% of the T_1 plants showed GUS expression. In the second part of the study tested the use of the pGreen binary vector with different selection cassettes *npt*II or *bar* under the nos or 35S promoter. In this experiment the highest transformation efficiency of 5.3% was obtained for the cultivar Akt with nos:*npt*II. Analysis of the T_0 plants showed a mixture of transgenic, chimeric-transgenic and non-transgenic individuals (Gasparis *et al.,* 2008).

4.1.10. Project aim

Before candidate genes of interest could be identified and prepared for transformation it was important to set up and an efficient and reliable system with plasmids already proven to work. Both biolistic transformation and *Agrobacterium*-mediated transformation take a long lead time from the initial plasmid design and preparation, plant tissue culture, transformation and finally selection and regeneration and stable transformation. Therefore, it would be preferable to have a reliable and efficient transformation protocol for oat to act as a template for future experiments which useful genes of interest to be applied to. This project aimed to test two different transformation methods, biolistic transformation and *Agrobacterium*-mediated transformation.

For biolistic transformation, two constructs pIOM6 and pJQ5, and a selection construct pUBA containing the *bar* gene were recommended by Sue Dalton (personal communication) as a starting point. The constructs pIOM6 (Buanafina *et al.,* 2015) and pJQ5 (Buanafina *et al.,* 2010) have been used successfully for plant transformation in the grass tall fescue *(Festuca arundinacea)* and had shown promising results in previous oat transformation experiments by Sue Dalton (personal communication, results not shown). The pUBA construct containing the *bar* gene would be used for screening regenerants selected on culture medium containing the herbicide bialaphos. Successful transformation with the *bar* gene would make the plant resistant to bialaphos. Successful transformation with the plasmids pLOM6 and pJQ5 would be tested with a PCR assay.

For *Agrobacterium*-mediated transformation two different vectors with different *A. tumefaciens* strains with different plasmids were selected which use *uidA* or *gus* as a selectable reporter gene which can be screened for by histochemical staining assay to reveal β-glucuronidase presence in the plant leaves. Both also contain respective antibiotic resistance SMGs (*hpt* or *bar*) to select successfully transformed plants.

4.2. Materials and Methods

4.2.1. Plant material

Mature seed of the cultivar Assiniboia harvested from 2016 was used for all of the experiments. The seed had been stratified at -18 °C to reduce the risk of grain weevil and improve germination homogeneity.

4.2.2. General culture media

All media was autoclaved at 120 °C for 15 min. After autoclaving 200 µl of sterile 1M NaOH was added per one litre of media to set the pH to 5.8. All Murashige & Skoog (MS) media was sourced from Duchefa Biochemie (Netherlands).

All medium recipes are from Sue Dalton, which are based media used for grass and cereal transformation. All petri dishes and containers were sealed with parafilm.

4.2.3. Seed germination and callus culture

4.2.3.1. Seed germination

The oat genotype Assiniboia was selected for use based on its positive record in tissue culture with DH. Seed was used in batches of 200. On the first day the seed was dehusked and surface sterilised in approximately 40 mL 70 % bleach 30 % sterile deionised water in a Falcon tube (Fisher Scientific UK) for 20 minutes and then rinsed in sterile distilled water approximately five times. The seed was then left over night in approximately 40 mL of sterile distilled water for imbibition at 4 °C in darkness. The next day the wash and rinse step was repeated. The seeds were sown on germination medium (**Table 4.1.**) evenly spread, approximately 15-17 seeds per plate, and were placed with the scutellum in contact with the medium. The germination plates were stored in the dark at 25 °C for two – four days.

Ingredient	**Amount per litre**
MS M0222	4.4 g
Gelrite	3 g
Sucrose	30 g
H2O	to 1000 mL
pH 5.6	

Table 4.1. *Composition of seed germination medium.*

4.2.3.2. Callus initiation

After 3-4 days the majority of seed had germinated and the shoot tip had emerged. When the shoot tip was 1-2 cm long the shoot section containing the meristem was removed by manual excision and placed onto callus initiation medium (**Table 4.2.**) containing 5 mg/L 2,4-D for three weeks, then moved to 3 mg/L 2,4-D for two weeks, then sub-cultured on fresh 3 mg/L 2,4-D for one week. Any shoots which grew during this period were manually excised.

Ingredient	Amount	Amount per litre
MS M0234	75 %	3.25 g
Maltose	3 %	30 g
2,4-D	5 mg and 3 mg/L	50 mL and 30 mL of 10 mg/100 mL stock
BAP	0.05 mg/L	500 µL of 10 mg/100 mL stock
Additive R18 (Sue Dalton, unpublished)		5 mL
N6 Vitamins (Sue Dalton, unpublished)	X5000 stock	200 µL
Proline	750 mg/L	750 mg
Glutamine	1000 mg/L	1000 mg
H2O		to 1000 mL
pH 5.6		

***Table 4.2**. Composition of the callus initiation medium.*

4.2.4. Particle bombardment

4.2.4.1. Plasmid material

The plasmid pUBA was used as a selection marker, fungal genes with potential effects on cell wall composition were provided by plOM6 and pJQ5. The plasmid pUBA is a 2-kb HindIII-BamHI fragment containing the maize Ubi- 1 promoter (Cornejo *et al.,* 1993) ligated into the 3.6-kb chimeric bar structural gene (15)-nopaline synthase terminator

(2)-pUC12 sequence (Toki *et al.,* 1992). The herbicide-resistance gene named *bar* encodes the enzyme phosphinothricin acetyl transferase (PAT) which acetylate L-phosphinothricin, the active isomer of glufosinate-ammonium herbicides such as phosphinothricin (PPT) or bialaphos. The plasmid pJQ5 contains a genomic clone of ferulic acid esterase (*FAEA*) from *Aspergillus niger* which had been used for the construction of vectors in pCOR105 plasmids was placed under the *Lolium multiflorum* senescence-enhanced gene promoter and intron (*LmSee1*) with the barley aleurain vacuolar target sequence modified for apoplast targeting (Buanafina *et al.,* 2010) based on the pCOR105 plasmid. The plasmid pIOM6 is a *Trichoderma reesei* b-1,4 endoxylanase gene (*xyn2*) gene placed under the control of a *Lolium multiflorum* senescence-enhanced gene promoter and intron (*LmSee1*) targeted to the apoplast (Buanafina *et al.,* 2015).

4.2.4.2. Biolistic delivery gene gun

The particle bombardment device used was based on the Particle Inflow Gun (PIG) (Finer *et al.,* 1992), and used 13 mm Swinney Filter Holders (Pall, UK) in place of rupture disks. The filters were washed and autoclaved after each use. A vacuum pump was used to create a vacuum of 18'' Hg, and helium was used as the propellant at a psi of 101 (7 bar).

4.2.4.3. Osmotic treatment

The calli were placed together in the centre of the petri dish in a single layer in a 3-4 cm diameter target containing osmotic treatment medium (**Table 4.3.**). The callus was left on this medium for a minimum of 4 hours prior to bombardment to plasmolyse the cells.

Ingredient	Amount	Amount per litre
MS M0222		4.4 g
Maltose	3 %	30 g
Sorbitol	9 %	90 g
2,4-D	3 mg/L	30 mL of 10mg/100 mL stock
H2O		to 1000 mL
pH 5.6		

Table 4.3*. Composition of high osmolarity medium.*

4.2.5. Preparation of gold particle stock solutions

0.6 µm gold particles (Bio-Rad, Richmond, CA, USA) were prepared at 100 mg/mL stock solution in water. DNA was isolated from plasmid preparations and the concentration was generally lower than 1 µg/µL. $CaCl_2.2H_2O$ solution was prepared at 2.5 M or 5 M and used depending on the DNA concentration. All were stored at -20 °C. Spermidine solution in water was prepared one hour before transformation at 14.5 mg/mL.

4.2.6. Preparation of gold particle solution

All components were thawed and kept on ice. Generally gold particles for several bombardments were prepared in one Eppendorf. For each bombardment 10 µl gold particles were used. The gold particles were vortexed before pipetting to ensure a homogeneous suspension. 10 µl of the selective DNA, 10 µl DNA of the gene of interest (GOI) and 25 µl $CaCl_2.H_2O$ were pipetted to a sterile Eppendorf tube and vortexed before adding 10 µl spermidine. The solution was left to stand for 5-10 min on ice before centrifuging. After centrifuging the supernatant was removed and the pellet was rinsed twice, immediately with ethanol. 10 µl of ethanol was added to the pellet per bombardment and vortexed until the pellet was re-suspended. The Eppendorf tube was kept on ice until it was time to use.

4.2.7. Delivery of DNA-coated gold particles

The gun chamber was sterilised with 70 % (v/v) ethanol, and the steel gauze components, stopping screen and filter holders were autoclaved. For each sample, the coated gold particles were briefly vortexed, and then 10 µl was placed onto the filter base in the Swinney filter holder and screwed into place. The target petri dish support was inserted with a 150 grade steel gauze baffle 0.5 cm above the tissues. The tissue was approximately 13 cm below the Swinney filter holder. After closing the chamber door, a vacuum pump was used to draw a vacuum of 28 mmHg. The pump was switched off and the gene gun immediately fired to deliver a 0.01 second pulse of helium gas at 7 bar

through the filter holder. The bombarded callus cultures were left overnight on high osmolarity medium in darkness at 25 °C.

4.2.8. Selection and regeneration of transformed callus

The following the day the callus was transferred to callus selection medium (**Table 4.4**.), sealed with Parafilm (Bemis Company, INC., USA) and stored at 25 °C. The herbicide Bialaphos was sterilised by filtration and added to the medium after autoclaving.

Ingredient	Amount	Amount per litre
M0235		3.35 g
Sucrose	3 %	30 g
2,4-D	3 mg/L	30 mL of 10 mg/100 mL stock
BAP	0.05 mg/L	500 uL of 10 mg/100 mL stock
Additive R18 (Sue Dalton, unpublished)		5mL
Thiamine	1 mg/L	1 mL of 100 mg/100 mL stock
Proline	750 mg/L	750 mg
Glutamine	1000 mg/L	1000 mg
H2O		to 1000 mL
Bialaphos	3 mg/L	1500 µL of 100 mg/50 mL stock
pH 5.6		

Table 4.4. *Composition of callus selection medium.*

Ingredient	Amount	Amount per litre
M0235		3.35 g
Sucrose	3 %	30 g
NAA	1 mg/L	1 mL of 100 mg/100 mL stock
BAP	1 mg/L	10 mL of 10 mg/100 mL stock
Additive R18 (Sue Dalton, unpublished)		5 mL
Thiamine	1 mg/L	1mL of 100 mg/100 mL stock
H2O		to 1000 mL
pH 5.6		
Bialaphos	3 mg/L	1500 µL of 100 mg/50 mL stock

***Table 4.5**. Composition of regeneration selection media.*

Over four to five weeks, non-transformed callus gradually turned brown. Callus which still looked pale and alive was transferred to regeneration selection medium (**Table 4.5**.) and cultured under approximately 100 µM/m2/s.

Plants which successfully regenerated were moved to rooting media (**Table 4.6.**). Leaf material for DNA isolation and PCR screening was sampled from plants over 5 cm in height.

Ingredient	Amount	Amount per litre
MS0222		4.4 g
Sucrose	3 %	30 g
H2O		to 1000 mL
Bialaphos	4 mg/L	2000 µl of 100 mg/50 mL stock
pH 5.6		

***Table 4.6.** Components of the rooting media*

Plants which rooted were transferred to the greenhouse and planted in soil under lights when the roots were approximately 2+ cm long.

4.2.9. *Agrobacterium*-mediated transformation

4.2.9.1. Agrobacterium strains and plasmids

Two different *Agrobacterium* strains and plasmids were tested. Both contained the β-glucuronidase reporter gene (*uidA*) and a selectable marker gene. The first, referred to in this work as AGRO1, was the binary vector pTF102 (Frame *et al.,* 2002) in the *Agrobacterium tumefaciens* strain EHA105. pTF102 contains the selectable marker gene *bar* (phosphinothricin acetyltransferase) driven by the cauliflower mosaic virus (CaMV35S) promoter and a P35S-*gus*-int reporter gene cassette (*gus* gene with an intron driven by the CaMV35S promoter). The second, referred to as AGRO2, was an *A. tumefaciens* strain AGL1. This construct contains the hygromycin phosphotransferase resistance gene (*hpt*) under the CaMV promoter pBRACT204 and the *gus* reporter gene under the maize ubiquitin promoter.

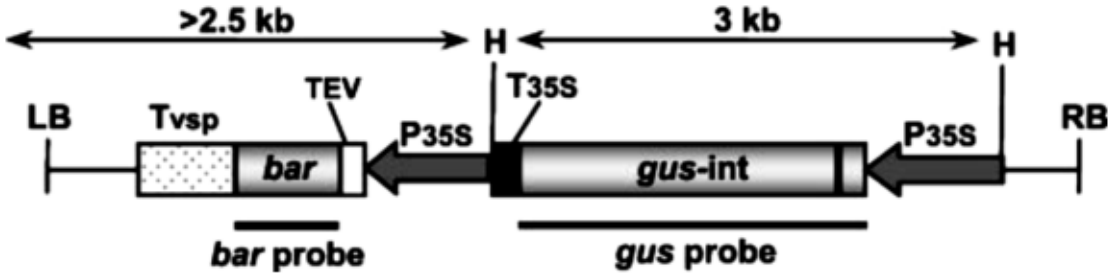


Figure 4.2. *T-DNA region of the standard binary vector pTF102. LB, Left border; RB, right border; bar, phosphinothricin acetyltransferase gene; gus-int, β-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer ; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator; H, HindIII. Adapted from Frame* et al. *(2002).*

4.2.9.2. Growth of Agrobacterium inoculum

The *A. tumefaciens* strain EHA105, harbouring the vector pTF102 (**figure 4.2.**), was pre-cultivated for two days on solid YEP medium containing 100 mg/L spectinomycin (for maintenance of pTF102) and 25 mgL^{-1} rifampicin (for EHA105) at 25 °C in the dark. The *A. tumefaciens* strain AGL1 harbouring the vector pBRACT204 was pre-cultivated for at least two days on solid YEP medium containing 100 mg/L kanamycin (for maintenance of GUS) and 25 mg/L rifampicin (for AGL1) at 25 °C in the dark. Two plates per strain were grown.

4.2.9.3. Agrobacterium-mediated transformation of callus

Inoculation began by using an inoculation loop to scrape a sample of the bacteria into a Falcon tube containing 50 mL *Agrobacterium* infection medium (4.3 g/L MS medium without vitamins, 68.4 g/L sucrose, 36 g/L glucose, 0.7 g/L proline, 3 mg/L 2,4-D, 100 µM glutamine, 200 µL/L N6 vitamins, 0.01 % Pluronic F68 and 300 µM acetosyringone, pH5.2). The tube was shaken at approximately 100 rpm for at least 90 minutes. The optical density (OD_{600}) was measured using a Biochrom WPA CO8000 (Biochrom, UK). Approximately 3-4 mL of callus tissue was placed in a 35 mm petri dish (Greiner Bio-one, Austria), with 4 mL of *Agrobacterium* solution. Plates were shaken to ensure the calli were coated in the solution, and then left to stand for 30 minutes. A sterile Pasteur pipette was used to carefully remove the *Agrobacterium* solution. Calli were moved to 90 mm petri dishes containing three dry filter papers and left to dry for 40 min. Callus was then transferred to fresh petri dishes containing three filter papers wetted with 3 mL of co-cultivation medium (4.3 g/L MS medium without vitamins, 60 g/L maltose, 300 mg/L cysteine, 3 mg/L 2,4-D, 100 µM glutamine, 200 µL/L N6 vitamins, 300 µM acetosyringone, pH5.2). The plates were sealed with Parafilm (Bemis Company, INC., USA) and stored at 25 °C for three days.

Ingredient	Amount	Amount per litre
M0235		3.35 g
Sucrose	3 %	30 g
2,4-D	3 mg/L	30 mL of 10 mg/100 mL stock
BAP	0.05 mg/L	500 µl of 10 mg/100 mL stock
Additive R18(Sue Dalton, unpublished)		5mL
Thiamine	1 mg/L	1 mL of 100 mg/100 mL stock
Proline	750 mg/L	750 mg
Glutamine	1000 mg/L	1000 mg
H2O		1000 mL
pH 5.6		
Meropenum antibiotic	50 mg/L	2 mL of 1 g/40 mL stock
Timentin antibiotic	100 mg/L	2 mL of 2 g/40 mL stock
Bialaphos	3 mg/L	1500 µL of 100 mg/50 mL stock
Hygryomycin B	40 mg/L	1600 µL/L 1g/40ml stock

Table 4.7. *Composition of the Agrobacterium callus regeneration media*.

4.2.9.4. Selection and regeneration of transformed callus

After the three day co-cultivation period the calli were moved to selection media (**table 4.7.**) containing either Hygromycin B or Bialaphos herbicide to kill non-transgenic cells and the antibiotics timentim and meropenum to eliminate the *Agrobacterium*.

Regenerated plants were moved to rooting selection medium (**Table 4.8.**). Plants which rooted were transferred to the transgenic greenhouse and transferred to soil under lights when the roots were over 2 cm long.

Ingredient	Amount	Amount per litre
M0235	75 %	3.35 g
Sucrose	3 %	30 g
NAA	1 mg/L	1 mL of 100 mg/100 mL stock
BAP	1 mg/L	10 mL of 10mg/100 mL stock
Additive R18 (Sue Dalton, unpublished)		5 mL
Thiamine	1 mg/L	1 mL of 100 mg/100 mL stock
H2O		to 1000 mL
pH 5.6		
Timentin	100 mg/L	2 mL of 2 g / 40 mL stock
Bialaphos	4 mg/L	

Table 4.8. *Composition of Agrobacterium rooting media.*

4.2.10. GUS staining

Explants transformed with constructs containing the *gus* gene were assayed by histological GUS staining, to screen for successful transformation. Leaf samples of a minimum 1cm^2 from explants were placed in 3 cm petri dishes containing 1 mL X-Gluc solution pH 7.4 (**table 4.9.**). The plates were incubated overnight at 37 °C in a ENKAB-1 dual voltage incubator (Cherwell Laboratories Ltd. England) on a Innova 2100 platform shaker (New Brunswick Scientific, USA).

Compound	Amount per 50 mL
NaPO4	25 mL
K4Fe(n6).3H2O	250 µL
K3Fe(N6)	250 µL
Triton	100 µL
ddH2O	24 mL
X-gluc	500 µL

Table 4.9. *Composition of X-gluc solution.*

4.3. Results

This section presents the results from the transformation experiments conducted with biolistics and *Agrobacterium*-mediated transformation. The oat genotype Assiniboia had been successful for use in anther culture where it had been shown to regenerate plants from embryogenic callus relatively efficiently, therefore it was chosen for use in all experiments.

Unless specified, all callus was induced and cultured in the strategy shown in **figure 4.3.** This callus induction and culture strategy is based on a strategy used for *Brachypodium* (Thole & Vain, 2012). A total of twenty replicates of 200 seed were prepared, with replicates staggered 1-2 weeks apart. Sub-culturing on fresh medium ensured that the callus would grow vigorously and be more likely to survive the transformation process.

With a few exceptions, each replicate of 200 seed was used to test a single transformation experiment with either particle bombardment or *Agrobacterium* which meant 13 separate cultures for each treatment could be produced.

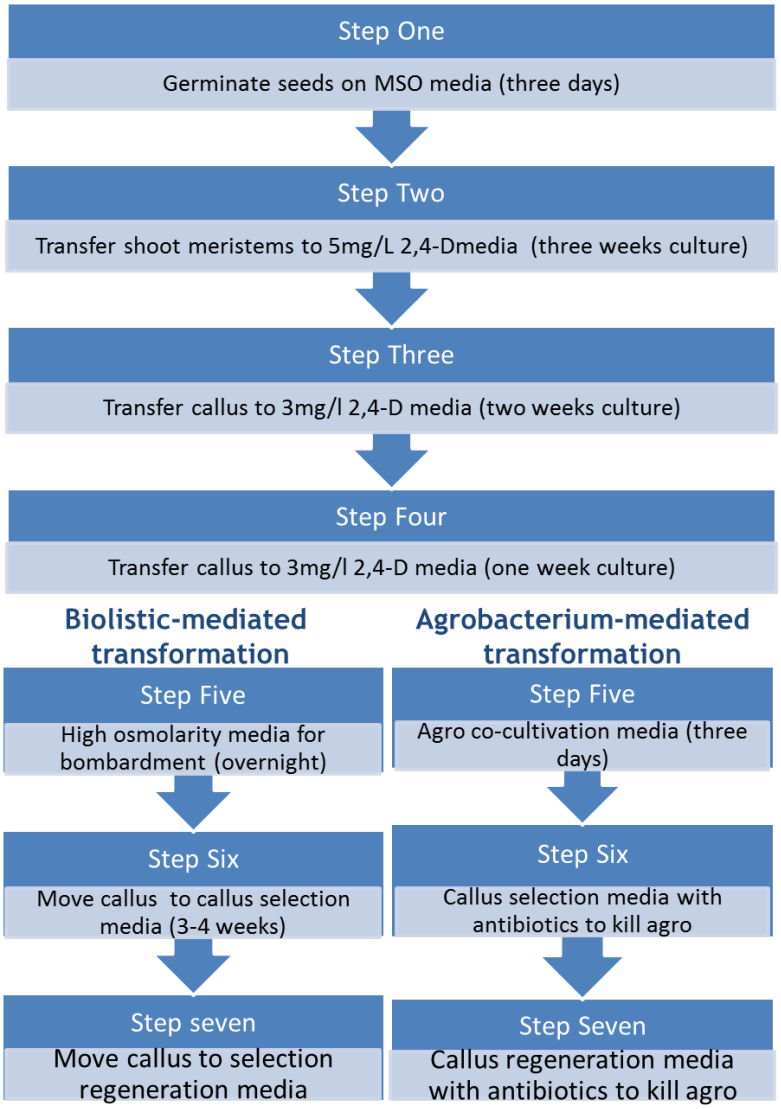


Figure 4.3. *Shows the strategy for transformation. After seed germination on phytohormone free medium the meristems are initially cultured on 5 mg/L 2,4-D to callus growth. The second and third sub-culture on 3 mg/L 2,4-D promotes steady callus growth*

4.3.1 Biolistics transformation

With the exception of replicate one which was split between the two construct combinations, all replicates started with 13 callus cultures as can be seen in **table 4.10.** Not all 13 callus cultures were used for transformation in every case. High rates of contamination were observed in callus cultures during the germination and callus

initiation and maintenance culture schedule (shown in **figure 4.3**.). All contaminated cultures were disposed of. Contamination with various bacteria and fungi was observed throughout the experiments, and does not show a particular pattern through the period that callus cultures were prepared.

Genes	Replicate	Biolistic shots	Explants			
			Regenerants	Rooted	pUBA present	GOI present
pUBA/pJQ5	1	6	No	0	0	0
	6	12	No	0	0	0
	10	7	No	0	0	0
	11	13	no	0	0	0
	14	6	yes	1	0	0
Total		44	0	1	0	0
pUBA/plOM6	1	6	No	0	0	0
	3	4	yes	0	0	0
	7	8	Yes	1	0	0
	13	12	Yes	2	0	0
	15	6	Yes	1	0	0
	16	7	No	0	0	0
Total		43	0	4	0	0

Table 4.10. *Results from the transformation experiments using biolistics with the construct combinations of pUBA/pIOM6 and pUBA/pJQ5.*

A total of 73 bombardments were conducted, 44 for the pUBA/pJQ5 and 43 for the pUBA/pIOM6 combinations (**table 4.10.**). Only one plant successfully rooted from the pUBA/pJQ5 bombardments, and four plants from the pUBA/pLOM6. The regenerants were slow growing and it was many weeks until they rooted and were ready to move into soil (**figure 4.4.**). Several other weak plants did regenerate, however these never rooted and slowly died. Most of these plants grew on the top of calli, and died when they were moved to be in contact with the medium directly (**figure 4.5**).

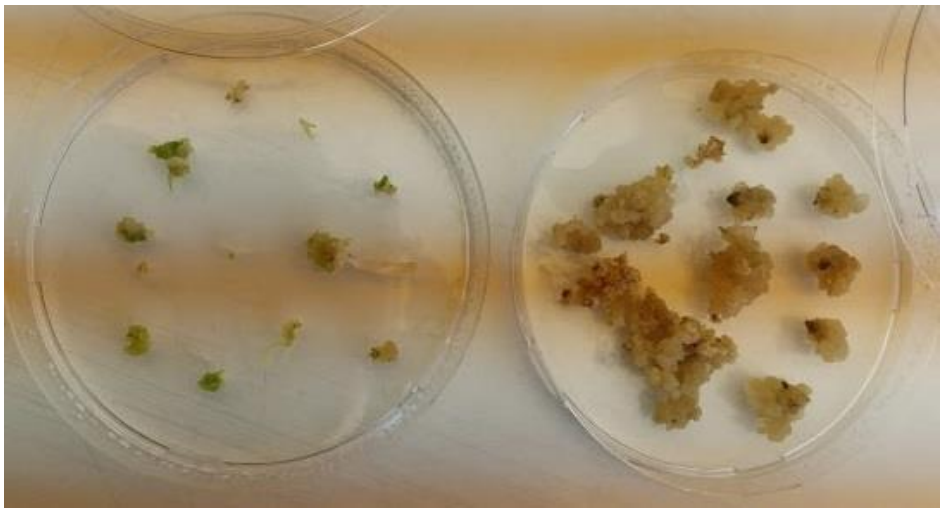


Figure 4.4. *Green regenerants emerging on the top of calli are transferred to fresh medium to test if they are transformed by putting them in direct contact with the selection medium.*

The results from the PCR assay for the presence of the *bar* gene showed that the regenerants were not transformed with the *bar* gene (not shown).

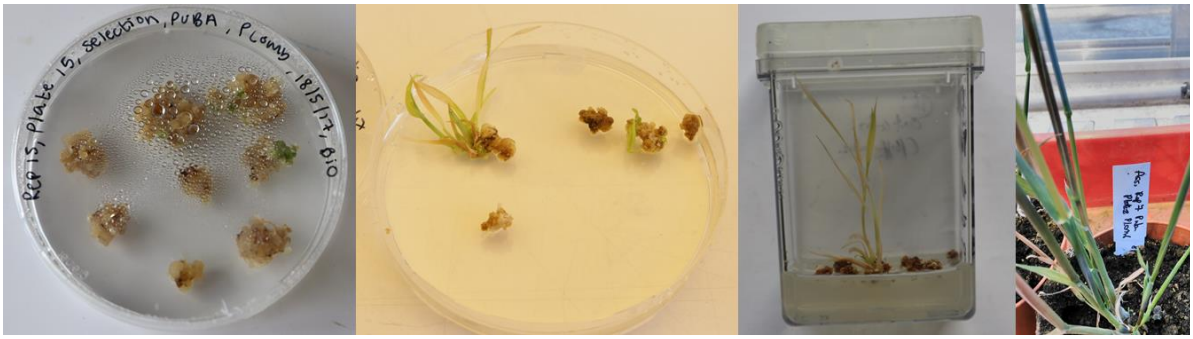


Figure 4.5. *Regenerated plants at various stages of regeneration on selection medium and establishment in soil.*

4.3.2. *Agrobacterium*-mediated transformation

The *Agrobacterium*-mediated transformation experiments were carried out in two stages. The first was made up of two batches using the AGRO1 and the standard transformation protocol, while the second tested three different batches with two different vectors, AGRO1 and AGRO2, with three different callus induction periods.

The initial aim was to test the protocol from start to finish and identify any problems or issues. The second part was intended to test two different vectors which would test two different SMGs *bar* and *hpt* with bialaphos and hygromycin B respectively. It would also test the reporter gene *uidA* by exposing leaf samples of antibiotic resistant plants to X-gluc to check for the characteristic blue spots, determining the expression of β-glucuronidase. The final factor would test the effect of different culture periods on 5 mg/L 2,4-D. In standard experiments the period is three weeks; results from the standard protocol were to be compared with four and five weeks culture periods.

Weeks on 5 mg/L 2,4-D	Vector	Batch	Cultures	Explants		
				Regenerants	Rooted	gus expression
3	AGRO1	4	4	no	0	no
3	AGRO1	5	8	no	0	no
3	AGRO1	18	3	yes	0	no
3	AGRO2	18	4	no	0	no
5	AGRO1	19	5	yes	0	no
5	AGRO2	19	6	no	0	no
4	AGRO1	20	4	no	0	no
4	AGRO2	20	3	no	0	no
Total			37			

Table 4.11. *Results from the Agrobacterium-mediated transformation of oat with different vectors and callus induction periods.*

The results from the *Agrobacterium*-mediated transformation were poor, and largely failed. For the first two replicates, a total of 12 callus cultures failed. The callus showed signs of degradation after a week, and no plants regenerated from the callus after it was transferred to regeneration medium. Changes were made in the second set of experiments. Callus was cut into roughly 0.5 mm diameter pieces as they were plated onto the final induction media to increase the surface area of callus in contact with the medium, and encourage growth. This increased surface area may also improve the chance of transformation during the inoculation stage as it would increase the chance of infection. In **figure 4.6.** the smaller fragments of callus on regeneration selection medium can be seen, after 2-3 weeks on regeneration medium.

However, after four to five weeks the plants showed no sign of rooting, and as with the biolistics experiment, most plants grew on top of callus rather than in direct contact with the medium. In **figure 4.7.** it can be seen that the *Agrobacterium* started to grow on the medium.

Leaf samples were taken from regenerants in **figure 4.8.** for GUS histological staining, and compared with known positive control samples (provided by Sue Dalton).

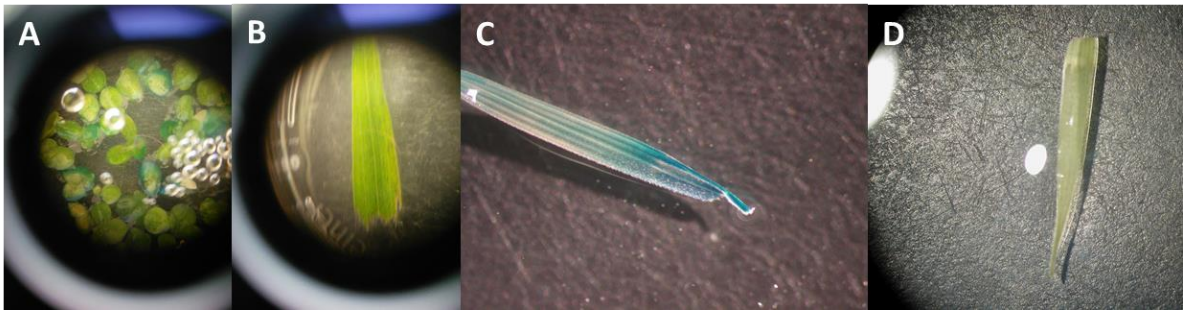


Figure 4.8. *Examples of gus expression screening with x-gluc. A and C are known controls, and B and D are oat regenerant samples.*

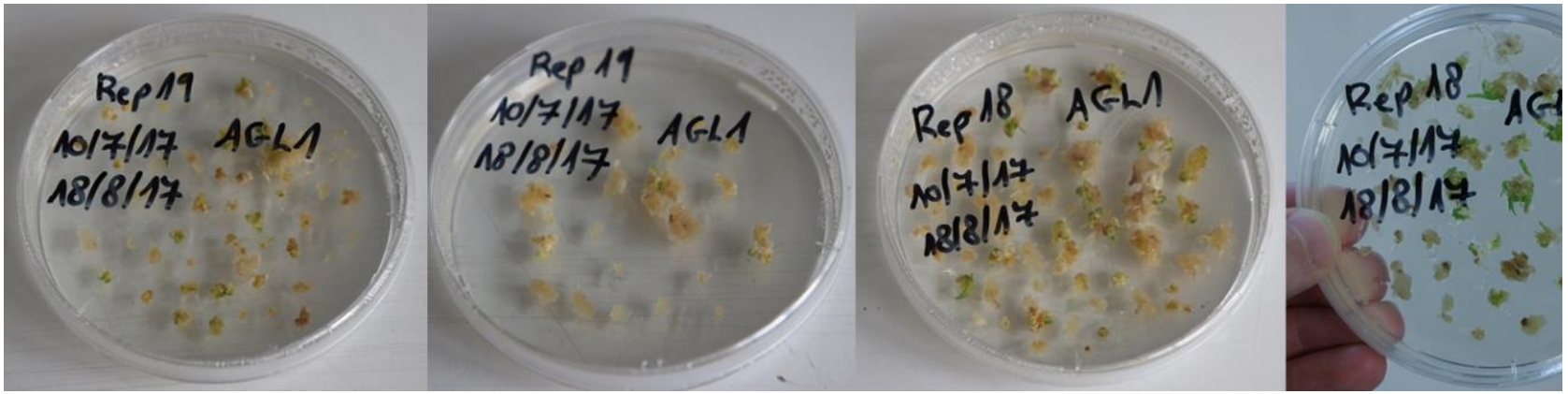


Figure 4.6. *Plantlets regenerating from callus on selection medium after Agrobacterium-mediated transformation with the AGRO1 vector.*

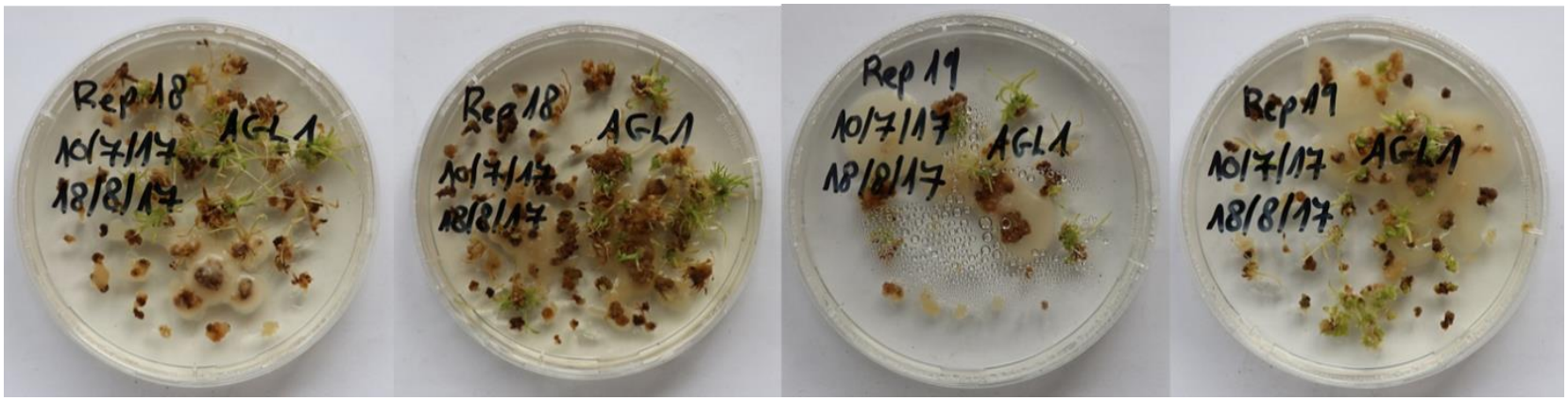


Figure 4.7. *Regenerant cultures four to five weeks after inoculation with Agrobacterium. Overgrowth is clearly seen on the medium of the AGRO1 vector.*

4.4. Discussion

This section discusses the results from the transformation experiments of oats and examines the potential for protocol optimisation in future work.

The *Brachypodium* callus induction protocol (Thole & Vain, 2012) was chosen due to its success in a related species. It is generally accepted that callus should not be left too long before regeneration, as it increases the chance of somaclonal variation and reduces the regeneration competency. Based on observations during these experiments, oat could perform better with a slightly longer callus induction period due to slow callus growth. Three subculture stages of three weeks each, or a higher concentration of 2,4-D (5 mg/L for each subculture) throughout the induction period could help with callus proliferation.

Contamination was prevalent throughout the experiments conducted. This is likely due to the many subculture stages each of which comes with a risk of culture contamination. The oat seed were dehusked, and washed with a strong bleach solution twice, but this would not kill endophytes. Further study of callus cultures to identify the contaminant species could lead to the use of a different sterilisation strategy or addition of agents to the culture medium to inhibit contaminant action (Leifert & Waites, 1994).

The genotype Assiniboia showed very positive regeneration ability in the DH experiments and had not been previously reported to be used for transformation. It was anticipated that its amenability to tissue culture would make it an ideal candidate for optimising a transformation protocol for oat. However, despite the successful regeneration of Assiniboia under different selection treatments in both the biolistics and *Agrobacterium*-mediated transformation experiments no evidence for stable incorporation of the transforming constructs was found. The reason why the callus was not affected by antibiotic selection is not clear. Bialaphos has been shown to be an effective herbicide in a range of species. For example, a study of different phosphinothricin (PPT) agents evaluated with the *bar* gene in maize found that bialaphos was a more powerful selection agent than glufosinate (Dennehey *et al.,* 1994), with bialaphos also being free from interactions with proline content of the media that affected glufosinate. The *bar* gene has

been used extensively as a source of resistance to PPT agents in many species, including maize (Spencer *et al.,* 1990), barley (Yao *et al.,* 1997), Arabidopsis, rice, tobacco (Nakamura *et al.,* 2010) and oat (Zhang *et al.,* 1999; Cho *et al.,* 2003; Maqbool *et al.,* 2009). The concentration of bialaphos used for selection in oat transformation studies has been reported to range from 2 mg/L (Cho *et al.,* 2003) to 3-5 mg/L (Maqbool *et al.,* 2009) with 3 mg/L being used in the maize study described above (Dennehey *et al.,* 1994). 3 mg/L was used in this work for the initial selection, and increased to 4 mg/L in the rooting medium. This was on the advice of Sue Dalton, who had used these concentrations for oat transformation previously.

Therefore it more likely the problem lies in the conduct of the experiment. Antibiotics are particularly prone to degradation by heat. When preparing tissue culture medium, the nutrient broth and gelling agent are autoclaved to ensure sterility and the antibiotic is added after when the medium has cooled to ensure the heat of the medium does not denature the antibiotic. It is possible that the antibiotic was added too soon and some was denatured, which would lower the effective concentration of the antibiotic. The other reason is that the antibiotic breaks down in the medium over time. The selection medium was made in small batches and stored in the dark at 4 °C, however there was not a strict best before use so the medium may not have been used on the same or the next day but could in theory be kept for over a week. Antibiotics break down over time, therefore the reason for non-transformed plants regenerating 3-4 weeks after plating may be due to this. It could also explain why the plants which did regenerate did so on top of the callus, and perished slowly when in contact with the medium. Another possibility is the influence of glutamine added to the tissue culture medium. The bar gene encodes for the enzyme phosphinothricin acetyl transferase (PAT).L-phosphinothricin (PPT) or glufosinate is the active compound of the herbicide Basta. L-phosphinothricin is a glutamate analogue that inhibits glutamine synthetase (Manderscheid & Wild, 1986). Glutamine synthetase is important for incorporating ammonium with glutamate to form glutamine, which is a major route for removing ammonium produced during processes including photorespiration. Inhibition of glutamine synthetase by PPT results in a build-up

of ammonium to toxic levels, leading to the plant's death. The enzyme PAT encoded by the bar gene inactivates PPT by acetylating it (Botterman *et al.,* 1991). Glutamine is added to all the tissue culture media as it has shown to have a positive effect on callus growth. The addition of glutamine should not inhibit the build-up of ammonium to toxic levels, but it might have otherwise interfered with PPT activity. The carbon source is known to affect the metabolic status of plant tissue. On glucose medium PPT selection is largely due to glutamine deprivation, while on sucrose medium the selection is due to ammonium toxicity (De Block *et al.,* 1995). Maltose was used as the carbohydrate source in experiments, which is broken down into two glucose molecules. Therefore it is possible that the use of sucrose source, and the addition of glutamine could be 'nursing' the oat callus and regenerants, at least temporally, which could explain escapes occurring. Further experimentation with different carbohydrate sources, and with and without glutamine are necessary to understand this relationship. Using a higher concentration of bialaphos may overcome the effect of additional glutamine addition. Alternatively, oat may be better at detoxifying or blocking uptake of the antibiotic. A liquid layer of medium could overcome this. Being stricter with the production, storage and period of use of medium containing antibiotics could also reduce the problem of antibiotic degradation. As preliminary transformation experiments with bialaphos as a selective agent had been promising (personal communication; Sue Dalton), non-transformed control callus was not used, but would be beneficial in future optimisation experiments.

The biolistics-mediated transformation experiments tested the effect of two different vectors, with two different SMGs and three different callus induction periods. The first set of experiments, made up of two replicates, tested one vector with the standard protocol. The vector pUBA/PIOM6 regenerated more plants than the pUBA/PJQ5 vector, but as both vectors were tested in a balanced quantity and the selection marker and antibiotic were the same it is unlikely that the difference is significant.

The first two batches of *Agrobacterium*-mediated transformation experiments were primarily to test the protocol, and identify any issues. The second part was to test two different combinations of *Agrobacterium* strain and vector. Unfortunately the

Agrobacterium-mediated transformation experiments failed to successfully transform oat. There were however some noticeable differences with the response to the different vector combinations. Only the AGRO1 vector combination regenerated plants, while AGRO2 did not. AGRO2 used selection with a different antibiotic and gene combination so it is possible that the hygromycin killed the callus as they had not been successfully transformed while the different action of bialaphos as discussed above. The conditions were suitable for plant regeneration and for the *Agrobacterium* to start colonising the medium. The *Agrobacterium* strains had been grown on selective medium to maintain vectors before inoculation. The evidence of *Agrobacterium* growing on the medium of AGRO1 would suggest that the inoculation process was completed with a living sample of *Agrobacterium*, which cannot be said for the AGRO2 inoculation, which could explain why the AGRO2 treatment failed to regenerate plants, and showed no signs of *Agrobacterium* on the culture medium. It is important to note that the different treatments of each vector combination were completed at the same time using a single culture of each. Therefore if there was a problem with the *Agrobacterium* culture or inoculation, it is likely to have affected all treatments. The result of these experiments produced no regenerants, but highlighted the problem with the callus induction. Callus induced from meristems was cultured and sub-cultured as per the protocol, but was not broken up during subcultures, so *Agrobacterium* inoculation was conducted on large pieces of callus. Observations with a microscope (not shown) revealed that the oat callus was more embryogenic than previously thought, so chopping up the callus before the final subculture stage could be beneficial, as it would increase the amount of callus in contact with the medium, and potentially increase the amount of embryogenic callus. It would also increase the surface area during *Agrobacterium* inoculation which could increase the chance of successful transformation. The regeneration response visually looked to have improved with this method, as many small plants can be seen (**figure 4.7.**) One of the most recently published papers by Gasparis *et al.,* (2008) showed a transformation rate of 12.3% was obtained for immature embryos of cv. Bajka. About 79% of the selected plants proved to be transgenic. Bajka had been used in the DH anther experiment (**chapter 2.**)

but was not tested in this project. Only Assiniboia was used in this project due to it having a good regeneration response in DH anther culture. The results from the second batch of *Agrobacterium*-mediated transformations where the callus was divided into small pieces before the final subculture particularly stood out. Based on the results here regeneration from callus does not appear to be a problem. However, Bajka may be more amenable to transformation than Assiniboia. Testing other genotypes such as Bajka could prove more successful as well as trying other strains of *Agrobacterium*. It is unfortunate that no plants were successfully transformed; however there is clearly a good foundation to build on. Optimisation of the callus culture to provide better material to transform and investigation into the glutamine issue would be the first place to start. Measuring transient expression shortly after transformation could shed light on the action of the vector activity.

Chapter 5: General discussion

The aim of this project was to investigate and optimise DH and GM protocols for oat so that these technologies can be applied to oat breeding.

For DH, a number of different media and genotypes were initially used to test the potential of several isolated microspore and anther culture protocols. The genotype Assiniboia and the medium MDHM were identified as the most promising. MDHM was then used with a range of media additives, which had been reported to improve DH protocols of other species were tested with Assiniboia and additional genotypes. Flow cytometry was used to confirm that at least some of the regenerated plants were haploid, while F2 progeny of elite crosses were used as donor plants to provide heterozygous material that would allow discrimination between somatic and microspore embryogenesis. A small number of regenerants from elite cross progeny were generated, providing the initial stock of a DH population, which could have potential uses in further work such as QTL mapping.

For GM, Assiniboia was chosen as a candidate genotype due to its callus growth and plant regeneration ability in DH studies, and was tested with both *Agrobacterium*-mediated and Biolistics-mediated transformation methods, using improvements to the media tested in the DH experiments.

With the identification of the genotype Assiniboia and the media MDHM, it was possible to increase the DH efficiency from a starting point of effectively zero in the first year, to 12 regenerants per 100 anthers with the genotype Assiniboia in the best instance by incorporating glutamine and casein hydrolysate into the media. This effect was also shown in the genotype Lisbeth, a previously recalcitrant genotype where the anther response rate increased from 0.95 ± 0.95 to 12.38 ± 5.72, and the regenerants per hundred anthers increased from 0.24 ± 0.24 to 4.52 ± 2.29. The anther response per hundred anthers of Aslak also increased, from 0.71 ± 0.51 to 3.33 ± 2.11 suggesting that the addition of glutamine in particular could help to improve the DH efficiency of more genotypes. This was further proved by the use of glutamine in the media with progeny of

the F2 cross Assiniboia x Firth. From the culturing of 11,004 anthers of 51 progeny, a total of 70 plants were regenerated of which 30 were green. While not meeting the levels needed for commercial use, 0.27 green regenerants per 100 anthers is close to the average rate recorded by Tanhuanpää *et al.,* (2008), of 0.7 green regenerants per 100 anthers (of a total of 26,070 anthers cultured) to produce the first published QTL map from an oat DH population. It is important to note that overall, the rate varied from the lowest at 0.8 to 1.7 green regenerants per 100 anthers when conducted with the same conditions (Tanhuanpää et al., 2008). The protocols developed here are therefore close to the efficiencies required to produce DH populations for experimental use, if not yet approaching the levels required for routine commercial use. Further modifications of the protocol are expected to improve efficiencies, in particular, the addition of high concentrations of copper to the pre-treatment solution and the cultured media. The study of panicle phenotypic traits and panicle structure have shown there is scope to improve efficiency by better panicle and anther selection. The media improvements developed with anther culture should be tested with IMC culture as this is the approach of choice for future high throughput protocols.

Assiniboia was selected as a candidate genotype for GM protocol development due to its regeneration efficiency with DH. Unfortunately no plants were successfully transformed, but with further optimisation of the oat callus induction stages and transformation procedure, particularly with use of transient expression for initial characterisation, there is a possibility to identify the limiting factors. In particular, modification of the selection procedure is likely to reduce 'escape' growth of callus and allow selection of genuine transformants. A particular target here is the reduction of glutamine concentration during initial selection.

The results here show that with methodical investigation it is possible to identify and apply incremental improvements which will eventually provide oat breeders with the tools necessary to maintain the crop's competitiveness. The reliable baseline frequency of the DH protocol developed here is a critical first step in this process.

References

Acebedo, M. M., Lavee, S., Liñán, J., & Troncoso, A. (1997). In vitro germination of embryos for speeding up seedling development in olive breeding programmes. *Scientia Horticulturae*, *69*(3–4), 207–215. http://doi.org/10.1016/S0304-4238(97)00004-6

Al-Khayri, J. M. (2011). Influence of yeast extract and casein hydrolysate on callus multiplication and somatic embryogenesis of date palm (Phoenix dactylifera L.). *Scientia Horticulturae*, *130*(3), 531–535. http://doi.org/10.1016/j.scienta.2011.07.024

Alemanno, L., & Guiderdoni, E. (1994). Increased doubled haploid plant regeneration from rice (Oryza sativa L.) anthers cultured on colchicine-supplemented media. *Plant Cell Reports*, *13*(8), 432–6. http://doi.org/10.1007/BF00231961

Ali, S., Khan, M. S., & Iqbal, J. (2012). In vitro direct plant regeneration from cultured young leaf segments of sugarcane (Saccharum officiniarum L.). *The Journal of Animal and Plant Sciences*, *22*(4), 1107–1112.

Almouslem, A., & Jauhar, P. (1998). Haploid durum wheat production via hybridization with maize. *Crop Science*, *38*(4), 1080. http://doi.org/10.2135/cropsci1998.0011183X003800040033x

Aloni, R., & Plotkin, T. (1985). Wound-induced and naturally occurring regenerative differentiation of xylem in Zea mays L. *Planta*, *163*(1), 126–132. http://doi.org/10.1007/BF00395906

Altman, a, Kaur-Sawhney, R., & Galston, a W. (1977). Stabilization of Oat Leaf Protoplasts through Polyamine-mediated Inhibition of Senescence. *Plant Physiology*, *60*, 570–574. http://doi.org/10.1104/pp.60.4.570

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., … Visser, R. (2004).

Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding*, *15*, 305–327. http://doi.org/10.1007/s11032-004-8001-y

Antoine-Michard, S., & Beckert, M. (1997). Spontaneous versus colchicine-induced chromosome doubling in maize anther culture. *Plant Cell, Tissue and Organ Culture*, *48*(3), 203–207. http://doi.org/10.1023/A:1005840400121

Aragão, F. A. S., Ribeiro, C. S. da C., Casali, V. W. D., & Giordano, L. de B. (2002). Tomato embryo culture for introgression of genes of Lycopersicon peruvianum in L. esculentum. *Horticultura Brasileira*, *20*(4), 605–610. http://doi.org/10.1590/S0102-05362002000400019

Ardebili, S., & Shariatpanahi, M. (2011). Effect of 2, 4-D as a Novel Inducer of Embryogenesis in Microspores of Brassica napus L. *Czech J Genet …*, *2011*(3), 114–122.

Arnison, P., & Donaldson, P. (1990). The influence of various physical parameters on anther culture of broccoli (Brassica oleracea var. italica). *Plant Cell, Tissue and Organ Culture*, *20*, 147–155.

Azouaou, Z., & Souvre, A. (1993). Effects of copper deficiency on pollen fertility and nucleic acids in the durum wheat anther. *Sexual Plant Reproduction*, *6*(3), 199–204. http://doi.org/10.1007/BF00228649

Bakos, F., Jäger, K., & Barnabás, B. (2005). Regeneration of haploid plants after distant pollination of wheat via zygote rescue. *Acta Biol. Cracov. Ser*, *47*(1), 167–171.

Ball, E. (1946). Development in Sterile Culture of Stem Tips and Subjacent Regions of Tropaeolum Majus L. And of Lupinus Albus L. *American Journal of Botany*, *33*(5), 301. http://doi.org/10.2307/2437117

Basu, C., Kausch, A. P., Luo, H., & Chandlee, J. M. (2003). Promoter analysis in transient assays using a GUS reporter gene construct in creeping bentgrass (Agrostis palustris). *Journal of Plant Physiology*, *160*(10), 1233–1239. http://doi.org/10.1078/0176-1617-01104

Baum, B. (1977). *Oats: wild and cultivated. A monograph of the genus Avena L.(Poaceae). book*.

Baum, B. R., & Rajhathy, T. (1976). A study of Avena macrostachya. *Canadian Journal of Botany*, *54*(21), 2434–2439. http://doi.org/10.1139/b76-258

Beck, S. L., Visser, G., & Dunlop, R. W. D. A.-N. (2005). A comparison of direct (flow cytometry) and indirect (stomatal guard cell lengths and chloroplast numbers) techniques as a measure of ploidy in black wattle, Acacia mearnsii (de Wild). *South African Journal of Botany*, *71*(3–4), 354–358. http://doi.org/10.1016/S0254-6299(15)30109-5

Bevan, M. W., Flavell, R. B., & Chilton, M.-D. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature*, *304*(5922), 184–187. http://doi.org/10.1038/304184a0

Bhaskar, P. B., Venkateshwaran, M., Wu, L., Ané, J.-M., & Jiang, J. (2009). Agrobacterium-Mediated Transient Gene Expression and Silencing: A Rapid Tool for Functional Gene Assay in Potato. *PLoS ONE*, *4*(6), e5812. http://doi.org/10.1371/journal.pone.0005812

Bhat, J. G., & Murthy, H. N. (2007). Factors affecting in-vitro gynogenic haploid production in niger (Guizotia abyssinica (L. f.) Cass.). *Plant Growth Regulation*, *52*, 241–248.

Bohanec, B. (2003). Ploidy determination using flow cytometry. In M. Maluszynski, M. Kasha, K. J. Forsrster, & I. Szarejko (Eds.), *Doubled Haploid Production in Crop Plants: A Manual* (pp. 397–403). Dordrecht: Kluwer Academic Publishers.

Bonet, F., Azbaid, L., & Olmedilla, A. (1998). Pollen embryogenesis: atavism or totipotency? *Protoplasma*, 115–121.

Borderies, G., le Béchec, M., Rossignol, M., Lafitte, C., Le Deunff, E., Beckert, M., … Elisabeth, M.-R. (2004). Characterization of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. *European Journal of Cell Biology*, *83*(5), 205–12.

Botterman, J., Gosselé, V., Thoen, C., & Lauwereys, M. (1991). Characterization of phosphinothricin acetyltransferase and C-terminal enzymatically active fusion proteins. *Gene*, *102*(1), 33–7.

Bouvier, L., Fillon, F. R., & Lespinasse, Y. (1994). Oryzalin as an Efficient Agent for Chromosome Doubling of Haploid Apple Shoots in vitro. *Plant Breeding*, *113*(4), 343–346. http://doi.org/10.1111/j.1439-0523.1994.tb00748.x

Braaten, J. T., Wood, P. J., Scott, F. W., Wolynetz, M. S., Lowe, M. K., Bradley-White, P., & Collins, M. W. (1994). Oat beta-glucan reduces blood cholesterol concentration in hypercholesterolemic subjects. *European Journal of Clinical Nutrition*, *48*(7), 465–74.

Braun, A. C. (1947). Thermal Studies on the Factors Responsible for Tumor Initiation in Crown Gall. *Source: American Journal of Botany*, *34*(4), 234–240.

Bregitzer, P., Dahleen, L. S., & Campbell, R. D. (1998). Enhancement of plant regeneration from embryogenic callus of commercial barley cultivars. *Plant Cell Reports*, *17*(12), 941–945. http://doi.org/10.1007/s002990050514

Broughton, S. (2008). Ovary co-culture improves embryo and green plant production in anther culture of Australian spring wheat (Triticum aestivum L.). *Plant Cell, Tissue and Organ Culture*, *95*(2), 185–195. http://doi.org/10.1007/s11240-008-9432-7

Buanafina, M. M. d. O., Dalton, S., Langdon, T., Timms-Taravella, E., Shearer, E. A., & Morris, P. (2015). Functional co-expression of a fungal ferulic acid esterase and a β-1,4 endoxylanase in Festuca arundinacea (tall fescue) modifies post-harvest cell wall deconstruction. *Planta*, *242*(1), 97–111. http://doi.org/10.1007/s00425-015-2288-2

Buanafina, M. M. de O., Langdon, T., Hauck, B., Dalton, S., Timms-Taravella, E., & Morris, P. (2010). Targeting expression of a fungal ferulic acid esterase to the apoplast, endoplasmic reticulum or golgi can disrupt feruloylation of the growing cell wall and increase the biodegradability of tall fescue (Festuca arundinacea). *Plant Biotechnology Journal*, *8*(3), 316–331. http://doi.org/10.1111/j.1467-7652.2009.00485.x

Bullock, W. P., Baenziger, P. S., Schaeffer, G. W., & Bottino, P. J. (1982). Anther culture of wheat (Triticum aestivum L.) F1's and their reciprocal crosses. *Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *62*(2), 155–9. http://doi.org/10.1007/BF00293350

Burch-Smith, T. M., Anderson, J. C., Martin, G. B., & Dinesh-Kumar, S. P. (2004). Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal*, *39*(5), 734–746. http://doi.org/10.1111/j.1365-313X.2004.02158.x

Büter, B., Schmid, J. E., & Stamp, P. (1991). Effects of L-proline and post-plating temperature treatment on Maize (Zea mays L.) anther culture. *Plant Cell Reports*, *10*(6–7), 325–8. http://doi.org/10.1007/BF00193151

Buyser, J., Henry, Y., Lonnet, P., Hertzog, R., & Hespel, A. (1987). "Florin": A Doubled Haploid Wheat Variety Developed by the Anther Culture Method. *Plant Breeding*, *98*(1), 53–56. http://doi.org/10.1111/j.1439-0523.1987.tb01089.x

Caredda, S., Doncoeur, C., Devaux, P., Sangwan, R. S., & Cl??ment, C. (2000). Plastid differentiation during androgenesis in albino and non-albino producing cultivars of barley (Hordeum vulgare L.). *Sexual Plant Reproduction*, *13*(2), 95–104. http://doi.org/10.1007/s004970000043

Castillo, A. M., Sánchez-Díaz, R. A., & Vallés, M. P. (2015). Effect of ovary induction on bread wheat anther culture: ovary genotype and developmental stage, and candidate gene association. *Frontiers in Plant Science*, *6*(June), 1–12. http://doi.org/10.3389/fpls.2015.00402

Castillo, A., Vallés, M., & Cistué, L. (2000). Comparison of anther and isolated microspore cultures in barley. Effects of culture density and regeneration medium. *Euphytica*, *3*(3), 1–8.

Cesaro, T., Baggio, M., & Zanetti, S. (2009). Haplodiploid androgenetic breeding in oat: genotypic variation in anther size and microspore development stage. *Scientia*

Agricola, (February), 118–122.

Chauhan, H., & Khurana, P. (2011). Use of doubled haploid technology for development of stable drought tolerant bread wheat (Triticum aestivum L.) transgenics. *Plant Biotechnology Journal*, *9*(3), 408–417. http://doi.org/10.1111/j.1467-7652.2010.00561.x

Chen, C., & Lin, M. (1976). Induction of rice plantlets from anther culture. *Bot. Bull. Acad. Sin*.

Chen, H., & Xiong, L. (2005). Pyridoxine is required for post-embryonic root development and tolerance to osmotic and oxidative stresses. *The Plant Journal*, *44*(3), 396–408. http://doi.org/10.1111/j.1365-313X.2005.02538.x

Chen, Y., Hausner, G., Kenaschuk, E., Procunier, D., Dribnenki, P., & Penner, G. (1998). Identification of microspore-derived plants in anther culture of flax (Linum usitatissimum L.) using molecular markers. *Plant Cell Reports*, *18*, 44–48. http://doi.org/10.1007/s002990050529

Chen, Z., Zhuge, Q., & Sundqvist, C. (1995). Oat leaf base: tissue with an efficient regeneration capacity. *Plant Cell Reports*, *14*(6), 354–358. http://doi.org/10.1007/BF00238596

Chilton, M.-D., Drummond, M. H., Merlo, D. J., & Sciaky, D. (1978). Highly conserved DNA of Ti plasmids overlaps T-DNA maintained in plant tumours. *Nature*, *275*(5676), 147–149. http://doi.org/10.1038/275147a0

Chilton, M. D., Drummond, M. H., Merlo, D. J., Sciaky, D., Montoya, A. L., Gordon, M. P., & Nester, E. W. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell*, *11*(2), 263–271. http://doi.org/10.1016/0092-8674(77)90043-5

Chiu, W. L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., & Sheen, J. (1996). Engineered GFP as a vital reporter in plants. *Current Biology*, *6*(3), 325–330. http://doi.org/10.1016/S0960-9822(02)00483-9

Cho, M.-J., Choi, H., Okamoto, D., Zhang, S., & Lemaux, P. (2003). Expression of green fluorescent protein and its inheritance in transgenic oat plants generated from shoot meristematic cultures. *Plant Cell Reports*, *21*(5), 467–474. http://doi.org/10.1007/s00299-002-0542-0

Cho, M. J., Jiang, W., & Lemaux, P. G. (1999). High-frequency transformation of oat via microprojectile bombardment of seed-derived highly regenerative cultures. *Plant Science*, *148*(1), 9–17. http://doi.org/10.1016/S0168-9452(99)00082-5

Christie, P. J., & Gordon, J. E. (2014). The Agrobacterium Ti Plasmids. *Microbiology Spectrum*, *2*(6). http://doi.org/10.1128/microbiolspec.PLAS-0010-2013

Christou, P. (1995). Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. *Euphytica*, *85*(1–3), 13–27. http://doi.org/10.1007/BF00023926

Cisneros, A., & Tel-Zur, N. (2010). Embryo rescue and plant regeneration following interspecific crosses in the genus Hylocereus (Cactaceae). *Euphytica*, *174*(1), 73–82. http://doi.org/10.1007/s10681-010-0135-x

Cistué, L., Romagosa, I., Batlle, F., & Echávarri, B. (2009). Improvements in the production of doubled haploids in durum wheat (Triticum turgidum L.) through isolated microspore culture. *Plant Cell Reports*, *28*(5), 727–735. http://doi.org/10.1007/s00299-009-0690-6

Clough, S. J., & Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *The Plant Journal : For Cell and Molecular Biology*, *16*(6), 735–43.

Cohen, S. N. (2013). DNA cloning: a personal view after 40 years. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(39), 15521–9. http://doi.org/10.1073/pnas.1313397110

Colinas, M., Eisenhut, M., Tohge, T., Pesquera, M., Fernie, A. R., Weber, A. P. M., & Fitzpatrick, T. B. (2016). Balancing of B $_6$ Vitamers Is Essential for Plant Development

and Metabolism in Arabidopsis. *The Plant Cell*, *28*(2), 439–453. http://doi.org/10.1105/tpc.15.01033

Conn, H. J. (1942). Validity of the Genus Alcaligenes. *Journal of Bacteriology*, *44*(3), 353–60.

Cornejo, M.-J., Luth, D., Blankenship, K. M., Anderson, O. D., & Blechl, A. E. (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Molecular Biology*, *23*(3), 567–581. http://doi.org/10.1007/BF00019304

Costa-Font, M., Gil, J. M., & Traill, W. B. (2008). Consumer acceptance, valuation of and attitudes towards genetically modified food: Review and implications for food policy. *Food Policy*, *33*(2), 99–111. http://doi.org/10.1016/J.FOODPOL.2007.07.002

Dahleen, L. S. (1995). Improved plant regeneration from barley callus cultures by increased copper levels. *Plant Cell, Tissue and Organ Culture*, *43*(3), 267–269. http://doi.org/10.1007/bf00039954

Dahleen, L. S. (1999). Donor-Plant Environment Effects on Regeneration from Barley Embryo-Derived Callus. *Crop Science*, *39*(3), 682. http://doi.org/10.2135/cropsci1999.0011183X003900020013x

Darko, E., Heydarizadeh, P., Schoefs, B., & Sabzalian, M. R. (2014). Photosynthesis under artificial light: the shift in primary and secondary metabolism. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *369*(1640), 20130243. http://doi.org/10.1098/rstb.2013.0243

Datta, S. (2005). Androgenic haploids: factors controlling development and its application in crop improvement. *CURRENT SCIENCE-BANGALORE-*, *89*(11), 1870–1878.

Davies, P. A., & Morton, S. (1998). A comparison of barley isolated microspore and anther culture and the influence of cell culture density. *Plant Cell Reports*, *17*(3), 206–210. http://doi.org/10.1007/s002990050379

Day, A., & Ellis, T. H. N. (1984). Chloroplast DNA deletions associated with wheat plants

regenerated from pollen: possible basis for maternal inheritance of chloroplasts. *Cell*, *39*(2), 359–368. http://doi.org/10.1016/0092-8674(84)90014-X

Day, A., & Ellis, T. H. N. (1985). Deleted forms of plastid DNA in albino plants from cereal anther culture. *Current Genetics*, *9*(8), 671–678. http://doi.org/10.1007/BF00449820

De Block, M., De Sonville, A., & Debrouwer, D. (1995). The selection mechanism of phosphinothricin is influenced by the metabolic status of the tissue. *Planta*, *197*(4), 619–626. http://doi.org/10.1007/BF00191569

de Framond, A. J., Barton, K. A., & Chilton, M.-D. (1983). Mini–Ti: A New Vector Strategy for Plant Genetic Engineering. *Nature Biotechnology*, *1*(3), 262–269. http://doi.org/10.1038/nbt0583-262

Dell, B. (1980). Male Sterility and Anther Wall Structure in Copper-deficient Plants. *Annals of Botany*, (48), 599–608.

Dennehey, B. K., Retersen, W. L., Ford-Santino, C., Pajeau, M., & Armstrong, C. L. (1994). Comparison of selective agents for use with the selectable marker gene bar in maize transformation. *Plant Cell, Tissue and Organ Culture*, *36*(1), 1–7. http://doi.org/10.1007/BF00048308

Droppa, M., & Horváth, G. (1990). The role of copper in photosynthesis. *Critical Reviews in Plant Sciences*, *9*(2), 111–123. http://doi.org/10.1080/07352689009382284

Eder, J., & Chalyk, S. (2002). In vivo haploid induction in maize. *TAG Theoretical and Applied Genetics*, *104*(4), 703–708. http://doi.org/10.1007/s00122-001-0773-4

El-Hennawy, M. A., Abdalla, A. F., Shafey, S. A., & Al-Ashkar, I. M. (2011). Production of doubled haploid wheat lines (Triticum aestivum L.) using anther culture technique. *Annals of Agricultural Sciences*, *56*(2), 63–72. http://doi.org/10.1016/j.aoas.2011.05.008

Emershad, R. L., & Ramming, D. W. (1994). Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (Vitis vinifera L.).

Plant Cell Reports, *14*(1), 6–12. http://doi.org/10.1007/BF00233289

Esteves, P., Clermont, I., Marchand, S., & Belzile, F. (2014). Improving the efficiency of isolated microspore culture in six-row spring barley: II-exploring novel growth regulators to maximize embryogenesis and reduce albinism. *Plant Cell Reports*, *33*(6), 871–9. http://doi.org/10.1007/s00299-014-1563-1

Evans, D. A., Sharp, W. R., & Flick, C. E. (1981). Growth and behaviour of cell cultures: Embryogenesis and organogenesis. In *Plant tissue culture: Methods and applications in agriculture* (pp. 45–113). New York: Academic Press.

Evans, L. T. (1998). *Feeding the ten billion : plants and population growth*. Cambridge University Press.

FAOSTAT. (2016). FAOSTAT. Retrieved from http://www.fao.org/faostat/en/#home

Farrand, S. K., van Berkum, P. V., & Oger, P. (2003). Agrobacterium is a definable genus of the family Rhizobaiceae. *International Journal of Systematic and Evolutionary Microbiology*, *53*(5), 1681–1687. http://doi.org/10.1099/ijs.0.02445-0

Ferrie, A. M. R. (2003). Microspore culture of Brassica species. In M. M, K. KJ., B. Forster, & I. Szarejko (Eds.), *Doubled Haploid Production in Crop Plants – A Manual* (pp. 205–215). Dordrecht/Boston/London: Kluwer.

Ferrie, A. M. R., Irmen, K. I., Beattie, a. D., & Rossnagel, B. G. (2013). Isolated microspore culture of oat (Avena sativa L.) for the production of doubled haploids: effect of pre-culture and post-culture conditions. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *116*(1), 89–96. http://doi.org/10.1007/s11240-013-0385-0

Finer, J. J., Vain, P., Jones, M. W., Mcmullen, M. D., & Widholm, J. M. (1992). Development of the particle inflow gun for DNA delivery to plant cells *. *Plant Cell Reports*, *11*, 323–328.

Fitzsimmons, R. (2012). Oh, What Those Oats Can Do. Quaker Oats, the Food and Drug Administration, and the Market Value of Scientific Evidence 1984 to 2010.

Comprehensive Reviews in Food Science and Food Safety, *11*(1), 56–99. http://doi.org/10.1111/j.1541-4337.2011.00170.x

Fouletier, B. (1974). Conditions favorisant la neoformation de cals haploides a partir d'antheres de riz cultivees in vitro. *C R Acad Sci Paris*, *378*, 2917–2920.

Fraley, R. T., Rogers, S. G., Horsch, R. B., Eichholtz, D. A., Flick, J. S., Fink, C. L., … Sanders, P. R. (1985). The SEV System: A New Disarmed Ti Plasmid Vector System for Plant Transformation. *Nature Biotechnology*, *3*(7), 629–635. http://doi.org/10.1038/nbt0785-629

Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., … F, D. A. B. R. (2002). Agrobacterium tumefaciens -Mediated Transformation of Maize Embryos Using a Standard Binary Vector System 1. *Journal of Plant Physiology*, *129*(11), 13–22. http://doi.org/10.1104/pp.000653.tumefaciens

Frame, B. R., Zhang, H., Cocciolone, S. M., Sidorenko, L. V., Dietrich, C. R., Pegg, S. E., … Wang, K. (2000). Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cellular & Developmental Biology - Plant*, *36*(1), 21–29. http://doi.org/10.1007/s11627-000-0007-5

Frank, B. (1889). Ueber die Pilzsymbiose der Leguminosen. *Berichte Der Deutschen Botanischen Gesellschaft*, *7*, 332–346.

Fu, Y.-B. (2018). Oat evolution revealed in the maternal lineages of 25 Avena species. *Scientific Reports*, *8*(1), 4252. http://doi.org/10.1038/s41598-018-22478-4

Gana, J. A., Sharma, G. C., Zipf, A., Saha, S., Roberts, J., & Wesenberg, D. M. (1995). Genotype effects on plant regeneration in callus and suspension cultures of Avena, (Table 1), 217–224.

Garciallamas, C. (2004). Effect of pollinator on haploid production in durum wheat crossed with maize and pearl millet. *Plant Breeding*, *123*(2), 201–203. http://doi.org/10.1046/j.1439-0523.2003.00904.x

Gasparis, S., Bregier, C., Orczyk, W., & Nadolska-Orczyk, A. (2008). Agrobacterium-mediated transformation of oat (Avena sativa L.) cultivars via immature embryo and leaf explants. *Plant Cell Reports*, *27*(11), 1721–9. http://doi.org/10.1007/s00299-008-0593-y

Gautheret, R. (1939). Sur la possibilité de réaliser la culture indéfinie des tissues de tubercules de carotte. *C. R. Soc. Biol. Paris*, *208*, 118–120.

Gelvin, S. B. (2017). Integration of *Agrobacterium* T-DNA into the Plant Genome. *Annual Review of Genetics*, *51*(1), 195–217. http://doi.org/10.1146/annurev-genet-120215-035320

Gemayel, R., Cho, J., Boeynaems, S., & Verstrepen, K. J. (2012). Beyond junk-variable tandem repeats as facilitators of rapid evolution of regulatory and coding sequences. *Genes*, *3*(3), 461–480. http://doi.org/10.3390/genes3030461

Germanà, M. A. (2011). Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Reports*, *30*(5), 839–57. http://doi.org/10.1007/s00299-011-1061-7

Gless, C., Lörz, H., & Jähne-Gärtner, A. (1998). Transgenic oat plants obtained at high efficiency by microprojectile bombardment of leaf base segments. *Journal of Plant Physiology*, *152*(2–3), 151–157. http://doi.org/10.1016/S0176-1617(98)80126-0

Glowacka, K., & Jezowski, S. (2009). Genetic and nongenetic factors influencing callus induction in Miscanthus sinensis (Anderss.) anther cultures. *Journal of Applied Genetics*, *50*(4), 341–5. http://doi.org/10.1007/BF03195692

Göllner, E. M., Blaschek, W., & Classen, B. (2010). Structural Investigations on Arabinogalactan-Protein from Wheat, Isolated with Yariv Reagent. *Journal of Agricultural and Food Chemistry*, *58*(6), 3621–3626. http://doi.org/10.1021/jf903843f

Göllner, E. M., Ichinose, H., Kaneko, S., Blaschek, W., & Classen, B. (2011). An arabinogalactan-protein from whole grain of Avena sativa L. belongs to the wattle-

blossom type of arabinogalactan-proteins. *Journal of Cereal Science*, *53*(2), 244–249. http://doi.org/10.1016/j.jcs.2011.01.004

Graham, P. H. (1964). The Application of Computer Techniques to the Taxonomy of the Root-nodule Bacteria of Legumes. *Journal of General Microbiology*, *35*(3), 511–517. http://doi.org/10.1099/00221287-35-3-511

Graner, A., Jahoor, A., Schondelmaier, J., Siedler, H., Pillen, K., Fischbeck, G., … Herrmann, R. G. (1991). Construction of an RFLP map of barley. *Theoretical and Applied Genetics*, *83*(2), 250–6. http://doi.org/10.1007/BF00226259

Grillet, L., Ouerdane, L., Flis, P., Hoang, M. T. T., Isaure, M.-P., Lobinski, R., … Mari, S. (2014). Ascorbate efflux as a new strategy for iron reduction and transport in plants. *The Journal of Biological Chemistry*, *289*(5), 2515–25. http://doi.org/10.1074/jbc.M113.514828

Guo, Y.-D., Sewón, P., & Pulli, S. (1999). Improved embryogenesis from anther culture and plant regeneration in timothy. *Plant Cell, Tissue and Organ Culture*, *57*(2), 85–93. http://doi.org/10.1023/A:1006392411276

Guzy-Wrobelska, J., & Szarejko, I. (2003). Molecular and agronomic evaluation of wheat doubled haploid lines obtained through maize pollination and anther culture methods. *Plant Breeding*, *122*(4), 305–313. http://doi.org/10.1046/j.1439-0523.2003.00858.x

Haberlandt, G. (1902). Kulturversuche mit isolierten Pflanzenzellen. *Sitzungsber. Akad. Wiss. Wien., Math.-Naturwiss. Kl., Abt., 1*(111), 69–92.

Hao, L., Zhou, L., Xu, X., Cao, J., & Xi, T. (2006). The role of salicylic acid and carrot embryogenic callus extracts in somatic embryogenesis of naked oat (Avena nuda). *Plant Cell, Tissue and Organ Culture*, *85*(1), 109–113. http://doi.org/10.1007/s11240-005-9052-4

Harwood, W. A. (2012). Advances and remaining challenges in the transformation of barley and wheat. *Journal of Experimental Botany*, *63*(5), 1791–1798.

http://doi.org/10.1093/jxb/err380

He, X., & Bjørnstad, Å. (2012). Diversity of North European oat analyzed by SSR, AFLP and DArT markers. *Theoretical and Applied Genetics*, *125*(1), 57–70. http://doi.org/10.1007/s00122-012-1816-8

Heller, R. (1953). *Recherches sur la nutrition minérale des tissus végétaux cultivés in vitro*. Paris : Masson.

Hervás, J. P., Fernández-Gómez, M. E., & Giménez-Martín, G. (1974). Colchicine Effect on the Mitotic Spindle: Estimate of Multipolar Anaphase Production. *Caryologia*, *27*(3), 359–368. http://doi.org/10.1080/00087114.1974.10796589

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., & Schilperoort, R. A. (1983). A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. *Nature*, *303*(5913), 179–180. http://doi.org/10.1038/303179a0

Hoekstra, S., van Zijderveld, M., Heidekamp, E., & van der Mark, E. (1993). Microspore culture of Hordeum vulgate L.: the influence of density and osmolality. *Plant Cell Reports*, *12*, 661–665.

Hofinger, B. J., Huynh, O. A., Jankowicz-Cieslak, J., Müller, A., Otto, I., Kumlehn, J., & Till, B. J. (2013). Validation of doubled haploid plants by enzymatic mismatch cleavage. *Plant Methods*, *9*(1), 43. http://doi.org/10.1186/1746-4811-9-43

Holme, I. B., Krogstrup, P., & Hansen, J. (1997). Embryogenic callus formation, growth and regeneration in callus and suspension cultures of Miscanthus x ogiformis Honda Giganteus' as affected by proline. *Plant Cell, Tissue and Organ Culture*, *50*(3), 203–210. http://doi.org/10.1023/A:1005981300847

Honys, D., & Twell, D. (2004). Transcriptome analysis of haploid male gametophyte development in Arabidopsis. *Genome Biology*, *5*(11), R85. http://doi.org/10.1186/gb-2004-5-11-r85

Hood, E. (2004). RNAi: What's all the noise about gene silencing? *Environmental Health Perspectives*, *112*(4), A224-9.

Hoseini, M., Ghadimzadeh, M., Ahmadi, B., & Teixeira da Silva, J. a. (2013). Effects of ascorbic acid, alpha-tocopherol, and glutathione on microspore embryogenesis in Brassica napus L. *In Vitro Cellular & Developmental Biology - Plant*, *50*(1), 26–35. http://doi.org/10.1007/s11627-013-9579-8

Hosp, J., de Maraschin, S. F., Touraev, A., & Boutilier, K. (2007). Functional genomics of microspore embryogenesis. *Euphytica*, *158*(3), 275–285. http://doi.org/10.1007/s10681-006-9238-9

Houten, J. G., Quak, F., & Meer, F. A. (1968). Heat treatment and meristem culture for the production of virus-free plant material. *Netherlands Journal of Plant Pathology*, *74*(1), 17–24. http://doi.org/10.1007/BF01988884

Hul, T., & Kasha, K. J. (1997). Improvement of isolated microspore culture of wheat (Triticum aestivum L.) through ovary co-culture. *Plant Cell Reports*, *16*(8), 520–525. http://doi.org/10.1007/BF01142316

Hunter, C. (1985). The effect of anther orientation on the production of microspore-derived embryoids and plants of Hordeum vulgare cv. Sabarlis. *Plant Cell Reports*, (I 985), 267–268.

Hunter, C. P. (1987). Plant generation method. European patent application, No. 87200773.7 Ilic-Grubor.

Ibrahim, A. M., Kayat, F. B., Hussin, Z. E. S. M., Susanto, D., & Ariffulah, M. (2014). Determination of suitable microspore stage and callus induction from anthers of kenaf (Hibiscus cannabinus L.). *TheScientificWorldJournal*, *2014*, 284342. http://doi.org/10.1155/2014/284342

Ikeuchi, M., Ogawa, Y., Iwase, A., & Sugimoto, K. (2016). Plant regeneration: cellular origins and molecular mechanisms. *Development*, *143*(9), 1442–1451. http://doi.org/10.1242/dev.134668

Imin, N., Kerim, T., Rolfe, B. G., & Weinman, J. J. (2004). Effect of early cold stress on the maturation of rice anthers. *Proteomics*, *4*(7), 1873–82. http://doi.org/10.1002/pmic.200300738

Indrianto, A., Heberle-Bors, E., & Touraev, A. (1999). Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. *Plant Science*, *143*(1), 71–79. http://doi.org/10.1016/S0168-9452(99)00022-9

Initiative, T. A. G. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature*, *408*(6814), 796–815. http://doi.org/10.1038/35048692

Islam, S. M. S. (2010). The effect of colchicine pretreatment on isolated microspore culture of wheat (Triticum aestivum L.). *AJCS*, *4*(9), 660–665.

J.Gubiša R.Vaňkováb V.Červenáa M.Dragúňovác M.Hudcovicováa H.Lichtnerovác T.Dokupilc, & Z.Jurekovád. (2007). Transformed tobacco plants with increased tolerance to drought. *South African Journal of Botany*, *73*(4), 505–511. http://doi.org/10.1016/J.SAJB.2007.03.011

Jacquard, C., Asakaviciute, R., Hamalian, a M., Sangwan, R. S., Devaux, P., & Clément, C. (2006). Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. *Plant Cell Reports*, *25*(5), 375–81. http://doi.org/10.1007/s00299-005-0070-9

Jacquard, C., Nolin, F., Hécart, C., Grauda, D., Rashal, I., Dhondt-Cordelier, S., … Clément, C. (2009). Microspore embryogenesis and programmed cell death in barley: effects of copper on albinism in recalcitrant cultivars. *Plant Cell Reports*, *28*(9), 1329–39. http://doi.org/10.1007/s00299-009-0733-z

Jahne, A., & Lorz, H. (1995). Cereal microspore culture. *Plant Science*, *109*, 1–12.

Jauhar, P. P. (2003). Haploid and doubled haploid production in durum wheat by wide hybridization. In M. Maluszynski, K. J. Kasha, B. P. Forster, & I. Szarejko (Eds.), *Doubled Haploid Production in Crop Plants* (pp. 161–166). Springer Netherlands.

Jellen, E. N., & Beard, J. (2000). Geographical Distribution of a Chromosome 7C and 17 Intergenomic Translocation in Cultivated Oat. *Crop Science*, *40*(1), 256. http://doi.org/10.2135/cropsci2000.401256x

Joy, R. W., Patel, K. R., & Thorpe, T. A. (1988). Ascorbic acid enhancement of organogenesis in tobacco callus. *Plant Cell, Tissue and Organ Culture*, *13*(3), 219–228. http://doi.org/10.1007/BF00043670

Kahrizi, D. (2009). Study of Androgenesis and Spontaneous Chromosome Doubling in Barley (Hordeum vulgare L.) Advanced Lines Using Isolated Microspore Culture. *International Journal of Plant Breeding*, *3*, 111–114.

Kakkar, R. K., & Sawhney, V. K. (2002). Polyamine research in plants - a changing perspective. *Physiologia Plantarum*, *116*, 281–292.

Kanta, K., Rangaswamy, N. S., & Maheshwari, P. (1962). Test-tube fertilization in a flowering plants. *Nature*, *194*(4835), 1214–1217. http://doi.org/10.1038/1941214a0

Kasha, K. J., Hu, T. C., Oro, R., Simion, E., & Shim, Y. S. (2001). Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (Hordeum vulgare L.) microspores. *Journal of Experimental Botany*, *52*(359), 1227–38.

Kasha, K. J., & Maluszynsky, M. (2003). *Production of doubled haploids in crop plants*. (M. Maluszynski, K. J. Kasha, B. P. Forster, & I. Szaejko, Eds.)*Doubled Haploid Production in Crop Plants. A manual.* Dordrecht/Vienna: Kluwer Academic Publishers.

Kavi Kishor, P. B., Hima Kumari, P., Sunita, M. S. L., & Sreenivasulu, N. (2015). Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. *Frontiers in Plant Science*, *6*, 544. http://doi.org/10.3389/fpls.2015.00544

Keller, W. (1984). Anther culture of Brassica. In I. Vasil (Ed.), *Cell culture and somatic cell genetics of plants vol. 1* (pp. 302–310). Orlando: Academic Press.

Kelley, R. Y., Zipf, A. E., Wesenberg, D. E., & Sharma, G. C. (2002). Putrescine-enhanced somatic embryos and plant numbers from elite oat (Avena spp. L.) and reciprocal

crosses. *In Vitro Cellular & Developmental Biology - Plant*, *38*(5), 508–512. http://doi.org/10.1079/IVP2002339

Kemble, R. J., Barsby, T. L., & Yarrow, S. A. (1988). Transformation of plant mitochondria with mitochondrial DNA plasmids via protoplast fusion. *MGG Molecular & General Genetics*, *213*(2–3), 202–205. http://doi.org/10.1007/BF00339582

Kernan, Z., & Ferrie, A. M. R. (2006). Microspore embryogenesis and the development of a double haploidy protocol for cow cockle (Saponaria vaccaria). *Plant Cell Reports*, *25*(4), 274–280. http://doi.org/10.1007/s00299-005-0064-7

Key, S., Ma, J. K.-C., & Drake, P. M. (2008). Genetically modified plants and human health. *Journal of the Royal Society of Medicine*, *101*(6), 290–8. http://doi.org/10.1258/jrsm.2008.070372

Khush, G., & Virmani, S. (1996). Haploids in plant breeding. *In Vitro Haploid Production in Higher Plants*, 11–33.

Kinoshita, A., Okamoto, Y., Ishimura, S., & Satake, T. (2005). Enhancement of Culture Efficiency by Reducing the Density of Anthers and by Blotting the Liquid Medium Adhering to the Calli with a Filter Paper in Floating Anther Culture of Rice. *Breeding Research*, *7*(2), 103–111. http://doi.org/10.1270/jsbbr.7.103

Kiviharju, E. (2009). Anther culture derived doubled haploids in oat. In *Advances in Haploid Production in Higher Plants*.

Kiviharju, E., Laurila, J., & Lehtonen, M. (2004). Anther culture properties of oat x wild red oat progenies and a search for RAPD markers associated with anther culture ability. *Agricultural and Food Science*, *13*(May 2003), 151–162.

Kiviharju, E. M., & Tauriainen, a. a. (1999). 2,4-Dichlorophenoxyacetic acid and kinetin in anther culture of cultivated and wild oats and their interspecific crosses: plant regeneration from A. sativa L. *Plant Cell Reports*, *18*(7–8), 582–588. http://doi.org/10.1007/s002990050626

Kiviharju, E., Moisander, S., & Laurila, J. (2005). Improved green plant regeneration rates from oat anther culture and the agronomic performance of some DH lines. *Plant Cell, Tissue and Organ Culture*, *81*(1), 1–9. http://doi.org/10.1007/s11240-004-1560-0

Kiviharju, E., & Pehu, E. (1998). The effect of cold and heat pretreatments on anther culture response of Avena sativa and A. sterilis. *Plant, Cell, Tissue and Organ Culture*, 97–104.

Kiviharju, E., Puolimatka, M., & Pehu, E. (1997). Regeneration of anther-derived plants of Avena sterilis. *Plant Cell, Tissue and Organ Culture*, *48*, 147–152.

Kiviharju, E., Puolimatka, M., Saastamoinen, M., & Pehu, E. (2000). Extension of anther culture to several genotypes of cultivated oats. *Plant Cell Reports*, *19*(7), 674–679. http://doi.org/10.1007/s002999900165

Klein, T. M., & Fitzpatrick-McElligott, S. (1993). Particle bombardment: a universal approach for gene transfer to cells and tissues. *Current Opinion in Biotechnology*, *4*(5), 583–90.

Knox, J. P. (1995). The extracellular matrix in higher plants. 4. Developmentally regulated proteoglycans and glycoproteins of the plant cell surface. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *9*(11), 1004–12.

Kobayashi, R. S., Sinden, S. L., & Bouwkamp, J. C. (1993). Ovule culture of sweet potato (Ipomoea batatas) and closely related species. *Plant Cell, Tissue and Organ Culture*, *32*(1), 77–82. http://doi.org/10.1007/BF00040119

Kohler, F., & Wenzel, G. (1985). Regeneration of Isolated Barley Microspores in Conditioned Media and Trials to Characterize the Responsible Factor. *Journal of Plant Physiology*, *121*(2), 181–191.

Komari, T., Ishida, Y., Hiei, Y., Komari, T., Ishida, Y., & Hiei, Y. (2004). Plant Transformation Technology: *Agrobacterium* -Mediated Transformation. In *Handbook of Plant Biotechnology*. Chichester, UK: John Wiley & Sons, Ltd.

http://doi.org/10.1002/0470869143.kc014

Kreuger, M., & Van Hoist, G. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of Daucus carota L . *Planta*, *189*, 243–248.

Kuai, B., Perret, S., Wan, S. M., Dalton, S. J., Bettany, A. J. E., & Morris, P. (2001). Transformation of oat and inheritance of bar gene expression. *Plant Cell, Tissue and Organ Culture*, *66*(2), 79–88. http://doi.org/10.1023/A:1010613704824

Kumar, A. A., Mishra, P., Kumari, K., & Panigrahi, K. C. S. (2012). Environmental stress influencing plant development and flowering. *Frontiers in Bioscience (Scholar Edition)*, *4*, 1315–24.

Kumari, M., Clarke, H. J., Small, I., & Siddique, K. H. M. (2009). Albinism in Plants: A Major Bottleneck in Wide Hybridization, Androgenesis and Doubled Haploid Culture. *Critical Reviews in Plant Sciences*, *28*(6), 393–409. http://doi.org/10.1080/07352680903133252

Kuriakose, B., Du Toit, E. S., & Jordaan, A. (2012). Transient gene expression assays in rose tissues using a Bio-Rad Helios® hand-held gene gun. *South African Journal of Botany*, *78*, 307–311. http://doi.org/10.1016/J.SAJB.2011.06.002

Kynast, R. G., Riera-Lizarazu, O., Vales, M. I., Okagaki, R. J., Maquieira, S. B., Chen, G., … Phillips, R. L. (2001). A complete set of maize individual chromosome additions to the oat genome. *Plant Physiology*, *125*(3), 1216–27.

Lacroix, B., Li, J., Tzfira, T., & Citovsky, V. (2006). Will you let me use your nucleus? How *Agrobacterium* gets its T-DNA expressed in the host plant cellThis paper is one of a selection of papers published in this Special Issue, entitled The Nucleus: A Cell Within A Cell. *Canadian Journal of Physiology and Pharmacology*, *84*(3–4), 333–345. http://doi.org/10.1139/y05-108

Ladizinsky, G. (1998). *Plant Evolution under Domestication*. Dordrecht: Kluwer Academic Publishers.

Ladizinsky, G. (2012). *Studies in Oat Evolution A Man's Life with Avena*. Springer International Publishing. http://doi.org/10.1007/978-3-642-30547-4

Ladizinsky, G., & Zohary, D. (1971). Notes on species delimination, species relationships and polyploidy in Avena L. *Euphytica*, *20*(3), 380–395. http://doi.org/10.1007/BF00035663

Lane, C., Nr, N., Xu, Z., Huang, B., & Sunderland, N. (1981). Culture of barley anthers in conditioned media. *Journal of Experimental Botany*, *32*(129), 767–778.

Lantos, C., & Páricsi, S. (2006). Isolated microspore culture of wheat (Triticum aestivum L.) with Hungarian cultivars. *Acta Biologica Szegediensis*, *50*, 31–35.

Lantos, C., Weyen, J., Orsini, J. M., Gnad, H., Schlieter, B., Lein, V., … Pauk, J. (2013). Efficient application of in vitro anther culture for different European winter wheat (Triticum aestivum L.) breeding programmes. *Plant Breeding*, *132*(2), 149–154. http://doi.org/10.1111/pbr.12032

Larkin, P. J., & Scowcroft, W. R. (1981). Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *60*(4), 197–214. http://doi.org/10.1007/BF02342540

Larsen, E. T., Tuvesson, I. K., & Andersen, S. B. (1991). Nuclear genes affecting percentage of green plants in barley (Hordeum vulgare L.) anther culture. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *82*(4), 417–20. http://doi.org/10.1007/BF00588593

Lawrence, P. K., & Frey, K. J. (1975). Backcross variability for grain yield in oat species crosses (Avena sativa L. x A. sterilis L.). *Euphytica*, *24*(1), 77–85. http://doi.org/10.1007/BF00147171

Legget, J. M., & Thomas, H. (1995). Oat evolution and cytogenetics. In R. W. Welch (Ed.), *The Oat Crop. Production and Utilization* (pp. 121–149). London: Chapman & Hall.

Leifert, C., & Waites, W. M. (1994). Dealing with microbial contaminants in plant tissue and cell culture: hazard analysis and critical control points. In *Physiology, Growth and Development of Plants in Culture* (pp. 363–378). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-011-0790-7_42

Lemonnier-Le Penhuizic, C., Chatelet, C., Kloareg, B., & Potin, P. (2001). Carrageenan oligosaccharides enhance stress-induced microspore embryogenesis in Brassica oleracea var. italica. *Plant Science : An International Journal of Experimental Plant Biology*, *160*(6), 1211–1220.

Lentini, Z., Reyes, P., Martínez, C., & Roca, W. (1995). Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. *Plant Science*, *110*, 127–138.

Letarte, J., Simion, E., Miner, M., & Kasha, K. J. (2006). Arabinogalactans and arabinogalactan-proteins induce embryogenesis in wheat (Triticum aestivum L.) microspore culture. *Plant Cell Reports*, *24*(12), 691–8. http://doi.org/10.1007/s00299-005-0013-5

Letham, D. S. (1974). Regulators of Cell Division in Plant Tissues: The Cytokinins of Coconut Milk. *Physiologia Plantarum*, *32*(1), 66–70. http://doi.org/10.1111/j.1399-3054.1974.tb03728.x

Li, C. C.-D., Rossnagel, B. B. G., & Scoles, G. J. G. (2000). Tracing the phylogeny of the hexaploid oat with satellite DNAs. *Crop Science*, *40*(6), 1755–1763. http://doi.org/10.2135/cropsci2000.4061755x

Li, D., & Devaux, P. (2001). Enhancement of microspore culture efficiency of recalcitrant barley genotypes. *Plant Cell Reports*, *20*(6), 475–481. http://doi.org/10.1007/s002990100368

Li, H., & Devaux, P. (2003). High frequency regeneration of barley doubled haploid plants from isolated microspore culture. *Plant Science*, *164*(3), 379–386. http://doi.org/10.1016/S0168-9452(02)00424-7

Li, H., & Devaux, P. (2005). Isolated microspore culture overperforms anther culture for

green plant regeneration in barley (Hordeum vulgare L.). *Acta Physiologiae Plantarum*, *27*(4), 611–619.

Lindstrom, K., & Young, J. P. W. (2011). International Committee on Systematics of Prokaryotes * Subcommittee on the taxonomy of Agrobacterium and Rhizobium: Minutes of the meeting, 7 September 2010, Geneva, Switzerland. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *61*(12), 3089–3093. http://doi.org/10.1099/ijs.0.036913-0

Link, G. K. K., & Eggers, V. (1941). Hyperauxiny in Crown Gall of Tomato. *Botanical Gazette*. The University of Chicago Press. http://doi.org/10.2307/2472184

Liu, G., & Godwin, I. D. (2012). Highly efficient sorghum transformation. *Plant Cell Reports*, *31*(6), 999–1007. http://doi.org/10.1007/s00299-011-1218-4

Liu, W., Zheng, M., Polle, E., & Konzak, C. (2002). Highly Efficient Doubled-Haploid Production in Wheat (L.) via Induced Microspore Embryogenesis. *Crop Science*, 686–692.

Luthar, Z., & Bohanec, B. (1999). Induction of direct somatic organogenesis in onion (Allium cepa L.) using a two-step flower or ovary culture. *Plant Cell Reports*, *18*(10), 797–802. http://doi.org/10.1007/s002990050664

Lux, H., Herrmann, L., & Wetzel, C. (1990). Production of haploid sugar beet (Beta vulgaris L.) by culturing unpollinated ovules. *Plant Breeding*, *104*(3), 177–183. http://doi.org/10.1111/j.1439-0523.1990.tb00420.x

Lv, B., Nitcher, R., Han, X., Wang, S., Ni, F., Li, K., … Fu, D. (2014). Characterization of FLOWERING LOCUS T1 (FT1) Gene in Brachypodium and Wheat. *PLoS ONE*, *9*(4), e94171. http://doi.org/10.1371/journal.pone.0094171

Makowska, K., & Oleszczuk, S. (2014). Albinism in barley androgenesis. *Plant Cell Reports*, *33*(3), 385–392. http://doi.org/10.1007/s00299-013-1543-x

Makowska, K., Oleszczuk, S., & Zimny, J. (2017). The Effect of Copper on Plant

Regeneration in Barley Microspore Culture. *Czech Journal of Genetics and Plant Breeding*, *53*(1), 17–22. http://doi.org/10.17221/82/2016-CJGPB

Maksymiec, W. (1997). Effect of copper on cellular processes in higher plants. *Photosynthetica*, *34*(3), 321–342. http://doi.org/10.1023/A:1006818815528

Malik, M. R., Wang, F., Dirpaul, J. M., Zhou, N., Polowick, P. L., Ferrie, A. M. R., & Krochko, J. E. (2007). Transcript profiling and identification of molecular markers for early microspore embryogenesis in Brassica napus. *Plant Physiology*, *144*(1), 134–54. http://doi.org/10.1104/pp.106.092932

Maluszynska, M. (2003). Cytogenetic tests for ploidy level anylses - chromosome counts. In K. J. Maluszynski, M Kasha, B. P. Forster, & I. Szarejko (Eds.), *Doubled Haploid Production in Crop Plants: A Manual* (pp. 391–395). Dordrecht: Kluwer Academic Publishers.

Maluszynski, M., Kasha, K. J., & Szarejko, I. (2003). Published doubled haploid protocols in plant species. In M. Maluszynski, K. J. Kasha, B. P. Forster, & I. Szarejko (Eds.), *Doubled Haploid Production in Crop Plants* (pp. 309–335). Springer Netherlands. http://doi.org/10.1007/978-94-017-1293-4_46

Manderscheid, R., & Wild, A. (1986). Studies on the Mechanism of Inhibition by Phosphinothricin of Glutamine Synthetase Isolated from Triticum aestivum L. *Journal of Plant Physiology*, *123*(2), 135–142. http://doi.org/10.1016/S0176-1617(86)80134-1

Manninen, O. (2008). Optimizing anther culture for barley breeding. *Agricultural and Food Science*, *6*(5–6), 389–398.

Maqbool, S. B., Zhong, H., El-Maghraby, Y., Ahmad, A., Chai, B., Wang, W., … Sticklen, M. B. (2002). Competence of oat (Avena sativa L.) shoot apical meristems for integrative transformation, inherited expression, and osmotic tolerance of transgenic lines containing hva1. *Theoretical and Applied Genetics*, *105*(2–3), 201–208. http://doi.org/10.1007/s00122-002-0984-3

Maqbool, S. B., Zhong, H., Oraby, H. F., & Sticklen, M. B. (2009). Transgenic Wheat, Barley and Oats, *478*. http://doi.org/10.1007/978-1-59745-379-0

Maqbool, S. B., Zhong, H., & Sticklen, M. B. (2004). Genetic Engineering of Oat (Avena Sativa L.) Via the Biolistic Bombardment of Shoot Apical Meristems. In *Transgenic Crops of the World* (pp. 63–78). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-1-4020-2333-0_5

Maraschin, S. F., de Priester, W., Spaink, H. P., & Wang, M. (2005). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. *Journal of Experimental Botany*, *56*(417), 1711–26. http://doi.org/10.1093/jxb/eri190

Marcińska, I., Nowakowska, A., Skrzypek, E., & Czyczyło-Mysza, I. (2013). Production of double haploids in oat (Avena sativa L.) by pollination with maize (Zea mays L.). *Central European Journal of Biology*, *8*(3), 306–313. http://doi.org/10.2478/s11535-013-0132-2

María Rodríguez-Serrano, Ivett Bárány, Deepak Prem, María-José Coronado, M. C. R. and P. S. T., Rodríguez-Serrano, M., Bárány, I., Prem, D., Coronado, M.-J., Risueño, M. C., & Testillano, P. S. (2012). NO, ROS, and cell death associated with caspase-like activity increase in stress-induced microspore embryogenesis of barley. *Journal of Experimental Botany*, *63*(5), 2007–24. http://doi.org/10.1093/jxb/err400

Marinkovié, N., & Radojevié, L. (1992). The influence of bud length, age of the tree and culture media on androgenesis induction inAesculus carnea Hayne anther culture. *Plant Cell, Tissue and Organ Culture*, 51–59.

Marshall, A. H., Howarth, C., Cowan, A. A., Langdon, T., Valentine, J., Mcdevitt, J., … Bounds, M. P. (2010). The incorporation of important traits underlying sustainable development of the oat crop through combining “conventional” phenotypic selection with molecular marker technologies (OatLINK).

McCabe, P. F., Valentine, T. A., Forsberg, L. S., & Pennell, R. I. (1997). Soluble Signals from Cells Identified at the Cell Wall Establish a Developmental Pathway in Carrot. *The*

Plant Cell, *9*(12), 2225. http://doi.org/10.2307/3870581

Mejza, S., Morgant, V., DiBona, D., & Wong, J. (1993). Plant regeneration from isolated microspores of Triticum aestivum. *Plant Cell Reports*, 149–153.

Miller, C. O., Skoog, F., Von Saltza, M. H., & Strong, F. M. (1955). KINETIN, A CELL DIVISION FACTOR FROM DEOXYRIBONUCLEIC ACID. *Journal of the American Chemical Society*, *77*(5), 1392–1392. http://doi.org/10.1021/ja01610a105

Mitalipov, S., & Wolf, D. (2009). Totipotency, pluripotency and nuclear reprogramming. *Engineering of Stem Cells*, *114*, 185–99. http://doi.org/10.1007/10_2008_45

Moore-Colyer, R. J. (1995). Oats and oat production in history and pre-history. In *The Oat Crop* (pp. 1–33). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-011-0015-1_1

Moore, G. A., Jacono, C. C., Neidigh, J. L., Lawrence, S. D., & Cline, K. (1992). Agrobacterium-mediated transformation of Citrus stem segments and regeneration of transgenic plants. *Plant Cell Reports*, *11–11*(5–6), 238–42. http://doi.org/10.1007/BF00235073

Moore, L. W., Chilton, W. S., & Canfield, M. L. (1997). Diversity of opines and opine-catabolizing bacteria isolated from naturally occurring crown gall tumors. *Applied and Environmental Microbiology*, *63*(1), 201–207.

Morel, G. (1960). Producing virus-free Cymbidiums. *American Orchid Society Bulletin*, *29*, 495–497.

Morel, G., & Wetmore, R. H. (1951). Tissue Culture of Monocotyledons. *Source: American Journal of Botany*, *38*(2), 138–140.

Morgan, W. G. (1976). A technique for the production of polyploids in grasses. *Euphytica*, *25*(1), 443–446. http://doi.org/10.1007/BF00041577

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 473–497.

http://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Murovec, J., & Bohanec, B. (2012). Haploids and doubled haploids in plant breeding. *Plant Breeding*. http://doi.org/10.5772/29982

Nakamura, S., Mano, S., Tanaka, Y., Ohnishi, M., NAKAMORI, C., ARAKI, M., … Ishiguro, S. (2010). Gateway Binary Vectors with the Bialaphos Resistance Gene, bar , as a Selection Marker for Plant Transformation. *Bioscience, Biotechnology, and Biochemistry*, *74*(6), 1315–1319. http://doi.org/10.1271/bbb.100184

Newell, C. A. (2000). Plant Transformation Technology Developments and Applications. *Molecular Biotechnology*, *16*, 53–65.

Nitsch, J. P., & Nitsch, C. (1956). Auxin-Dependent Growth of Excised Helianthus Tuberosus Tissues . I . Author (s): J . P . Nitsch and Colette Nitsch Source : American Journal of Botany , Vol . 43 , No . 10 (Dec ., 1956), pp . 839-851 Published by : Botanical Society of America , Inc . *American Journal of Botany*, *43*(10), 839–851.

Nuutila, A. M., Hämäläinen, J., & Mannonen, L. (2000). Optimization of media nitrogen and copper concentrations for regeneration of green plants from polyembryogenic cultures of barley (Hordeum vulgare L.). *Plant Science*, *151*(1), 85–92. http://doi.org/10.1016/S0168-9452(99)00202-2

Ochatt, S., Pech, C., Grewal, R., Conreux, C., Lulsdorf, M., & Jacas, L. (2009). Abiotic stress enhances androgenesis from isolated microspores of some legume species (Fabaceae). *Journal of Plant Physiology*, *166*(12), 1314–28. http://doi.org/10.1016/j.jplph.2009.01.011

Okoń, S., Paczos-Grzęda, E., Ociepa, T., Koroluk, A., Sowa, S., Kowalczyk, K., & Chrząstek, M. (2016). *Avena sterilis* L. Genotypes as a Potential Source of Resistance to Oat Powdery Mildew. *Plant Disease*, *100*(10), 2145–2151. http://doi.org/10.1094/PDIS-11-15-1365-RE

Oleszczuk, S., Sowa, S., & Zimny, J. (2006). Androgenic response to preculture stress in microspore cultures of barley. *Protoplasma*, *228*(1–3), 95–100.

http://doi.org/10.1007/s00709-006-0179-x

Olsen, F. (1987). Induction of microspore embryogenesis in cultured anthers of Hordeum vulgare. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. *Carlsberg Research Communications*, *52*(8), 393–404.

Olszewska, D., Kisiala, A., Niklas-Nowak, A., & Nowaczyk, P. (2014). Study of in vitro anther culture in selected genotypes of genus Capsicum. *Turkish Journal of Biology*, *38*, 118–124. http://doi.org/10.3906/biy-1307-50

Ouyang, J., Jia, S., Zhang, C., Chen, X., & Feng, G. (1989). A new synthetic medium (W14) for wheat anther culture. *Annu Rep Inst Genet Acad Sin*, 91–92.

Ozsan, T., & Onus, A. (2017). In vitro Pepper (Capsicum annuum L.) Anther Culture: Can be Affected Via Vitamins B? *Biotechnology Journal International*, *20*(1), 1–13. http://doi.org/10.9734/BJI/2017/37102

Pandey, A., & Tamta, S. (2014). *In Vitro* Propagation of the Important Tasar Oak (*Quercus serrata* Thunb.) by Casein Hydrolysate Promoted High Frequency Shoot Proliferation. *Journal of Sustainable Forestry*, *33*(6), 590–603. http://doi.org/10.1080/10549811.2014.912587

Parra-Vega, V., Renau-Morata, B., Sifres, A., & Seguí-Simarro, J. M. (2012). Stress treatments and in vitro culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (Capsicum annuum L.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *112*(3), 353–360. http://doi.org/10.1007/s11240-012-0242-6

Pasternak, T. P., Rudas, V. A., Lörz, H., & Kumlehn, J. (1999). Embryogenic Callus Formation and Plant Regeneration from Leaf Base Segments of Barley (Hordeum vulgare L.). *Journal of Plant Physiology*, *155*(3), 371–375. http://doi.org/10.1016/S0176-1617(99)80119-9

Pauk, J., Mihaly, R., Monostori, T., & Puolimatka, M. (2003). Protocol of triticale (x Triticosecale Wittmack) microspore culture. In M. M, K. KJ, F. BP, & S. I (Eds.),

Doubled Haploid Production in Crop Plants – A Manual (pp. 129–134). Dordrecht/Boston/London: Kluwer.

Pawar, B., Kale, P., Bahurupe, J., Jadhav, A., KALE, A., & PAWAR, S. (2015). Proline and Glutamine Improve in vitro Callus Induction and Subsequent Shooting in Rice. *Rice Science*, *22*(6), 283–289. http://doi.org/10.1016/J.RSCI.2015.11.001

Pechan, P., & Keller, W. (1989). Induction of microspore embryogenesis inBrassica napus L. by gamma irradiation and ethanol stress. *In Vitro Cellular & Developmental Biology*, *25*(11), 98–99.

Pei, W., & Yu-rong, C. (1975). A STUDY ON THE APPLICATION OF C-17 MEDIUM FOR ANTHER CULTURE. *Acta Botanica Sinica*, *28*.

Percudani, R., & Peracchi, A. (2003). A genomic overview of pyridoxal-phosphate-dependent enzymes. *EMBO Reports*, *4*(9), 850–4. http://doi.org/10.1038/sj.embor.embor914

Perera, P., Yakandawala, D., & Hocher, V. (2008). Variations among anthers and their orientation during in vitro culture for inducing androgenesis in coconut, 104–110.

Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbart, F., & Tempé, J. (1983). Further extension of the opine concept: Plasmids in Agrobacterium rhizogenes cooperate for opine degradation. *MGG Molecular & General Genetics*, *190*(2), 204–214. http://doi.org/10.1007/BF00330641

Petolino, J. F., & Thompson, S. a. (1987). Genetic analysis of anther culture response in maize. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *74*(2), 284–6. http://doi.org/10.1007/BF00289982

Pilon, M., Abdel-Ghany, S. E., Cohu, C. M., Gogolin, K. A., & Ye, H. (2006). Copper cofactor delivery in plant cells. *Current Opinion in Plant Biology*, *9*(3), 256–263. http://doi.org/10.1016/J.PBI.2006.03.007

Pink, & Puddephat. (1999). Deployment of disease resistance genes by plant

transformation - a “mix and match” approach. *Trends in Plant Science*, *4*(2), 71–75.

Pollen Development — University of Leicester. (n.d.). Retrieved from https://www2.le.ac.uk/departments/genetics/people/twell/lab/pollenis/development

Ponitka, A., & Slusarkiewicz-Jarzina, A. (2009). Regeneration of oat androgenic plants in relation to induction media and culture conditions of embryo-like structures. *Acta Societatis Botanicorum Poloniae*, *78*(3), 209–213.

Powell, W., Borrino, E., & Goodall, V. (1988). The effect of anther orientation on microspore-derived plant production in barley (Hordeum vulgare L.). *Euphytica*, *163*, 159–163.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., & Cormier, M. J. (1992). Primary structure of the Aequorea victoria green-fluorescent protein. *Gene*, *111*(2), 229–33.

Preece, C., Livarda, A., Christin, P.-A., Wallace, M., Martin, G., Charles, M., … Osborne, C. P. (2017). How did the domestication of Fertile Crescent grain crops increase their yields? *Functional Ecology*, *31*(2), 387–397. http://doi.org/10.1111/1365-2435.12760

Raina, S., & Irfan, S. (1998). High-frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. *Plant Cell Reports*, 957–962.

Rajhathy, T., & D., H. T. P. (1974). *Cytogenetics of oats (Avena L.)*. Genetics Society of Canada.

Rech, E. L., Vianna, G. R., & Aragão, F. J. L. (2008). High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nature Protocols*, *3*(3), 410–418. http://doi.org/10.1038/nprot.2008.9

Redha, A., Attia, T., Buter, B., Stamp, P., & Schmid, J. E. (1998). Single and combined effects of colchicine, L-proline and post-inoculation low temperature on anther culture of wheat, Triticum aestivum L. *Plant Breeding*, *117*(4), 335–340.

http://doi.org/10.1111/j.1439-0523.1998.tb01950.x

Redha, A., & Suleman, P. (2011). Effects of exogenous application of polyamines on wheat anther cultures. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *105*(3), 345–353. http://doi.org/10.1007/s11240-010-9873-7

Regina, A., Berbezy, P., Kosar-Hashemi, B., Li, S., Cmiel, M., Larroque, O., … Morell, M. (2015). A genetic strategy generating wheat with very high amylose content. *Plant Biotechnology Journal*, *13*(9), 1276–1286. http://doi.org/10.1111/pbi.12345

Reinert, J. (1959). Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. *Planta*, *53*(4), 318–333. http://doi.org/10.1007/BF01881795

Riboni, M., Robustelli Test, A., Galbiati, M., Tonelli, C., & Conti, L. (2014). Environmental stress and flowering time: the photoperiodic connection. *Plant Signaling & Behavior*, *9*(7), e29036. http://doi.org/10.4161/psb.29036

Rines, H. W. (1983). Oat Anther Culture: Genotype Effects on Callus Initiation and the Production of a Haploid Plant. *Crop Science*, *23*(2), 268. http://doi.org/10.2135/cropsci1983.0011183X002300020022x

Rines, H. W. (2003). Oat haploids from wide hybridization. In *Doubled Haploid Production in Crop Plants* (pp. 155–159). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-017-1293-4_24

Rines, H. W., & Mccoy, T. J. (1981). Tissue Culture Initiation and Plant Regeneration in Hexaploid Species of Oats. *Crop Science*, *21*, 837–843.

Ritala, A., Mannonen, L., & Oksman-Caldentey, K.-M. (2001). Factors affecting the regeneration capacity of isolated barley microspores (Hordeum vulgare L.). *Plant Cell Reports*, *20*(5), 403–407. http://doi.org/10.1007/s002990100345

Ritala, A., Mannonen, L., & Oksman-Caldentey, K.-M. (2014). Factors affecting the regeneration capacity of isolated barley microspores (Hordeum vulgare L.). *Plant Cell*

Reports, *20*(5), 403–407. http://doi.org/10.1007/s002990100345

Rodrigues, L., & Forte, B. (2004). Effects of light conditions and 2, 4-D concentration in soybean anther culture. *Plant Growth Regulation*, *44*, 125–131.

Romano, A., van der Plas, L. H. W., Witholt, B., Eggink, G., & Mooibroek, H. (2005). Expression of poly-3-(R)-hydroxyalkanoate (PHA) polymerase and acyl-CoA-transacylase in plastids of transgenic potato leads to the synthesis of a hydrophobic polymer, presumably medium-chain-length PHAs. *Planta*, *220*(3), 455–464. http://doi.org/10.1007/s00425-004-1349-8

Ron, M., Kajala, K., Pauluzzi, G., Wang, D., Reynoso, M. A., Zumstein, K., … Brady, S. M. (2014). Hairy root transformation using Agrobacterium rhizogenes as a tool for exploring cell type-specific gene expression and function using tomato as a model. *Plant Physiology*, *166*(2), 455–69. http://doi.org/10.1104/pp.114.239392

Rudolf, K., Bohanec, B., & Hansen, M. (1999). Microspore culture of white cabbage, Brassica oleracea var. capitata L.: Genetic improvement of non-responsive cultivars and effect of genome doubling agents. *Plant Breeding*, 0–4.

Ruhlman, T. A. (2014). Plastid Transformation in Lettuce (Lactuca sativa L.) by Biolistic DNA Delivery (pp. 331–343). Humana Press, Totowa, NJ. http://doi.org/10.1007/978-1-62703-995-6_21

San, L., Gelebart, P., & Vasil, I. (1986). *Production of gynogenetic haploids*. (I. K. Vasil, Ed.)*Cell culture and somatic cell genetics of plants vol. 3*. New York: Academic.

San Noeum, L. H. (1976). NONFERTILIZED OVARIES OF HORDEUM-VULGARE CULTURED IN-VITRO. *Annales de l'Amelioration Des Plantes (Paris)*, *26*, 751–754.

Sandford, J. C., Klein, T. M., Wolf, E. D., & Allen, N. (1987). Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Technology*, *5*(1), 27–37. http://doi.org/10.1080/02726358708904533

Santra, M., Ankrah, N., Santra, D. K., & Kidwell, K. K. (2012). An Improved Wheat

Microspore Culture Technique for the Production of Doubled Haploid Plants. *Crop Science*, *52*(5), 2314. http://doi.org/10.2135/cropsci2012.03.0141

Sattler, M. C., Carvalho, C. R., & Clarindo, W. R. (2016). The polyploidy and its key role in plant breeding. *Planta*, *243*(2), 281–296. http://doi.org/10.1007/s00425-015-2450-x

Saurabh, S., Vidyarthi, A. S., & Prasad, D. (2014). RNA interference: concept to reality in crop improvement. *Planta*, *239*(3), 543–564. http://doi.org/10.1007/s00425-013-2019-5

Sayed, H., Kayyal, H., Ramsey, L., Ceccarelli, S., & Baum, M. (2002). Segregation distortion in doubled haploid lines of barley (Hordeum vulgare L.) detected by simple sequence repeat (SSR) markers. *Euphytica*, *125*(2), 265–272. http://doi.org/10.1023/A:1015861610226

Schilperoort, R. A., Veldstra, H., Warnaar, S. O., Mulder, G., & Cohen, J. A. (1967). Formation of complexes between DNA isolated from tobacco crown gall tumours and RNA complementary to Agrobacterium tumefaciens DNA. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis*, *145*(2), 523–525. http://doi.org/10.1016/0005-2787(67)90075-5

Schulze, J. (2007). Improvements in cereal tissue culture by thidiazuron: a review. *Fruit, Vegetable and Cereal Science and …*, *1*(1978), 64–79.

Seifert, G. J., & Roberts, K. (2007). The biology of arabinogalactan proteins. *Annual Review of Plant Biology*, *58*, 137–161. http://doi.org/10.1146/annurev.arplant.58.032806.103801

Shah, K. H., Almaghrabi, B., & Bohlmann, H. (2013). Comparison of Expression Vectors for Transient Expression of Recombinant Proteins in Plants. *Plant Molecular Biology Reporter*, *31*(6), 1529–1538. http://doi.org/10.1007/s11105-013-0614-z

Shahnewaz, S., & Bari, M. (2004). Effect of concentration of sucrose on the frequency of callus induction and plant regeneration in anther culture of rice (Oryza sativa L.). *Plant Tissue Cult*, *14*(1), 37–43.

Shannon, P., Nicholson, A., Dunwell, J., & DR, D. (1985). Effect of anther orientation on microspore-callus production in barley (Hordeum vulgare L.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *4*, 271–280.

Shariatpanahi, M. E., Belogradova, K., Hessamvaziri, L., Heberle-Bors, E., & Touraev, A. (2006). Efficient embryogenesis and regeneration in freshly isolated and cultured wheat (Triticum aestivum L.) microspores without stress pretreatment. *Plant Cell Reports*, *25*(12), 1294–1299. http://doi.org/10.1007/s00299-006-0205-7

Sharma, S. K., & Millam, S. (2004). Somatic embryogenesis in Solanum tuberosum L.: a histological examination of key developmental stages. *Plant Cell Reports*, *23*(3), 115–9. http://doi.org/10.1007/s00299-004-0814-y

Sharma, V. K., H�nsch, R., Mendel, R. R., & Schulze, J. (2005). Seasonal effect on tissue culture response and plant regeneration frequency from non-bombarded and bombarded immature scutella of barley (Hordeum vulgare) harvested from controlled environment. *Plant Cell, Tissue and Organ Culture*, *81*(1), 19–26. http://doi.org/10.1007/s11240-004-2617-9

Sharp, W. R., Raskin, R. S., & Sommer, H. E. (1972). The use of nurse culture in the development of haploid clones in tomato. *Planta*, *104*(4), 357–361. http://doi.org/10.1007/BF00386318

Shim, Y. S., & Kasha, K. J. (2003). The influence of pretreatment on cell stage progression and the time of DNA synthesis in barley (Hordeum vulgare L.) uninucleate microspores. *Plant Cell Reports*, *21*(11), 1065–1071. http://doi.org/10.1007/s00299-003-0635-4

Shrestha, S. L., & Kang, W.-H. (2015). Stomata length and Density as an Indicator of Ploidy level in Sweet Pepper (Capsicum annuum L.). *Research Journal of Recent Sciences Res. J. Recent. Sci*, *5*, 4–10.

Sidhu, P. K., & Davies, P. a. (2009). Regeneration of fertile green plants from oat isolated microspore culture. *Plant Cell Reports*, *28*(4), 571–7. http://doi.org/10.1007/s00299-

009-0684-4

Skoog, F., & Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposia of the Society for Experimental Biology*, *11*, 118–30.

Slama Ayed, O., De Buyser, J., Picard, E., Trifa, Y., & Amara, H. S. (2010). Effect of pre-treatment on isolated microspores culture ability in durum wheat (Triticum turgidum subsp. durum Desf.). *Journal of Plant Breeding and Crop Science*, *2*(2), 30–38.

Ślusarkiewicz-Jarzina, A., & Ponitka, A. (2003). EFFICIENT PRODUCTION OF SPONTANEOUS AND INDUCED DOUBLED HAPLOID TRITICALE PLANTS DERIVED FROM ANTHER CULTURE. *Cereal Research Communications*. Akadémiai Kiadó. http://doi.org/10.2307/23786969

Ślusarkiewicz-Jarzina, A., & Ponitka, A. (2007). The effect of physical medium state on anther culture response in Polish cultivated oat (Avena sativa L.). *Acta Biologica Cracoviensia Series Botanica*, 27–31.

Smith, E. F., & Townsend, C. O. (1907). A plant-tumour of bacterial origin. *Science*, *25*(643), 671–673. http://doi.org/10.1126/science.25.643.671

Smykal, P. (2000). Smykal (2000).pdf. *Biologia Plantarnum*, *43*(4), 481–489.

Smykal, P., & Pechan, P. M. (2000). Stress, as assessed by the appearance of sHsp transcripts, is required but not sufficient to initiate androgenesis. *Physiologia Plantarum*, *110*(1), 135–143. http://doi.org/10.1034/j.1399-3054.2000.110118.x

Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F., & Bushnell, W. R. (1992). Fertile, Transgenic Oat Plants. *Nature Biotechnology*, *10*(12), 1589–1594. http://doi.org/10.1038/nbt1292-1589

Sood, P., Bhattacharya, A., & Sood, A. (2011). Problems and possibilities of monocot transformation. *Biologia Plantarum*, *55*(1), 1–15. http://doi.org/10.1007/s10535-011-0001-2

Spencer, T. M., Gordon-Kamm, W. J., Daines, R. J., Start, W. G., & Lemaux, P. G. (1990). Bialaphos selection of stable transformants from maize cell culture. *Theoretical and Applied Genetics*, *79*(5), 625–31. http://doi.org/10.1007/BF00226875

Stevens, J. B., & Brinkman, M. A. (1986). Performance of <i>Avena sativa<i/> L./<i>Avena fatua<i/> L. Backcross lines. *Euphytica*, *35*(3), 785–792. http://doi.org/10.1007/BF00028586

Steward, F. C., Mapes, M. O., & Mears, K. (1958). Growth and Organized Development of Cultured Cells. II. Organization in Cultures Grown from Freely Suspended Cells. *American Journal of Botany*, *45*(10), 705. http://doi.org/10.2307/2439728

Subhashini, K., & Reddy, G. M. (1991). Role of proline in callus growth and plant regeneration under salt stress in rice. *Proceedings of the Indian National Science Academy*, 81–84.

Sun, C. S., Lu, T. G., & Sondahl, M. R. (1991). Anther culture of naked oat and the establishment of its haploid suspension. *Acta Botanica Sinica*, *33*, 417–420.

Sunderland, N. (1974). Anther Culture as a Means of Haploid Induction. In *Haploids in Higher Plants-Advances and Potential* (pp. 91–122).

Tang, F., Tao, Y., Zhao, T., & Wang, G. (2006). In vitro production of haploid and doubled haploid plants from pollinated ovaries of maize (Zea mays). *Plant Cell, Tissue and Organ Culture*, *84*(2), 233–237. http://doi.org/10.1007/s11240-005-9017-7

Tanhuanpää, P., Kalendar, R., Schulman, A. H., & Kiviharju, E. (2008). The first doubled haploid linkage map for cultivated oat. *Genome / National Research Council Canada = Génome / Conseil National de Recherches Canada*, *51*(8), 560–9. http://doi.org/10.1139/G08-040

Tanhuanpää, P., Manninen, O., Beattie, A., Eckstein, P., Scoles, G., Rossnagel, B., & Kiviharju, E. (2012). An updated doubled haploid oat linkage map and QTL mapping of agronomic and grain quality traits from Canadian field trials. *Genome*, *55*, 289–301. http://doi.org/10.1139/G2012-017

Tanhuanpää, P., Manninen, O., & Kiviharju, E. (2010). QTLs for important breeding characteristics in the doubled haploid oat progeny. *Genome*, *493*, 482–493. http://doi.org/10.1139/G10-022

Telmer, C., Newcomb, W., & Simmonds, D. (1995). Cellular changes during heat shock induction and embryo development of cultured microspores ofBrassica napus cv. Topas. *Protoplasma*, 106–112.

Terho, P. (2009). Flowing Software.

Thole, V., & Vain, P. (2012). Agrobacterium-Mediated Transformation of Brachypodium distachyon. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 847, pp. 137–149). http://doi.org/10.1007/978-1-61779-558-9_12

Thomas, J., Chen, Q., & Howes, N. (1997). Chromosome doubling of haploids of common wheat with caffeine. *Genome*, *40*(4), 552–8.

Till, B. J., Zerr, T., Comai, L., & Henikoff, S. (2006). A protocol for TILLING and Ecotilling in plants and animals. *Nature Protocols*, *1*(5), 2465–2477. http://doi.org/10.1038/nprot.2006.329

Toki, S., Takamatsu, S., Nojiri, C., Ooba, S., Anzai, H., Iwata, M., … Uchimiya, H. (1992). Expression of a Maize Ubiquitin Gene Promoter-bar Chimeric Gene in Transgenic Rice Plants'. *Plant Physiol*, *100*, 1503–1507.

Torbert, K. A., Rines, H. W., & Somers, D. A. (1995). Use of paromomycin as a selective agent for oat transformation. *Plant Cell Reports*, *14*(10), 635–640. http://doi.org/10.1007/BF00232728

Touraev, A., Ilham, A., Vicente, O., & Heberle-Bors, E. (1996). Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. *Plant Cell Reports*, 561–565.

Touraev, A., Indrianto, A., Wratschko, I., Vicente, O., & Heberle-Bors, E. (1996). Efficient microspore embryogenesis in wheat (Triticum aestivum L.) induced by starvation at

high temperature. *Sexual Plant Reproduction*, *9*(4), 209–215. http://doi.org/10.1007/BF02173100

Touraev, A., Pfosser, M., & Heberle-Bors, E. (2001). The microspore: A haploid multipurpose cell. *Advances in Botanical Research*, *35*, 53–109. http://doi.org/10.1016/S0065-2296(01)35004-8

Trottier, M., Collin, J., & Comeau, A. (1993). Comparison of media for their aptitude in wheat anther culture. *Plant Cell, Tissue and Organ Culture*, (Kao 1981), 59–67.

Ueki, S., Lacroix, B., Krichevsky, A., Lazarowitz, S. G., & Citovsky, V. (2008). Functional transient genetic transformation of Arabidopsis leaves by biolistic bombardment. *Nature Protocols*, *4*(1), 71–77. http://doi.org/10.1038/nprot.2008.217

Ueki, S., Magori, S., Lacroix, B., & Citovsky, V. (2013). Transient gene expression in epidermal cells of plant leaves by biolistic DNA delivery. *Methods in Molecular Biology (Clifton, N.J.)*, *940*, 17–26. http://doi.org/10.1007/978-1-62703-110-3_2

Uspenski, E. E., & Uspenskaia, W. J. (1925). Reinkultur und ungeschlechtliche Fortpflanzung der Volvox globator in einer synthetischen Nährlösung. *Z. Bot.*, *17*, 273–308.

Valentine, J., Cowan, A. A., & Marshall, A. H. (2011). Oat breeding. In F. H. Webster & P. J. Woods (Eds.), *Oats chemistry and technology* (2nd ed., pp. 11–30). AACC International, Inc. USA.

Van Hengel, A. J., & Roberts, K. (2003). AtAGP30, an arabinogalactan-protein in the cell walls of the primary root, plays a role in root regeneration and seed germination. *Plant Journal*, *36*(2), 256–270. http://doi.org/10.1046/j.1365-313X.2003.01874.x

Van Larebeke, N., Genetello, C., Schell, J., Schilperoort, R. A., Hermans, A. K., Hernalsteens, J. P., & Van Montagu, M. (1975). Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. *Nature*, *255*(5511), 742–743. http://doi.org/10.1038/255742a0

Van Meeteren, U., Van Gelder, H., & Van Ieperen, W. (2000). Reconsideration of the use of deionized water as vase water in experiments on cut flowers. *Postharvest Biology and Technology*, *18*(2), 175–187.

Van Overbeek, J., Conklin, M. E., & Blakeslee, A. F. (1941). FACTORS IN COCONUT MILK ESSENTIAL FOR GROWTH AND DEVELOPMENT OF VERY YOUNG DATURA EMBRYOS. *Science*, *94*(2441), 350–351. http://doi.org/10.1126/science.94.2441.350

Velázquez, E., Peix, A., Zurdo-Piñiro, J. L., Palomo, J. L., Mateos, P. F., Rivas, R., … Martínez-Molina, E. (2005). The Coexistence of Symbiosis and Pathogenicity-Determining Genes in *Rhizobium rhizogenes* Strains Enables Them to Induce Nodules and Tumors or Hairy Roots in Plants. *Molecular Plant-Microbe Interactions*, *18*(12), 1325–1332. http://doi.org/10.1094/MPMI-18-1325

Virtanenb, E., & Mälkkia, Y. (2001). Gastrointestinal Effects of Oat Bran and Oat Gum: A Review. *LWT - Food Science and Technology*, *34*(6), 337–347. http://doi.org/10.1006/FSTL.2001.0795

Wang, J., Su, Y., Jia, F., & Jin, H. (2013). Characterization of casein hydrolysates derived from enzymatic hydrolysis. *Chemistry Central Journal*, *7*(1), 62. http://doi.org/10.1186/1752-153X-7-62

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta*, *218*(1), 1–14. http://doi.org/10.1007/s00425-003-1105-5

Wang, Y. Y., Kuang, A., Russell, S. D., & Tian, H. Q. (2006). In vitro fertilization as a tool for investigating sexual reproduction of angiosperms. *Sexual Plant Reproduction*, *19*(3), 103–115. http://doi.org/10.1007/s00497-006-0029-1

Wędzony, M. (2003). Protocol for anther culture in hexaploid triticale (x Triticosecale Wittm.). In M. Maluszynski, K. J. Kasha, B. P. Forster, & I. Szarejko (Eds.), *Doubled Haploid Production in Crop Plants* (pp. 123–128). Dordrecht/Boston/London: Springer Netherlands. http://doi.org/10.1007/978-94-017-1293-4

Wędzony, M., Forster, B., Żur, I., Golemiec, E., Szechyńska-Hebda, M., Dubas, E., & Gotębiowska, G. (2009). Progress in doubled haploid technology in higher plants. In A. Touraev, B. P. Forster, & S. M. Jain (Eds.), *Advances in Haploid Production in Higher Plants*. Springer Netherlands.

Wernicke, W., & Kohlenbach, H. W. (1977). Versuche zur kultur isolierter mikrosporen von Nicotiana und Hyoscyamus. *Zeitschrift Für Pflanzenphysiologie*, *81*(4), 330–340. http://doi.org/10.1016/S0044-328X(77)80100-1

Wetherell, D. F., & Dougall, D. K. (1976). Sources of Nitrogen Supporting Growth and Embryogenesis in Cultured Wild Carrot Tissue. *Physiologia Plantarum*, *37*(2), 97–103. http://doi.org/10.1111/j.1399-3054.1976.tb03939.x

Wheatley, W., Marsolais, A., & Kasha, K. (1986). Microspore growth and anther staging in barley anther culture. *Plant Cell Reports*, *3*, 47–49.

White, E. (1995). Structure and development of oats. In R. Welch (Ed.), *The Oat Crop. Production and Utilization* (pp. 88–118). London: Chapman & Hall.

White, P. R. (1943). *A Handbook of Plant Tissue Culture*. Lancaster, PA: The Jacques Cattell Press.

White, P. R., & Braun, A. C. (1941). Crown gall production by bacteria-free tumor tissues. *Science*, *94*(2436), 239–241. http://doi.org/10.1126/science.94.2436.239

Wiley, P. R., Tosi, P., Evrard, A., Lovegrove, A., Jones, H. D., & Shewry, P. R. (2007). Promoter analysis and immunolocalisation show that puroindoline genes are exclusively expressed in starchy endosperm cells of wheat grain. *Plant Molecular Biology*, *64*(1–2), 125–136. http://doi.org/10.1007/s11103-007-9139-x

Willems, a, & Collins, M. D. (1993). Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology*, *43*(2), 305–313. http://doi.org/10.1099/00207713-43-2-305

Williams, K. P., Sobral, B. W., & Dickerman, A. W. (2007). A Robust Species Tree for the

Alphaproteobacteria □. *JOURNAL OF BACTERIOLOGY*, *189*(13), 4578–4586. http://doi.org/10.1128/JB.00269-07

Wojnarowiez, G., Jacquard, C., Devaux, P., Sangwan, R. S., & Clément, C. (2002). Influence of copper sulfate on anther culture in barley (Hordeum vulgare L.). *Plant Science*, *162*(5), 843–847. http://doi.org/10.1016/S0168-9452(02)00036-5

Wu, B., & Cheng, K. (1982). Cytological and embryological studies on haploid plant production from cultured unpollinated ovaries of Nicotiana tabacum L. *Acta Botanica Sinica*, *24*, 125–129.

Würschum, T., Tucker, M. R., & Maurer, H. P. (2013). Stress treatments influence efficiency of microspore embryogenesis and green plant regeneration in hexaploid triticale (× Triticosecale Wittmack L.). *In Vitro Cellular & Developmental Biology - Plant*, *50*(1), 143–148. http://doi.org/10.1007/s11627-013-9539-3

Würschum, T., Tucker, M. R., Reif, J. C., & Maurer, H. (2012). Improved efficiency of doubled haploid generation in hexaploid triticale by in vitro chromosome doubling. *BMC Plant Biology*, *12*(1), 109. http://doi.org/10.1186/1471-2229-12-109

Xie, J., Gao, M., Cai, Q., & Cheng, X. (1995). Improved isolated microspore culture efficiency in medium with maltose and optimized growth regulator combination in japonica rice (Oryza sativa). *Plant Cell, Tissue and Organ Culture*, (Zhuang 1993), 245–250.

Xie, J. H., Gao, M. W., Liang, Z. Q., Shu, Q. Y., Cheng, X. Y., & Xue, Q. Z. (1997). The effect of cool-pretreatment on the isolated microspore culture and the free amino acid change of anthers in Japonica rice (Oryza sativa L). *Journal of Plant Physiology*, *151*(1), 79–82. http://doi.org/10.1016/S0176-1617(97)80040-5

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T., & Wu, R. (1996). Expression of a Late Embryogenesis Abundant Protein Gene, HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. *Plant Physiology*, *110*(1), 249–257.

Xu, Y., Li, P., Zou, C., Lu, Y., Xie, C., Zhang, X., … Olsen, M. S. (2017). Enhancing genetic gain

in the era of molecular breeding. *Journal of Experimental Botany*, *68*(11), 2641–2666. http://doi.org/10.1093/jxb/erx135

Xu, Z. H., & Sunderland, N. (1982). INOCULATION DENSITY IN THE CULTURE OF BARLEY ANTHERS. *SCIENTIA SINICA SERIES B-CHEMICAL BIOLOGICAL AGRICULTURAL MEDICAL & EARTH SCIENCES*, *25*(9), 961–969.

XU, Z. H., & Sunderland, N. (1982). Inoculation density in the culture of barley anthers. *Science in China Series B-Chemistry, Biological, Agricultural, Medical & Earth Sciences*, *25*(9), 961–969. http://doi.org/10.1360/YB1982-25-9-961

Yan, H., Martin, S. L., Bekele, W. A., Latta, R. G., Diederichsen, A., Peng, Y., & Tinker, N. A. (2016). Genome size variation in the genus Avena. *Genome*, *59(3)*, 209–220. http://doi.org/10.1139/gen-2015-0132

Yan, J. Q., Xue, Q. Z., & Wang, Y. X. (1996). Use of androgenesis in intersubspecific hybrid rice breeding. *Plant Breeding*, *115*(5), 301–304. http://doi.org/10.1111/j.1439-0523.1996.tb00923.x

Yang, Y., Li, R., & Qi, M. (2000). In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *The Plant Journal : For Cell and Molecular Biology*, *22*(6), 543–51.

Yao, J., & Cohen, D. (2000). Multiple gene control of plastome-genome incompatibility and plastid DNA inheritance in interspecific hybrids of Zantedeschia. *Theoretical and Applied Genetics*, 400–406.

Yao, Q. A., Simion, E., William, M., Krochko, J., & Kasha, K. J. (1997). Biolistic transformation of haploid isolated microspores of barley (Hordeum vulgare L.). *Genome*, *40*(4), 570–81.

Young, J. M., Young, J. M., Kuykendall, L. D., Kuykendall, L. D., Mart\'inez-Romero, E., Mart\'inez-Romero, E., … Sawada, H. (2001). A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of Agrobacterium conn 1942 and Allorhizobium undicola de Lajudie et al. 1998 as new

combinations: Rhizobium radiobacter, R. rhizogenes, R. rubi,. *International Journal of Systematic and Evolutionary Microbiology*, *51*(2001), 89–103. http://doi.org/10.1099/00207713-51-1-89

Zadoks, J., Chang, T., & Konzak, C. (1974). A decimal growth code for the growth stages of cereals. *Weed Research*, *14*, 415–421.

Zamani, I., Kovacs, G., Gouli-Vavdinoudi, E., Roupakias, D. G., & Barnabas, B. (2000). Regeneration of fertile doubled haploid plants from colchicine-supplemented media in wheat anther culture. *Plant Breeding*, *119*(6), 461–465. http://doi.org/10.1046/j.1439-0523.2000.00538.x

Zenkteler, M., & Nitzsche, W. (1984). Wide hybridization experiments in cereals. *Theoretical and Applied Genetics*, *68*(4), 311–5. http://doi.org/10.1007/BF00267883

Zhang, C., & Qifeng, C. (1993). Genetic studies of rice (Oryza sativa L.) anther culture response. *Plant Cell, Tissue and Organ Culture*, (Chen 1988), 177–182.

Zhang, S., Cho, M.-J., Koprek, T., Yun, R., Bregitzer, P., & Lemaux, P. G. (1999). Genetic transformation of commercial cultivars of oat (Avena sativa L.) and barley (Hordeum vulgare L.) using in vitro shoot meristematic cultures derived from germinated seedlings. *Plant Cell Reports*, *18*, 959–966. http://doi.org/10.1007/s002990050691

Zhang, S., Zhong, H., & Sticklen, M. B. (1996). Production of multiple shoots from shoot apical meristems of oat (Avena sativa L.). *Journal of Plant Physiology*, *148*(6), 667–671. http://doi.org/10.1016/S0176-1617(96)80365-8

Zhao, Q., Feng, Q., Lu, H., Li, Y., Wang, A., Tian, Q., … Huang, X. (2018). Pan-genome analysis highlights the extent of genomic variation in cultivated and wild rice. *Nature Genetics*, *50*(2), 278–284. http://doi.org/10.1038/s41588-018-0041-z

Zheng, M., Weng, Y., Liu, W., & Konzak, C. F. (2002). The effect of ovary-conditioned medium on microspore embryogenesis in common wheat (Triticum aestivum L.). *Plant Cell Reports*, *20*(9), 802–807. http://doi.org/10.1007/s00299-001-0411-2

Zivy, M., Devaux, P., Blaisonneau, J., Jean, R., & Thiellement, H. (1992). Segregation distortion and linkage studies in microspore-derived double haploid lines of Hordeum vulgare L. *Theoretical and Applied Genetics*, *83–83*(6–7), 919–924. http://doi.org/10.1007/BF00226716