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Comparisons of spring triticale (X *Triticosecale* Wittmack) microspore pretreatments to reduce doubled haploid production time

by

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Abstract

Currently, triticale microspores require a 20 to 28 day humid cold pretreatment to initiate the switch from gametophytic to sporophytic development. The objective of this thesis work was to decrease the time required to pretreat triticale microspores. The study was conducted on three dates and repeated three times within each date. A comparison of nine treatments consisting of three temperatures regimes and three liquid mediums to determine which technique could equal or increase microspore derived embryo (MDE) yields of the cold pretreatment. Donor plants were grown in a growth chamber set at 15/10°C day/night temperatures, 16.5-hour photoperiod and a light intensity of 350–400 μmol m^{-2} s^{-1}. Microspores were placed on solid induction media at 25°C for 21-28 days. Embryos were transferred to a modified MS media, inserted in an environment set at 22°C with an 8-hour photoperiod and light intensity of 120-180 μmol m^{-2} s^{-1}. Genotype dependence was evident. There was a consistent replicable MDE yield for AC Ultima, but other cultivar yields were neither consistent nor replicable. The traditional humid cold pretreatment provided greater MDE yields and green plant regeneration than all other pretreatments tested.

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List of Abbreviations

AC: Anther culture

AE: Androgenic embryo

BAP: benzylamino-purine

CaM: calmodulin

CE: Chromosome elimination

CHB: Induction media for isolated microspores (described by Chu *et al*, 1990)

CHO: Carbohydrate

DH: Doubled haploidy

DNA: Deoxyribonucleic acid

DRO: Double reverse osmosis

FCDC: Field Crop Development Centre

FHG: Inductions media for isolated microspores (described by Kasha *et al*, 2000)

G1: Gap 1

G2: Gap 2

GD: Genotypic difference

HSE: Heat shock element

HSF: Heat shock transcription factor

Hsp: Heat shock protein

IAA: Indoleacetic acid

IM: Isolated microspore

IMC: Isolated microspore culture

M: Mitotic

MC17: Induction media for isolated microspores

MCPA: 4-chloro-o-tolyloxyacetic acids

MDE: microspore-derived embryo

MS: Regeneration media (described by Kasha *et al*, 2000)

NAA: Napthaleneacetic acid

PAA: Phenylacetic acid

PEG: Polyethylene glycol

QTL: Quantitative trait loci

RNA: Ribonucleic acid

S: synthesis

SSD: Single seed descent

Tween 80: polyoxyethylenesorbitan monooleate

ZGS: Zadoks growth stage

2,4-D: 2,4-dichlorophenoxyacetic acid

2 4 5 T: 2,4,5-trichlorophenoyacetic acid

Chapter 1
Literature Review

1.0 Introduction

While studying *in vitro* pollen development in angel's trumpet (*Datura innoxia* L.), Guha and Maheshwari (1964,1966) discovered that some microspores switched to microspore-derived embryos (MDE). Such early discoveries inspired scientists around the world to develop and improve methods of doubled haploid plant production (Hu, 1997).

Double haploidy (DH) provided a method for plant breeding programs to reach homozygosity in a single generation, rather than the 6-8 generations with traditional breeding. This may increase selection efficiency, and lead to the rapid development of homozygous plants for gene mapping, isolation and transformation (Hu, 1997). The success of both *in vivo* and *in vitro* DH techniques depends on the crop species. *In vivo* techniques require the stimulation of incomplete fertilization within a cereal spike, while *in vitro* techniques require the stimulation of plant cells or organs like microspores, anthers and ovules (Hu, 1997). *In vitro* techniques such as isolated microspore culture (IMC) are potentially very efficient because there are many more microspores than ovaries per spike (Kasha *et al*, 1995). The following four conditions are required for IMC: (1) selecting microspores at the correct stage of development, including the cellular phase, development and order necessary for the switch from gametophytic to sporophytic development (Hu, 1997; Immonen and Robinson, 1999); (2) using pretreatments and biochemical changes that occur when microspores switch to gametic development. (Sunderland and Wicks, 1969; Sunderland and Wicks, 1971; Sunderland *et al*, 1974); (3) the health and vigor of the donor plant (Luckett and Darvey, 1992; Kasha *et al*, 1995), and; (4) using appropriate media to produce

maximum MDE and green plants (Hu, 1997; Finnie *et al*, 1989; Luckett and Darvey, 1992; Kasha *et al*, 1995; Kasha *et al*, 2000).

Microspore-derived plantlets can have various ploidy levels. Determining ploidy before maturity may be important. Not all haploids will spontaneously double, for example a study of wheat doubled haploids conducted by Hu and Kasha (1997b) found 82.7 % haploid plants spontaneously doubled and 78.8 % were completely fertile. Aneuploids are common within DH triticale populations (Ryöppy, 1997; Hu and Kasha, 1997b). Aneuploids can have chromosomes, or parts of chromosomes, added or deleted from the genome, resulting in an uneven number of chromosomes (Fehr, 1993). Such plants are often used for genetic studies but are rarely used in breeding programs. To ensure all plants are doubled, methods to determine ploidy levels have been studied. Methods to determine ploidy level include visual inspection of the inflorescence, chromosome counts of root tips or callus cells (Schaffer *et al*, 1979; Mix *et al*, 1978), chloroplast counts of stomatal guard cells (Lucas *et al*, 1991; Hanning, 1995), length of stomatal guard cells (Borrino and Powell, 1987) and flow cytometry (Hu and Kasha, 1997b). Chemical doubling agents such as colchicine can double haploids.

Early selection methods have the potential of eliminating plants in early stages of development, thus using less space and research funds. Early selection methods encompassed selection for specific traits such as plant height (Bozorgipour and Snape, 1992), erucic acid content (Albrecht *et al*, 1995), disease tolerance (Bansal *et al*, 1998) and chemical tolerance (Swanson *et al*, 1988). As more genes are mapped, the necessary tools for marker-assisted selection may become available (Pellegrineschi, *et al*, 2001; Hu, 1997).

1.1 Advantages and limitations of doubled haploidy in plant breeding programs

There are three self-pollinated crop-breeding methods: pure line selection, mass selection, and hybridization which may include methods such as pedigree, bulk, single seed descent (SSD) and double haploid (DH) production (Fehr, 1993). Traditional plant breeding methods like pedigree, bulk or SSD generally take five or six generations to produce a plant that is 94 to 97% percent homozygous at all loci (Snape, 1989; Hu, 1997; Kasha *et al*, 1990). With all breeding techniques, plant selection or genetic testing begins after the plant is almost or completely homozygous, therefore homozygosity in one generation would serve to shorten the plant breeding cycle (Hu, 1997). This is one of the most important direct applications of doubled haploidy to plant breeding programs (Kasha *et al*, 1995). Winter cereals generally require one year to complete a generation due to vernalization requirements. The time savings for generation advance by double haploidy in winter cereals are therefore much greater than in spring cereals (Hu, 1997).

Selection efficiency is greater with DH breeding. A homozygous plant at all loci eliminates the phenomenon of dominant alleles masking recessive alleles in early generations (Hu, 1997). When plants are homozygous in one generation, selections can be made from the entire population, thereby eliminating head or row selection of heterozygous plants. In an early study comparing offspring of androgenetically derived DH and pedigree populations, the DH population appeared to have more disease resistance than the same line produced using the pedigree method (Winzeler *et al*, 1987). It was hypothesized that this was because all the disease resistant genotypes were seen in one generation (Winzeler *et al*, 1987).

Developing homozygous plants and/or populations in one generation using doubled haploidy allows for genetic studies, including QTL mapping, gene isolation and transformation (Hu, 1997, Thompson *et al*, 1991).

Although DH breeding has timesaving benefits compared to pedigree conventional plant breeding, there are two disadvantages. When heterozygous offspring are doubled or fixed in one generation there will be a high rejection frequency (Hu, 1997). Unlike conventional breeding techniques, DH breeding does not have six or seven years of selection pressure before becoming close to homozygosity (Hu, 1997). An additional limitation is the reported cases of some DH recombinants not following Mendel's first law (Hu, 1997; Snape, 1989). Some literature suggests that lines doubled in early generations such as the F_2 might create linkage disequilibrium; genes that are tightly linked require more than one generation of recombination to produce all possible recombinants (Hu, 1997; Snape, 1989). Others disagree with the linkage disequilibrium theory, suggesting the same recombinants appear, but frequencies differ between the conventional and DH breeding methods (Winzeler *et al*, 1987; Friedt and Foroughi-Wehr, 1983; Charmet and Branlard, 1985).

1.2 Doubled haploid techniques

Haploids in nature are a result of abnormal fertilization. A study of jimsonweed (*Datura stramonium* L.) in 1922 is the first reported occurrence of haploids (Hu, 1997). The discovery of jimsonweed haploids was an inspiration for researchers to study the phenomena, and develop techniques to artificially produce haploid plants in many crop species. *In vivo* techniques require the stimulation of the whole plant, while *in vitro* techniques refer to modifying culture methods to stimulate explants like ovules, anther or microspores (Hu, 1997; Kasha *et al*, 1995).

Parthenocarpy, apogamy and semigamy produce haploid embryoids without fertilization from a male gamete, also called *in vivo* techniques (Khush and Virmani, 1996). DH embryos, which develop without fertilization, are often referred to as embryoids (Hu, 1997; Davies *et al*, 1998). Parthenocarpy refers to the development of an embryoid from the ovum through artificial

stimulation by foreign pollen, and/or chemical compounds like auxin analogues (Andersen *et al* 1997; Hu, 1997; Wędzony *et al*, 1998). Apogamy is similar to parthenocarpy except the embryo is formed from a gametic cell other than an ovum (Hu, 1997). Semigamy is also similar to parthenocarpy, but the sperm and egg nuclei do not unite and divide. Rather, they divide independently to form haploid embryoids (Khush and Virmani 1996; Kasha *et al*, 1995). Semigamy usually results in chimeral plants of both parental genotypes, but occasionally produces plants of one genotype (Khush and Virmani 1996). Parthenocarpy, apogamy and semigamy are techniques that produce low frequencies of DH plants and are only used when other more efficient techniques fail (Kasha *et al*, 1995; Khush and Virmani, 1996).

Chromosome elimination (CE) is an *in vivo* technique that involves pollination with a foreign pollen parent. This form of parthenogenesis requires the stimulation of the whole plant, creating haploid embryoids after the elimination of the foreign pollen chromosomes. Chromosome elimination is a very successful breeding tool and often distinguished from parthenogenesis as a separate category (Kasha, 1974; O'Donoughue and Bennett, 1994). Two commonly used CE techniques in cereals are the bulbosum and maize methods. The bulbosum method involves crossing barley (*Hordeum vulgare* L.) to its ancestral relative *Hordeum bulbosum* L. Cytological observations reveal the elimination of *Hordeum bulbosum* chromosomes immediately after hybridization (Kasha, 1974; O'Donoughue and Bennett, 1994). Since the bulbosum method was successful in barley, it was later applied to wheat (*Triticum aestivum* L.) (Hu, 1997). The bulbosum method was not as successful in wheat, because the *kr1* and *kr2* loci of many wheat genotypes prevent *Hordeum bulbosum* L. pollen tube growth (O'Donoughue and Bennett, 1994; Hu, 1997). Attempting to increase the amount of receptive genotypes by hybridizing dominant wheat cultivars with recessive *kr1* and *kr2*, like Chinese Spring, is time consuming and often results in the incorporation of undesirable genes (Picard *et al*, 1985; Sitch

and Snape, 1986). Crossing wheat (*Triticum aestivum* L.) to *Hordeum bulbosum* was discontinued after the discovery that crossing wheat to maize (*Zea mays* L.) induced haploid embryoids in many genotypes (Zenkteler and Nitzche, 1984). Crossing wheat with corn creates a karotypically unstable hybrid that eliminates the maize chromosomes, which are not visible after the first three divisions (Laurie and Bennett, 1989; Laurie and Bennett, 1986). Chromosome elimination methods are now proven, work in many laboratories and produce several haploids per plant (Zenkteler and Nitzche, 1984; Hu, 1997). Such methods are not, however, a method of choice for barley because *in vitro* methods are more efficient (Kasha *et al*, 1995).

In vitro techniques encompass ovule, anther and IM culture. Such techniques (anther or microspore culture) involve thousands of microspores per spike, and potentially yield thousands to hundreds of thousands of plants (Kasha *et al*, 1995). In contrast, *in vivo* techniques have a maximum yield of one haploid plant per ovary (Baenziger, 1996). Thus, all things being equal, researchers will choose *in vitro* rather than *in vivo* techniques, providing the crop species responds favorably to *in vitro* techniques. (Laurie, 1998; Laurie and Bennett, 1989; O'Donoughue and Bennett, 1994; Kasha *et al*, 1995; Hu, 1997).

Culturing the ovule before or immediately after fertilization generates callus tissue and subsequently plants (Bohanec, 1997). Ovule culture produces DH plants for many crops like barley, wheat, corn, rice, tobacco, lily, sunflower, buckwheat, but is only used when IMC is not efficient for the crop species (Kasha *et al*, 1995). Crop species that respond to anther or IMC are barley (Kasha *et al*, 2000), wheat (Hu, 1997), triticale (Ryöppy, 1997), rice (Gosal *et al*, 1997), sorghum (Liang *et al*, 1997), and rye (Deimling and Flehinghaus-Roux, 1997). Anther culture is as the name suggests: anthers cultured within induction media switch from gametophytic to the sporophytic development, producing embryoids and/or callus tissue, resulting in green doubled haploid plants (Hu, 1997). Isolated microspore culture requires the removal of mid-late

uninucleate microspores from the anther. Microspores are cultured within an induction media, and switch to sporophytic development, then embryoids and/or callus tissue develop doubled haploid plants (Hu, 1997).

1.3 Requirements of the microspore to switch to sporophytic cell development

The following section focuses on understanding initial cytological events, enhancing microspore-derived embryoids (MDE) or callus development. Finding the correct stage of microspores and knowing the developmental pathway, like the A, B, C or callus, that assist the switch from gametic to sporophytic development will thereafter be discussed. Also discussed is the MDE development from sporophytic to organelle development. Finally, this section will review the importance of pretreatments for MDE development.

1.3.1 Microspore development and staging

Plant cells are totipotent, but to maximize production of MDE, microspores must be selected at the proper stage of development (Hu, 1997). Staging is crucial for maximizing embryoid and green plant regeneration frequencies (Kasha *et al*, 1995). A plant life cycle is composed of two distinct phases, the gametophytic generation and the sporophytic generation (Hu, 1997). The gametophytic is a haploid generation that includes gametes (Hu, 1997). The gametes fuse to form a zygote resulting in the diploid sporophytic generation (Hu, 1997). The zygote develops into a plant during its reproductive phase, which produces gametes, and thus the cycle returns to the gametophytic generation (Hu, 1997). Microspore-derived embryos rely on the switch from the gametic pathway to the sporophytic pathway (Hu, 1997). To be able to select specific stages believed to be at switch points, it is necessary to understand normal gametophytic development

(Shultz-Schaeffer, 1980), and switch points in the eukaryotic cell cycle (Bouharmont, 1977; Sunderland and Wicks, 1971).

Pollen development starts with the production of microsporocytes, which undergo two meiotic divisions to produce a radial quartet cell (Shultz-Schaeffer, 1980). The radial quartet cell separates and releases four individual microspores (Shultz-Schaeffer, 1980). Microspore development includes the uninucleate, mid-late uninucleate, binucleate and finally, pollen at the tri-nucleate stage (Hu, 1997; Shultz-Schaeffer, 1980). The nucleus is in or near the centre of the microspore at the early uninucleate stage, migrates toward the wall opposite the pore at the mid-late uninucleate, and thereafter increases in size and enters prophase at the late-uninucleate stage (Hu, 1997; Schultz-Schaeffer, 1980). The binucleate stage is a result of an asymmetrical division, producing a large vegetative and a small condensed generative nucleus (Schultz-Schaeffer, 1980). In the final stage, the generative nucleus doubles, producing the third nuclei of the pollen grain (Schultz-Schaeffer, 1980).

Initial observations of microspore staging began with tobacco (*Nicotiana tabacum* L.) and angel's trumpet. Nitsch and Nitsch (1969) reported that microspores in the late uninucleate to bi-nucleate stage yielded more tobacco plantlets, and concluded that the first pollen mitosis was the critical switch point. Sunderland and Wicks (1969) reported that the uninucleate stage, after meiosis but prior to the bi-nucleate, produced a greater number of tobacco plantlets. More specific stages of microspores considered in later research by Sunderland and Wicks (1971), confirmed the late uninucleate was more effective than early uninucleate. Studies on angel's trumpet reveal an increase of embryoids when anthers were selected at the binucleate rather than meiotic microsporocyte or late binucleate microspores (Sunderland *et al*, 1974). Selecting microspores at later stages for monocotyledons, like wheat, increases albino regeneration (Logue, 1996). Plastid metamorphosis is later in dicotyledons, which rarely regenerate albino plants, thus

the theory that early plastid metamorphosis of monocots is associated with higher albino regeneration (Logue, 1996). The ideal stage or switching point for crop species like barley, wheat, triticale and rye is mid-to-late uninucleate (Wheatley *et al*, 1986; Hu and Kasha, 1997a; Sayed-Tabatabaei *et al*, 1998; Immonen, 1999).

Sunderland and Wicks (1971) continued their research to determine at which phase microspores switched from gametophytic to sporophytic, from the four possible phases of a eukaryotic cell. The eukaryotic cell cycle has four phases: synthesis (S), gap 1 (G1), mitotic (M) and gap 2 (G2) (Mathews and van Holde, 1990). The cell cycle has checkpoints, allowing the cell to rest in one phase or continue on its developmental pathway. There is one checkpoint in G1 prior to the S phase, a second in G2 prior to the M phase and the third at the end of the mitotic phase prior to G1. Depending on the crop species there is a switch in the cell cycle that corresponds to a certain stage of the microspore, allowing for embryoid development. Bouharmont (1977) found that barley anthers selected at G1 or S phase generally had a symmetrical first mitosis. Sunderland and Wicks (1971) concluded that tobacco differentiated a bicellular grain, which did not allow it to mature to a trinucleate pollen grain. Much of the accumulated gametic cytoplasm has to degrade before tobacco embryogenesis, which does not happen to angel's trumpet (Sunderland and Wicks, 1971). Sangwan-Norreel (1978) found that after the switch to embryogenesis the number of mitochondria cristae and golgi body ribosomes increased and dictyosomes produced more vesicles in the vegetative than in the generative cell of angel's trumpet. After embryogenesis, RNA rich bodies increase and these are thought to enhance the first stages of the switch (Sangwan-Norreel, 1978). Sunderland *et al* (1979) examined barley microspores at stage 2, around mid-uninucleate, and found DNA replication had not begun, leading to the conclusion that the spores were in the G1 phase when they switched to

the sporophytic generation. DNA replication was completed at the G2 inter phase in stage 3, late uninucleate, just prior to the first pollen mitosis (Sunderland *et al*, 1979).

After determining the ideal stage of microspores and the cellular switching points, researchers sought to determine the initial stages of sporophytic development and its chromosomal compliment (Sunderland and Wicks, 1969; Sunderland and Wicks, 1971). Anthers or microspores cultured *in vitro* at a specific stage, and in some cases with the correct pretreatment, can produce plantlets via embryoids or callus. After the initial discovery of microspore-derived embryoids by Guha and Maheshwari (1964, 1966), other research focused on cytological events that occur at the switch from the gametic to the sporophytic generation. Initial studies on tobacco revealed a normal asymmetrical first pollen mitosis, but the second pollen mitosis of cultured microspores was of the vegetative nucleus, unlike gametic pollen grains (Sunderland and Wicks, 1969). Sunderland and Wicks (1969) described spherical bodies or embryoids forming after the division of the vegetative nucleus and the eventual disappearance of the generative nucleus. Further research on tobacco by Sunderland and Wicks (1971) revealed a symmetrical division at first pollen mitosis, when the nucleus divided into two equal sized daughter vegetative nuclei, with no generative nuclei. Sunderland and Wicks (1971) found symmetrical division was less dominant in tobacco and occurred when the vacuole did not develop, or the orientation of the spindle axis was lateral instead of terminal. Sunderland *et al* (1974) found similar embryoid development in angel's trumpet. They labeled the three pathways A, B and C. The asymmetrical division of the vegetative cell and disappearance of the generative nucleus is called the A pathway. The symmetrical division of the two equal vegetative cells is called the B pathway. The C pathway was found to be a version of the A, with the exception of a persistent nuclear fused generative cell (Sunderland *et al*, 1974).

After the first mitotic division of the A pathway, progression is rapid with synchronous divisions at first, then divisions occur independently as the proembryoid cell numbers increase (Sunderland *et al*, 1974). The generative nucleus disappears rapidly after the first few divisions of the vegetative cell. Between day 4 and 6 embryoids have 50 to 60 haploid cells. Sunderland *et al* (1974) found the development of the B pathway virtually indistinguishable from the A, due to the speed of the degeneration of the generative cell. Dunwell and Sunderland (1976b) suggested that in the B pathway after the division of the two equal nuclei, there is a lack of a complete wall or barrier. This allows both sets of chromosomes to associate, and division may occur on the same spindle axis, producing a diploid nucleus.

The most visible difference between the A and C pathway is the persistent generative cell of the C pathway, which divides and fuses into a polyploid nucleus (Sunderland *et al*, 1974). Sunderland *et al* (1974) found the vegetative and endoreduplicated generative nucleus can divide on a common spindle, yielding a 3n proembryoid, or the endoreduplicated generative nucleus fuses to two vegetative nuclei, producing a 4n proembryoid. Most plantlets are haploid or diploid, and therefore most embryoids with greater ploidy levels do not develop properly (Sunderland *et al*, 1974). The characteristic events prior to microspores developing by the C pathway are the thinning of the generative cell wall prior to detachment from the intine, while the vegetative nucleus is still in interphase with very little cytoplasmic synthesis (Dunwell and Sunderland, 1976a). Further research suggested that wheat MDE are developed by the A pathway (Reynolds, 1993), but triticale MDE develops by the B pathway (Hassawi *et al*, 1990). Canola (*Brassica napus* L.) produces MDE via the A pathway with a heat shock pretreatment (Ilić-Grubor *et al*, 1998) and soybeans (*Glycine max* L.) by the B pathway with a cold pretreatment (Kaltchuk-Santos *et al*, 1997).

Even though later research by Sunderland *et al* (1979) revealed that barley microspores had the capability to develop into embryoids by the A, B and C pathways, early research suggested some barley microspore-derived plantlets (MDP) were produced by callus (Wilson *et al*, 1978; Sunderland and Wicks, 1971; Sunderland and Evans, 1980). Callus has three developmental stages: induction, division, and differentiation (Yeoman and Aitchison, 1973). The induction stage involves preparation for the division of undifferentiated tissue. Induction involves tissue with meristematic capabilities that can be induced into a synchronous division by the addition of the plant growth regulator auxin (Yeoman and Aitchison, 1973). The division phase increases the number of cells, which eventually enter the differentiation phase to produce plantlets. Anther and microspore culture media contain plant growth regulators such as auxin; hence callus pieces are transferred to a low auxin media for the differentiation phase (Sunderland and Wicks, 1971). It is important to transfer wound-free calli of equal weight, in sterile conditions, to promote equal growth potential (Yeoman and Aitchison, 1973). Wilson *et al* (1978) studied the effects of microspore culture on barley (cv. Dissa) and found microspores produced embryoids similar to the A and B pathways, with unusual callus development that was neither the A, B or C pathway as described by Sunderland *et al* (1974). Sunderland and Evans (1980) also noticed unusual barley calli development from the cultivar 'Sabarlis'. It had growing sectors and not just a mass of unorganized cells. The growing sectors differed in ploidy levels and may have developed from a generative cell, vegetative cells or by the uninucleate spore. Major problems with calli-derived plantlets are the variations in chromosome number and high frequencies of albino plantlets (Kasha and Reinbergs, 1981), which prompted scientists to find alternate methods of producing plantlets. Albinism is the most obvious example of gametoclonal variation (Baenziger, 1996), and is linked to length of culture time (Harwood *et al*, 1996). The

decrease of gametoclonal variation and genetic instability of the microspore technique might be the result of shorter culture time between isolation and plant regeneration (Harwood *et al*, 1996).

Sunderland's (1973) ultra structure examination of tobacco microspores suggested the steps necessary to ensure embryoid development. Features of embryogenic microspores include atypical plastids, vacuole development and lipid biosynthesis in later stages of development. Atypical plastids are electron dense, lack starch and are distributed equally throughout the cytoplasm (Sunderland, 1973). After seven days of culture a dense chromatic structure develops in the vegetative nucleus and the plastids localize around the nucleus (Sunderland, 1973). The plastids do not contain clearly defined ribosomes, but do have electron dense structures. After 14 days the vegetative nucleus divides, and double membrane vesicles appear and cluster around the daughter nuclei. Starch deposition then begins in the vesicles and plastids. Sunderland (1973) concluded that after the first division, development of new cellular components leads to two meristematic-like cells. Lipid biosynthesis is virtually nonexistent in early embryoid development, but increases in later stages. Sunderland (1973) hypothesized that the increase of RNA in the cytoplasm might affect the plastids at the time of induction. Incompletion of any of the previously mentioned steps results in non-embryoid structures, aborted embryoids or abnormal plants (Sunderland, 1973). After embryogenesis RNA rich bodies increase, and suggested to promote the first stages of sporophytic development (Sangwari-Norell, 1978). An examination of barley microspores at stage 2, around mid-uninucleate, revealed DNA replications do not occur, leading to the conclusion that the spores are in the G1 phase at the switch (Sunderland *et al*, 1979). DNA replication is complete at G2 inter-phase in stage 3, late uninucleate, just prior to first pollen mitosis. Sunderland *et al* (1979) concluded that stage 2 microspores have the possibility of contributions from the generative cell to callus formation, or

organized development, but stage 3 had the opportunity for direct development of A, B, or C pathways (Sunderland *et al*, 1979).

1.3.2 Pretreatments of anthers or microspores

The correct stage of microspore and pretreatments are required for the switch of cereal microspores from gametophytic to the sporophytic generation, and to maximize embryo and fertile green plants regeneration (Hu and Kasha, 1999; Luckett and Darvey, 1992). Chemical compounds, temperature or combinations of both induce this switch in developmental pathways.

There is limited research on biochemical regulation of the microspores during pretreatments. There has been much research on pretreatments in cereals, focusing on the pathway of development induced by a particular pretreatment. Sunderland *et al* (1979) found a cold pretreatment increased the number of MDE and calli, but stress did not favor one pathway over the other. Conversely, Hu and Kasha (1999) reported that the type of wheat microspore pretreatment favors one pathway of development. They chose tillers with microspores at the mid-to-late uninucleate stage and pre-treated by placing wheat spikes for 7 days in 0.4 M mannitol at 28°C or 28 days at 4°C (Hu and Kasha, 1999). The first pretreatment yielded symmetrical divisions (B-pathway), while the second pretreatment resulted in asymmetrical divisions (A-pathway). Since the B pathway is associated with spontaneously doubled haploids, the first pretreatment has the advantage of avoiding toxic chromosome doubling chemicals like colchicine (Hu and Kasha, 1999). A heat shock pretreatment of 32.5°C for 24 hours applied to canola (*Brassica napus* cv. Topas) microspores resulted in an asymmetrical division (Telmer *et al*, 1995). Sun *et al* (2000) studied the regulation of heat shock proteins in maize and presented the following two possible hypotheses: 1) Heat shock increases calcium ions that promote or enhance a reaction between calmodulin (CaM) and cytosolic heat shock protein 70 (Hsp 70), releasing the heat shock transcription factor (HSF) from the HSF-Hsp70 complex. The HSF then

binds to the heat shock element (HSE), which activates transcription of Hsp genes, (2) the binding of CaM and cystosolic Hsp 70 creates a conformational change of Hsp 70 by autophosphorylation that might release the HSF, resulting in an activation of Hsp genes. Either hypothesis suggests that calcium is an important factor for the transcription of Hsp genes. Hanning (1995) stated that antimicrotubule herbicides increase calcium ions in the cell. Antimicrotubule herbicides cause disruptions of microtubule development, and consequently the spindle apparatus, that subsequently prevents cytokinesis (Hanning, 1995). Colchicine is also an antimicrotubule agent, and when applied as a pretreatment to canola at 25°C, induces the B pathway of development (Twell *et al*, 1998). The common explanation for the effect of a colchicine pretreatment is the prevention of cytokinesis. Twell *et al* (1998) found that after a colchicine pretreatment, the *lat 52-gus* gene was activated in the two equal daughter vegetative nuclei of arabidopsis (*Arabidopsis thaliana* L.) microspores.

Plant growth regulators are often associated with triggering biochemical processes. Ziauddin *et al* (1990) reported that 80% of barley microspores spontaneously double when pretreated with 0.3M mannitol at 25 to 28°C in an ovary conditioned FHG media (Kasha *et al*, 2000) for 3-5 days in the dark. They concluded that such spontaneous doubling was probably the result of the mannitol, and unidentified elements of the ovary-conditioned media. Ziauddin *et al* (1992) performed a similar experiment, but replaced the ovary-conditioned media with phenylacetic acid (PAA) that promoted 70-80% spontaneously doubled microspore-derived embryoids (often associated with the B pathway). They suggested the plant growth-regulating hormone is endogenous to barley ovaries, which influences the signal resulting in the development of spontaneously doubled microspores. More research is required to fully understand the biochemical regulation of microspore development, and to help develop ideal conditions facilitating initial *in vitro* development of microspores.

Variations in pretreatments are necessary for different crops. For example, cold shocks applied to rice panicles (Raina, 1997), barley spikes (Kasha *et al*, 2000), wheat spikes (Hu and Kasha, 1999), corn tassels (Büter, 1997) or heat shocks to wheat spikes (Liu *et al*, 2002a), barley anthers (Kasha *et al*, 2000) and canola (Telmer *et al*, 1995) have been reported to induce embryoid development and green plant regeneration. A traditional pretreatment for wheat and barley is 4°C for 28 days, with a few milliliters water in a small petri plate, within a larger petri plate containing the spikes (Hu and Kasha, 1999; Kasha *et al*, 2000). Others studies indicate that spring triticale anthers or spikes placed in 3°C for 21 days, and winter triticale at 4°C for 14-21 days, regenerated fertile green plants (Bernard and Charmet, 1985; Immonen and Robinson, 2000). Decreasing the pretreatment time can decrease the time required to produce a DH plant. Shorter pretreatment protocols such as 4°C 0.3 M mannitol for 3 to 5 days are replacing traditional 4°C water 28-day pretreatments in barley IM culture (Kasha *et al*, 2000). It is not certain if such mannitol pretreatments provide nutritional requirements, stress induction or osmotic pressure (Luckett and Darvey, 1992), but many researchers refer to it as a 'starvation pretreatment'. Recent research suggests that adding nutrient solutions during the pretreatment does not affect the frequency of embryo or spontaneously doubled green plants. The microspores are therefore not starved, but must receive nutrients from other tissues like anther walls (Liu *et al*, 2002a). Isolated microspore culture (IMC) is genotype dependent, and there has been recent research on decreasing genotypic effects at pretreatment by adding inducing chemicals like 2-hydroxynicotinic acid (HNA) (Zheng *et al*, 2001). Incubating spikes in liquid media for 38-52 hours, and thereafter adding HNA, increased the frequency of embryos and green plants for the recalcitrant triticale cultivars Waldron and WPB926 (Zheng *et al*, 2001).

1.3.3 Media components and culture conditions

After pretreatment IMC continues with microspore isolation, by blending the entire spike, macerating anthers with a stir rod, or vortexing. The blending method is more consistent, and in some cases increases green plant regeneration frequencies (Ritala *et al*, 2001). After isolation, microspores are placed on induction media to facilitate the switch from the gametophytic to the sporophytic generation of the plant life cycle. There are several induction media to choose from, but FHG media modified with PAA works well for barley and wheat microspores (Kasha *et al*, 2000). FHG contains KNO_3, NH_4NO_3, KH_2PO_4 $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$, $FeNa_2$·EDTA, $MnSO_4 \cdot 5H_2O$, H_3BO_3, $ZnSO_4 \cdot 7\ H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $NaMoO_4 \cdot 2H_2O$, glutamine, myo-inositol, thiamine, maltose, PAA, benxylamino-purine (BAP) (Kasha *et al*, 2000) . Research suggests a CHB induction media (Chu *et al*, 1990) supports wheat and triticale embryo development (Sayed-Tabatabaei *et al*, 1998). The ingredients for the CHB triticale induction media are; KNO_3, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_20$, $CaCl_2 \cdot 2H_20$, , Fe-Na_2EDTA, $MnSO_4 \cdot 4H_20$, H_3BO_3, $ZnSO_4 \cdot 7H_20$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot H_2O$, $Na_2MoO_4 \cdot 2H_2O$, KI, glutamine, myo-inositol, thiamine-HCl, glucose or other tested sugars, 2,4-D, glycine, pyridoxine-HCl, nicotinic acid, biotin, calcium pantothenate, ascorbic acid, kinetin (Chu *et al*, 1990). Media components such as carbohydrates (CHO), plant growth-regulating hormones, nitrogen and gelling agents can affect the results of *in vitro* techniques. Other factors affecting *in vitro* techniques are culture conditions, such as temperature, light, and microspore density.

Carbohydrate (CHO) is one of many induction media components that may require modification depending on the crop or genotype. Initially, induction media contained sucrose, possibly because sucrose is an abundant CHO source in plants, and has been used successfully for *in vivo* techniques like the maize method. Increasing the sucrose levels in media results in an increase in callus or embryo development, but generates high frequencies of albino plants (Ono

and Larter, 1976; Ball *et al*, 1992; Hu, 1997). Initial research reported albinism to be genotype dependent, but further study found that changing the CHO source to maltose could decrease albinism (Finnie *et al*, 1989). Finnie *et al*, (1989) suggested maltose and malt extract increased green plant regeneration. Other studies suggested variations in maltose concentrations for various crop species; such as 135 g/L for wheat (Ziauddin *et al*, 1992), 120 g/L for rye (*Secale cereale* L.) anther culture and 60 g/L for rye microspore culture (Deimling, 1997), 20 mM for barley microspores (Scott and Lyne, 1994) or 175 mM for barley anthers (Finnie *et al*, 1991). There are a number of explanations in the literature regarding the beneficial effect of maltose: 1) The effect of maltose may be related to stabilizing osmolarity, or the slow metabolization to its glucose components, compared to sucrose (Cai *et al*, 1992); 2) Maltose could be an osmoticum promoting absorption of salts, amino acids or organic acids better than glucose (Kasha *et al*, 1990); 3) Maltose provides more than an osmotic effect, because green plant regeneration increases with the combination of maltose and an osmoticum (Zhi-Hong, 1990); 4) Maltose metabolizes slowly, creating a starvation effect rather than osmotic stress, because several other CHO sources do not increase green plant frequencies like maltose (Indrianto *et al*, 1999).

Plant growth regulators can also improve embryo and green plant frequencies. Different auxins and auxin-promoting compounds like napthaleneacetic acid (NAA), phenylacetic acid (PAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and dicamba can be used in induction media. Every crop species and plant organ does not respond to the same form of auxin or auxin-promoting compound. Phenylacetic acid increases green plant frequencies and spontaneously doubled haploids from wheat anthers and barley, but the effects are inhibitory when the concentration is higher than 100 mg/L (Ziauddin *et al*, 1992). The result of PAA is not consistent for all crops, for example it inhibits MDE and green plant frequencies of rice (*Oryza sativa* L.) (Lentini *et al*, 1995) and barley anthers (Ziauddin *et al*, 1992). There are reports of

NAA decreasing culture time by three weeks using a direct generation method, which eliminates the requirement for regeneration media (Liang *et al*, 1987).

Cytokinins are added to induction media to stimulate cell division and meristematic cell development. Cytokinin research has not received the attention auxins have, but benxylamino-purine (BAP) appears to increase green plant frequencies in barley anther or microspore cultures (Cai *et al*, 1992; Zhi-Hong, 1990; Dahleen and Bregitzer, 2002). Ethylene can promote or inhibit embryogenesis, depending on the pollen developmental stage and amount of pollen (Tiainen, 1996). Increased senescence or aging anthers increase the amount of ethylene in culture, which is inhibitory to embryogenesis. Removals of aging anthers, the addition of ethylene antagonists, or inhibitors like silver nitrate are suggested (Tiainen, 1996). Some research cautions that ethylene inhibitors do not always decrease ethylene concentrations, and the concentration of the ethylene inhibitors can inhibit callus development (Tiainen, 1996; Lentini *et al*, 1995). For example, 10 mg/L of silver nitrate promoted callus development, but 15 mg/L was inhibitive of rice callus development (Lentini *et al*, 1995). Ethylene inhibitors and antagonists do not decrease the volume of ethylene produced by a wounded plant tissue, therefore the only options are not wounding plant tissue, or removing damaged plant tissue (Tiainen, 1996; Kasha *et al*, 2000).

There are several nitrogen containing compounds such as potassium nitrate, ammonium nitrate, glutamine and inositol incorporated into differing induction and regeneration media. Glutamine is a universal additive to most media as a replacement for organic nitrogen (Luckett and Darvey, 1992). Studies indicate that the largest frequency of green wheat plant regeneration depends on proper quantities of glutamine, such as 20 mM in induction and 35 mM in regeneration media (Kasha *et al*, 1990). Other studies indicate that optimum ratios are important, for example the maximum embryo and green plant frequencies of barley isolate microspore (IM) cultures requires an ammonium to nitrate ratio of 90:10 (Mordhurst and Lorz, 1993).

Combinations of nitrogen compounds, like 2 mM ammonium nitrate with 5.1 mM glutamine, optimize embryogenic development of barley anthers (Olsen, 1987). Inositol promotes optimal embryogenesis for barley anthers at 1000 mg/L of media (Xu and Sunderland, 1981).

Zhou and Konzak (1989) and Luckett and Darvey (1992) reported that liquid media yields more MDE, because of the increased contact between microspores and media components. Liquid media also promotes embryo development by dispersing toxic substances, possibly from living or dying anthers, equally throughout the media. Some gelling agents like agar contain toxic substances inhibitory to MDE development, while other gelling agents like agarose, gellrite and phytagel do not inhibit embryo frequencies (Zhou and Konzak, 1989; Olsen, 1987).

From the literature, it is evident that ideal culture temperatures differ between crop species. For example, induction temperatures of 25°C for barley microspores (Kasha *et al*, 1995), 28-32°C for wheat microspores (Kasha *et al*, 1995) are well established, while rye anthers do not appear to respond differently to temperature changes (Flehinghaus *et al*, 1991). There are indications that different light regimes are required at different stages of DH development in culture. For example, continuous light at induction in IM or anthers reduced or inhibited barley, wheat and triticale embryogenesis (Zhi-Hong, 1990; Reynolds and Kitto, 1992; Bernard, 1980). A dark treatment during the same period increased embryo frequencies (Kasha *et al*, 1995; Liang *et al*, 1987, Bernard, 1980). Accepted practices for plant regeneration include a low light intensity environment at the beginning, followed by transfer of vigorous seedlings to the environmental conditions of the donor plants (Kasha *et al*, 1995; Liang *et al*, 1987).

1.3.4 Microspore donor plant

In vitro techniques such as microspore culture are genotype-dependent, where embryo and subsequent green plant production depends on the genotype of the microspore donor plant (Zhou *et al*, 1991; Zhou, 1996; Kasha *et al*, 1990). Although genotype appears to control embryo and

green plant regeneration, modifications to pretreatments and media components can increase regeneration (Kuhlmann and Foroughi-Wehr, 1989; Immonen, 1999; D'yachuk *et al*, 1986; Andersen *et al*, 1988; Kasha *et al*, 1995; Zhou *et al*, 1991; Zhou, 1996; Kasha *et al*, 1990). There has been research conducted on microspore donor plants, examining the effects of donor plant health, genetic instability and interactions between media and genotype on embryo, green and albino plant regeneration (Kuhlmann and Foroughi-Wehr, 1989; Immonen, 1999; D'yachuk *et al*, 1986; Andersen *et al*, 1988; Kasha *et al*, 1995).

The health of microspore donor plants influences the responsiveness of isolated microspores (IM) or anthers. If there is nutrient, moisture, temperature or pest related stress, IMC often fails or has reduced embryo and green plant regeneration (Kasha *et al*, 1995; Kasha *et al*, 1997; Luckett and Darvey, 1992; Hess and Carman, 1998). Spraying pesticides on donor plants at or close to anthesis prevents the switch from gametophytic to sporophytic development (Kasha *et al*, 1997; Luckett and Darvey, 1992). There is debate regarding where donor plants should be grown; either in the field or in controlled environments like green houses or growth cabinets (Andersen *et al*, 1988; Bjørnstad *et al*, 1989; Hou *et al*, 1994; Kasha *et al*, 2000; Ritala *et al*, 2001). It seems logical that there are differences in opinions since environments differ around the world and some may be more conducive in providing "optimal conditions" than others. For example, Andersen *et al* (1988) grew donor plants in growth chambers at 15-20°C days and 8-12°C nights, Hou *et al* (1994) at 12°C day/night with a 16-hour photoperiod and Bjørnstad *et al* (1989) at 18/12°C day/night temperatures with an 18-hour photoperiod. Andersen *et al* (1988) found field grown donor plants preformed better than the growth cabinets grown plants, Hou *et al* (1994) found no difference between the two environments and Bjørnstad *et al* (1989) found the growth cabinet grown donor plants preformed better than field grown donor plants. Following guidelines like 15-18C° day/night temperatures with 16-17 hour photoperiod and light intensity

of 350-400μmol is important (Kasha *et al*, 2000), but the health and vigor of the donor plant is more important than following a single strict growth protocol. The health and vigor of the donor also appears to be related to a seasonal effect (Ritala *et al*, 2001). Ritala *et al*, (2001) suggested not planting in artificial environments between November and February, because green plant regeneration is higher for the spring, summer and autumn plantings. The five main factors affecting donor plant health are moisture, nutrients, temperature, light, and seasonal effects. Ensuring all factors are at optimum levels for a specific crop species will promote healthy microspore development.

Research suggests a genotypic influence on albino regeneration (Deimling and Flehinghaus-Roux, 1997). F_1 regenerates with *S. cereale* and *S. vavilovii* crosses regenerate fewer albinos with more *S. vavilovii* in the background, but some regenerate 100% albinos (Deimling and Flehinghaus-Roux, 1997). Albinism of wheat and barley appears to be under genetic control (Deimling and Flehinghaus-Roux, 1997). Comparisons of wheat and barley plastid DNA suggest deletions or rearrangements of 40 – 80% in comparison to wild types (Deimling and Flehinghaus-Roux, 1997; Zhou, 1996). The deletions of plastid DNA also appear heterogeneous, suggesting deletions or rearrangements occur after sporophytic division (Huang, 1996). Organellar variants are rarely observed in female gametic doubled haploids, possibly suggesting an unstable microspore cytoplasm, or that direct contact with media increases mutations (Huang, 1996). Research suggests that albinism is under the control of both gene actions (cytoplasmic and nuclear) (Zhou, 1996). Although genotype influences albino ratios, other factors such as culture conditions can decrease albino regeneration (Deimling and Flehinghaus-Roux, 1997).

1.4 Methods to determine ploidy level

In many experiments or breeding programs it is necessary to determine chromosome number so all microspore-derived haploids can be tabulated or doubled prior to anthesis. Several methods are available for the determination of ploidy level, such as a visual examination of the inflorescence, root tip chromosome count, chloroplast counts in stomatal guard cells, length of stomatal guard cells and/or flow cytometry (Schaeffer *et al*, 1979; Mix *et al*, 1978; Lucas *et al*, 1991; Hanning, 1995; Hu and Kasha, 1997b).

Visual inspection of a sterile inflorescence is not a good method since it is usually too late to double the chromosome number to achieve fertile plants. The objective of chromosome counts is to find actively dividing cells such as root tip or callus, and count the chromosomes. Schaeffer *et al* (1979) used acetocarmine and Mix *et al* (1978) used feulgen stain for successful observation of chromosomes numbers. Lucas *et al* (1991) estimated the ploidy level of canola by the number of chloroplasts in stomatal guard cells. They found 0.1 chloroplasts per stomatal guard cell for haploids and 16.2 for diploids respectively. These researchers also determined that chloroplast counts in the stomatal guard cells were less precise for the identification of polyploids. Hanning (1995) employed chloroplast counts in canola stomatal guard cells, reporting 8 for haploids, 16 for diploids and 32 for tetraploids. This researcher did not appear to have any difficulty distinguishing between the haploid, diploids, or the tetraploid canola plants. Hanning (1995) also reported that the stomatal guard cells increased in size from haploid to tetraploid. Borrino and Powell, (1987) found a correlation between chromosome number and size of stomatal guard cell of 30μm for haploids, 47.4 μm for diploids and 58 μm for tetraploids, subsequently proven by root tip chromosome counts. Hu and Kasha (1997b) used flow cytometry to distinguish haploids from diploids, by comparing the fluorescence intensity peaks to a known wheat cultivar. They found difficulties distinguishing polyploids: finding a plant with a reading

slightly below the estimated diploid peak could indicate it had 41 instead of 42 chromosomes, or a case of sampling error (Hu and Kasha, 1997b). The afore-mentioned problem could be solved by chromosome counts in root tip cells. Flow cytometry is an expensive system for the average laboratory. Despite the disadvantages of flow cytometry, the most important advantage is that of being a quick method applicable to early tissue like embryoids and callus.

1.5 Early selection methods in plant breeding programs

Early selection of highly heritable traits can save time and space in plant breeding programs. Bozorgipour and Snape (1992) studied methods for *in vitro* selection of agronomic characteristics of barley. They compared embryo size, germination and coleoptile height to yield per tiller, tiller harvest index, plant harvest index, kernel weights and plant height. They found coleoptile height was highly correlated to plant height. Therefore, they proposed that a selection for plant height could be made prior to transplanting. Albrecht *et al* (1995) studied early selection of low erucic acid content in canola microspore derived embryoids, and concluded that erucic acid was only found in cotyledons of seed derived embryos, microspore derived embryoids and seed. They reported that the erucic acid content of the cotyledons of microspore-derived embryoids was correlated to the content in the seed produced by the same plant. Cegielska-Taras *et al* (1999) also reported a high correlation of erucic acid in microspore-derived embryo cotyledons and the seed produced by the same plant (Cegielska-Taras *et al*, 1999). Therefore selection for high or low erucic acid is possible at the early stages of the canola microspore culture process.

Bansal *et al* (1998) demonstrated an example of selection against disease. They exposed canola microspore-derived embryoids to virulent blackleg spores. Increased selection pressure was successful as there were more resistant varieties of the pre-selected group compared to the group of double haploids with no selection pressure (Bansal, *et al*, 1998). Swanson *et al* (1988)

selected for herbicide tolerant *Brassica napus in vitro* by adding a mutagen and exposing the microspores or protoplasts of MDE tissue to chlorosulfuron. The surviving microspores or protoplasts were resistance to chlorosulfuron.

Early selection of DH plants eliminates a large number of undesirable plant types within a plant-breeding program. Transformation of wheat (*Triticum aestivum*) with antifungal compounds such as thaumatin-like proteins from barley was reported to increase tolerance/resistance to *Alternaria triticina* (Pellegrineschi *et al*, 2001). After such genes were inserted, the plants were exposed to the fungal pathogen, and tolerant plants were selected (Pellegrineschi *et al*, 2001).

1.6 Double haploid performance

There has been some research comparing agronomic performance of DH lines to lines derived from traditional breeding methods. Double haploid lines must have agronomic performance equal to or greater than lines derived by traditional methods.

Some studies suggest there are agronomic differences between plants derived from anther culture (AC) compared to the maize and traditional plant breeding methods (Ma *et al*, 1999; Baenziger *et al*, 1989). Such researchers have reported that AC derived plants have lower yield than similar lines derived by maize DH or single seed descent (SSD) (Ma *et al*, 1999). This suggests that the lower yields of AC-derived plants could be the result of either gametoclonal variation or gamete selection (Ma *et al*, 1999). Gametoclonal variation is possibly linked with isolated genetic mutations within callus tissue, and that plants regenerated from the same callus segregate for agronomic characteristics (Ryöppy, 1997). Ma *et al* (1999) also concluded that maize pollinated plants have less gametoclonal variation than plants derived by AC, in theory supported by similar yields of the maize pollinated and SSD derived plants (Ma *et al*, 1999).

Contrary to the suggestion by Ma *et al* (1999), recent research using amplified fragment length polymorphism (AFLP) suggests that the same line produce by AC or the maize method have equal gametoclonal variation (Guzy-Wróbelska and Szarejko, 2003). Guzy-Wróbelska and Szarejko (2003) also suggested that plants derived by either AC, maize method or SSD have similar yields. In contrast, some studies have reported no significant difference between AC derived plants and plants derived by traditional methods (Charmet and Branlard, 1985; Friedt and Foroughi-Wehr, 1983). These researchers suggested that, although there is some variation in AC-derived plants, possibly due to gametoclonal variation, they are easily selected against in an otherwise genetically uniform population (Charmet and Branlard, 1985).

1.7 Triticale

Triticale (X *Triticosecale* Wittmack L.) is a relatively new species: a human-made crop with a history of only 128 years (Müntzing, 1979). The history of triticale began with A.S. Wilson reporting the successful hybridization of wheat and rye to the Botanical Society of Edinburgh in 1876 (Müntzing, 1979). Cells of the original triticale octoploids contained 56 somatic chromosomes, revealing it was a true hybrid of wheat (2n=6x=42) and rye (2n=2x=14) (Müntzing, 1979). Triticale is often referred to as a synthetic allopolyploid because it originated from a human-made cross, and has more than the one genome from the Gramineae family. The specific ploidy level of triticale depends on the genetic composition of the wheat parent. There are three main groups of triticale; (1) tetraploids (2n=4x=28 AARR) a cross between a diploid wheat and diploid rye; (2) hexaploids (2n=6x=42 AABBRR) a cross between tetraploid wheat (2n=4x=28) that has the A and B genomes from durum wheat (*Triticum durum*), and rye; (3) octoploids (2n=8x=56 AABBDDRR) a cross between hexaploid bread wheat (2n=6x=42) and rye (Ryöppy, 1997). Triticale research was virtually abandoned when genetically stable octoploid

varieties seemed impossible (Müntzing, 1980), but increased when the genetically stable hexaploid triticale was developed. In 1969, the University of Manitoba released Canada's first triticale variety (Müntzing, 1980). The development of 'Rosner' (2n=6x=42, AABBRR genomes) established the potential of triticale research in Canada. Development of octoploid triticale continued, but the focus switched to varietal development of hexaploids (Müntzing, 1980). The offspring of octoploid by hexaploid crosses perform better than either parent, which is attributed to slight differences in the A and B genomes of the hexaploid and tetraploid wheat (Müntzing, 1980).

The release of 'Rosner' proved triticale had considerable potential in the livestock, human food, distilling and brewing industries (Müntzing, 1980). At present, triticale still has considerable potential for such industrial applications. Silage yield are approximately 10 percent higher than barley or oat under dry land conditions, and triticale has greater digestibility than oats (Salmon *et al*, 2001). High lysine content in the grain suggests triticale may have considerable potential in the hog industry. Lysine, an essential amino acid, is an important precursor for protein development (Voet *et al,* 1990). Essential amino acids like lysine are vital in the diets of monogastric animals because, unlike ruminants, they do not have the capability of synthesizing it (Brandt *et al*, 2000). Recent research suggests that triticale can be fed in a coarse ground form, replacing wheat in the diet of four week old pigs without any significant difference in performance (Jaikaran *et al*, 2000). Plant breeders continue to develop new triticale varieties with improved quality to expand into new markets, in addition to breakfast flakes for the human food industry.

To incorporate traits for new and existing markets, plant breeders rely on all plant breeding tools such as DH and traditional methods. It takes up to 14 years to release a winter triticale variety using conventional breeding methods, but this may be reduced by almost half

using doubled haploid techniques. Triticale doubled haploid techniques began in the 1980's with anther culture, followed by chromosome elimination and isolated microspore culture (IMC) in the 1990's (Ryöppy, 1997). The development of *in vivo* techniques, like chromosome elimination, is similar to wheat *in vivo* development. The successful interspecific cross of *Hordeum bulbosum* L. x barley (*Hordeum vulgare* L) was applied to wheat and triticale with minimal or no success (Ryöppy, 1997). The intergeneric cross of corn (*Zea mays*) increased wheat haploid development and subsequently was applied to triticale (Wędzony *et al*, 1998).

The minimal success of the maize method in triticale prompted research to determine if altering factors such as auxin analogues could increase haploid regeneration (Wędzony *et al*, 1998). Wędzony *et al* (1998) examined the effect of seven auxin analogues on embryo viability and growth: (1) indoleacetic acid (IAA); (2) 2,4-dichlorophenoxyacetic acid (2,4-D); (3) phenyl acetic acid (PAA); (4) 4-chloro-o-tolyloxyacetic acid (MCPA); (5) 2,4,5-trichlorophenoyacetic acid (2,4,5 T); (6) 4-amino-3,5,6-trichloropicolinic acid (Picloram); (7) 3,6-dichloro-o-anisic acid (Dicamba). Picloram and dicamba resulted in the largest embryos and greatest embryo viability (Wędzony *et al*, 1998). There has been over 20 years of research into anther culture to establish the correct stage of microspore, appropriate pretreatment, optimum physical factors such as light and temperature and induction media (Ryöppy, 1997). Microspore research has received approximately eight years of study, and most early attempts of microspore research failed to develop embryos (Ryöppy, 1997). Within the last six years IMC research has resulted in the regeneration of both embryos and green plants. For example, Sayed-Tabatabaei *et al*, (1998) successfully pretreated mid-late uninucleate triticale microspores at 4°C for 21 days, followed by induction within a solid CHB media. They reported successfully regenerating 325 MDE and 267 green plants (Sayed-Tabatabaei *et al*, 1998). Further research of pretreatments, induction and regeneration media is needed. Barley and wheat microspore pretreatments require between 3 and

7 days, removing the plants from the facility 21-25 days faster than traditional cold pretreatments (Kasha *et al*, 2000). Triticale microspore culture still requires a longer pretreatment time. If this could be reduced it would improve the efficiency of triticale DH breeding programs.

1.8 Summary

In summary, efficient doubled haploid production requires proper donor plant staging to ensure a sufficient number of microspores switch from gametophytic to sporophytic development. The correct switch point can vary between species and genotype, for example tobacco at the late-uninucleate (Sunderland and Wicks, 1971), angel's trumpet at the binucleate (Sunderland *et al*, 1974) or cereal crops such as barley, wheat, triticale and rye in the mid-late uninucleate stage (Wheatley *et al*, 1986; Hu and Kasha, 1997a; Sayed-Tabatabaei *et al*, 1998; Immonen and Robinson, 1999). Initial research found that there was an A, B or C developmental pathway for microspore-derived embryoids. Such research indicated that callus could produce microspore-derived plantlets. Callus was a less desirable choice of plantlet development because it often has variation in chromosome number and high frequencies of albinos (Kasha and Reinbergs, 1981). Ultrastructural observations revealed the specific changes that must occur in the microspore during the switch to the sporophytic path. Early barley microspore research by Sunderland and Evans (1980) revealed the importance of pretreatments. Hu and Kasha (1999) indicated that a microspore does not follow a strict path of development. Since the B pathway of development is often associated with spontaneous chromosome doubling it would be advantageous to encourage microspore development via this pathway, as spontaneously doubled haploids do not require a chemical doubling. To fully understand how the pretreatment works, more biochemical research is necessary. Sun *et al* (2000) studied the biochemical effects of heat shock on *Brassica napus* cv. Topas. They concluded with two hypotheses to explain how a heat shock initiated heat shock

protein genes, with the effect of calcium ion concentration common to both. A treatment of colchicine at 25°C induced a B pathway of development in canola, which may have activated the *lat 52-gus* gene.

A group of microspore-derived plantlets can vary in ploidy levels; hence this requires methods of determining ploidy level, and necessitates the doubling of any haploids. Methods examined in this review include visual examination of the inflorescence, root tip chromosome count, chloroplast counts in stomatal guard cells, length of stomatal guard cells and flow cytometry. The method chosen will depend on how it can be adapted to a given research laboratory or breeding program. Early selection methods could allow a breeding program to eliminate undesirable plant types. Bozorgipour and Snape (1992) found a high correlation between coleoptile height and plant height, therefore selection for plant height can theoretically be performed in culture. Since there is a high correlation between the erucic acid content in the microspore-derived cotyledon and the seed from the same plant in canola, this trait may be selected in culture (Albrecht *et al*, 1995; Cegielska-Taras *et al*, 1999). Increasing selection pressure by adding a disease or herbicide has the potential to increase the number of canola microspore-derived embryoids tolerant to the given disease or herbicide (Bansal *et al*, 1998; Swanson *et al*, 1988). Such early selection methods could save a research lab or breeding program time and money by eliminating undesirable plant types early in the developmental process.

Lines derived from most DH methods have agronomic performance equal to or greater than the same line derived by traditional methods. Generally, studies concur that DH methods produce some lines with considerable potential, but there is debate about the differences between traditional versus DH derived lines. Other studies suggests AC derived lines have lower yields than the same line derived by traditional methods.

The history of triticale is more recent than wheat and barley. Since the development of triticale, it has entered several markets, including the animal, human food and brewing and distilling industries. Most DH research has focused on triticale anther culture, but recent research has examined the effectiveness of the maize method and IMC. The maize and IMC methods require more research before they can be applied to breeding programs. Genotype dependence continues to regulate responsiveness of lines to DH techniques, thereby affecting embryo and green plant frequencies.

1.8.1 Objective of thesis research

Objectives of the thesis research were to:

1) Determine the response of five spring triticale genotypes to isolated microspore culture.
2) Compare different pretreatments to determine the possibility of shortening the duration.

1.8.2 Hypotheses of thesis research

The underlying null hypotheses were:

1) There will be no genotypic differences between the five spring triticale cultivars.
2) There will be no difference between pretreatments.

Chapter 2

Preliminary research: the effect of genotype, culture technique and embryo co-culture on triticale microspore-culture-derived embryogenesis

2.0 Abstract

The objective of this preliminary study was to identify triticale genotypes exhibiting microspore-culture-derived embryogenesis (MDE). Five spring triticale (X *Triticosecale* Wittmack) genotypes ('AC Alta', 'AC Ultima', 'Sandro', 'Pronghorn', and '94S001008') were subjected to two microspore culture techniques (the filter paper and liquid drop methods of adding microspores to solid media), with and without ovary co-culture. A traditional 4°C humid pretreatment was applied to donor spikes grown at 15/10°C day/night temperatures, with a 16.5-hour photoperiod, and a light intensity of 350-400 μmol m^{-2} s^{-1}. Genotypes differed for microspore-derived embryogenesis. Three main conclusions were drawn from the study; 1) A genotype effect was evident as only two cultivars 'Sandro' and 'AC Ultima' exhibited significant MDE yields; 2) Ovary co-culture did not negate the effect of genotype, only increasing MDE yields of genotypes already responsive without ovary co-culture; 3) Of the two culture techniques evaluated, the filter paper method significantly increased MDE yields of genotypes responsive to the microspore culture.

2.1 Introduction

Five factors influencing androgenic embryo (AE) development and subsequent green plant regeneration from microspore culture include the stage of microspore at harvest, pretreatment conditions, media formulations, and both donor plant vigor and genotype.

Selecting the correct stage of microspore aids in the maximization of AE development and green plant regeneration (Kasha *et al*, 1995). The early uninucleate stage occurs when the nucleus is closest to the microspore pore whereas the late uninucleate is when the nucleus is opposite the pore (Kasha *et al*, 2000; Hu, 1997). The harvest of mid-late uninucleate microspores often results in increased green plant development (Logue, 1996). Harvesting later microspore stages of monocotyledonous plants like barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), rye (*Secale cereale*) or triticale (X *Triticosecale* Wittmack) may increase albino regeneration. Dicots rarely regenerate albino plants (Logue, 1996). Early plastid metamorphosis is a suggested theory for the increased albinism of monocots with later microspore stages (Logue, 1996). Previous research suggests successful triticale microspores harvested in the mid-late uninucleate stage often results in AE (Sayed-Tabatabaei *et al*, 1998).

Pretreatment affects the competence of the microspore. The function of a pretreatment is to arrest cell development, allowing the accumulation of necessary reserves of polysaccharides such as starch (Hu and Kasha, 1999; Luckett and Darvey, 1992). Pretreatment involves variation of temperature, humidity, osmoticum, nutrient media and/or chemical inducers (Sayed-Tabatabaei *et al*, 1998; Kasha *et al*, 2000; Hu *et al*, 1995; Zheng *et al*, 2001). Previous triticale microspore research suggests that a 4°C humid pretreatment for 21 days is sufficient for triticale cultivars with a high anther culture embryogenic response (Sayed-Tabatabaei *et al*, 1998). The traditional 4°C humid dark 28-day pretreatment has also been successful for cereal species like barley and wheat with unproven embryogenic response (Kasha *et al*, 2000; Luckett and Darvey, 1992).

Media formulations also influence AE development and subsequent green plant regeneration (Luckett and Darvey, 1992). There are several media formulations, and some triticale microspore studies suggest CHB solidified media (Chu *et al*, 1990) results in the regeneration of more green plants than MC17 (Luckett and Darvey, 1992) liquid or solidified

(Sayed-Tabatabaei *et al*, 1998). Li and Devaux (2001), and Liu *et al*, (2002b) reported that the addition of ovaries to media can increase low AE yields of recalcitrant wheat and barley cultivars.

Donor plant vigor also affects AE yields (Kasha *et al*, 2000; Hess and Carman, 1998; Guo and Pulli, 2000). Kasha *et al*, (2000), Hess and Carman, (1998), and Guo and Pulli, (2000) reported that a decrease in donor plant vigor can result in a subsequent decrease of microspore viability. Microspore donors should be grown in appropriate environments that include proper temperature and lighting, with low moisture, nutrient, and pest related stress (Hess and Carman, 1998; Kasha *et al*, 2000).

Androgenic embryo yield and subsequent green plant regeneration varies with cultivar, or genotype (Immonen and Robinson, 2000; Guo and Pulli, 2000; Kasha *et al*, 1995). Genotypic differences may range from extreme (as in rye, where the genotypic difference can have a greater effect than the treatment applied (Guo and Pulli, 2000)), to low (as in barley, where the genotype effect is not as influential as the treatment (Kasha *et al*, 2000)). Immonen and Robinson, (2000) suggested that triticale exhibits genotypic differences, and reported that the winter cultivar 'Pika' is recalcitrant.

The objectives of this study were to: 1) Examine AE yield differences between two techniques of microspore application to solid CHB media; 2) Determine if ovary co-culture influences AE yields; 3) Determine if genotypic differences exist between the spring triticale cultivars 'Alta', 'AC Ultima', 'Sandro', 'Pronghorn', and '94S001008'.

2.2 Materials and methods

Five spring triticale genotypes were selected to represent germplasm available to western Canadian triticale breeders: 'Alta', 'AC Ultima', 'Sandro', 'Pronghorn', and a triticale line designated as '94S001008'. Alberta Agriculture Food and Rural Development (AAFRD) Field

Crop Development Centre (FCDC) released 'Pronghorn' in 1995 as an early maturing cultivar (Salmon *et al*, 2001). 'AC Alta' was registered in 1994 with attributes of short straw and good lodging resistance (McLeod *et al*, 1995). 'AC Ultima' is an early maturing, spring cultivar released in 1999 (Salmon *et al*, 2001). 'Sandro' has elevated test weight, and is recommended for areas of western Canada with longer growing seasons (Salmon *et al*, 2001). The FCDC plant-breeding program is currently developing the line '94S001008'

The spring triticale cultivars were grown in an artificially controlled environment with a mixture of cool white fluorescent and incandescent lights set at 350-400 μmol m^{-2} s^{-1}. The photoperiod was 16.5-hour with 15/10°C day/night temperatures. Seeds were planted in 700 cm^3 pots containing a soil free medium containing 1 part peat moss, 1 part fine vermiculite, ½ part medium grade sand, 135 g ground limestone, 70 g superphosphate, 120 g 18-6-12 (N-P_2O_5-K_2O) controlled release fertilizer and 1 g fritted trace elements in 36 litres (Stringam, 1971). Initially the plants were watered on a daily basis as required, followed by twice daily applications of room temperature water as the spike emerged from the boot (Zadoks Growth Stage (ZGS) 50-59) (Zadoks *et al*, 1974)). After the six-leaf stage (ZGS 16), the plants were fertilized weekly using water soluble 20-20-20 (N-P_2O_5-K_2O), complete with micronutrients (Zadoks *et al*, 1974). Two seeds were planted into each of four pots of each genotype, replicated three times, for a total of sixty pots.

Spikes were selected when the microspores were at the mid-late uninucleate stages, (approximately 60 days after planting), at the stage of the greatest potential for AE and green plant development (Kasha *et al*, 2000; Sayed-Tabatabaei *et al*, 1998). Three spikes from each replication of the five cultivars were selected for each treatment. There were 20 treatments including 5 genotypes, 2 techniques of microspore preparation, with and without ovary co-culture. A cold pretreatment (4°C for 28 days) was applied to all treatments.

Sterile techniques were used to isolate microspores and plate on an induction media. Microspore isolation began with the removal of florets and grinding in a mortar with a pestle containing 0.4 M mannitol. The ground tissue was filtered through a 500μ-, then a 100μ- mesh to separate other spike tissue from the microspores. A solid induction media rather than a liquid was chosen for this study. Two techniques of microspore preparation for solid media were applied. The first technique involved re-suspending the microspores in liquid induction media, (Table 2.1), then adding to a piece of 42.5 mm filter in a Büchner funnel under vacuum drop-by-drop with a Pasteur pipette to form a mound of microspore in the centre of the filter paper. After all the microspores were added to the filter paper it was removed from the Büchner funnel and set on solid CHB (Chu *et al*, 1990), media (Kasha *et al*, 1997). The second technique involved re-suspending the microspore in liquid induction media, and randomly placing drops of the solution on the surface of the solid induction media (Ziauddin *et al*, 1990). Live ovaries were added to the surface of the solid media near microspore with the ovary co-culture treatments. The plates of microspores were incubated in the dark at 25°C for 21-28 days. Subsequently, embryos were transferred to a regeneration environment set at 22°C, 8-hour photoperiod, and a light intensity of 120-180 μmol m^{-2} s^{-1}. Regenerated green plants were transferred to soil and placed within the donor plant environment.

Table 2.1. Induction and regeneration media used for triticale embryoid and green plant regeneration.

Components	Induction media mg/L	MS regeneration/differentiation media mg/L
KNO_3	1415	1900
$(NH_4)_2SO_4$	232	-*
NH_4NO_3	-	165
KH_2PO_4	200	170
$CaCl_2 \cdot 2H_2O$	83	440
$MgSO_4 \cdot 7H_2O$	93	370
$MnSO_4 \cdot 4H_2O$	5	-
$MnSO_4 \cdot 5H_2O$	-	22.3
H_3BO_3	5	6.3
KI	0.4	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.0125	0.25
$CuSO_4 \cdot H_2O$	0.0125	-
$CuSO_4 \cdot 5H_2O$	-	0.025
$CoCl_2 \cdot 6H_2O$	0.0125	0.025
Glycine	1.0	-
Thiamine-HCl	2.5	100
Pyridoxine-HCl	0.5	500
Nicotinic acid	0.5	500
Biotin	0.25	-
Calcium pantothenate	0.25	-
Ascorbic Acid	0.5	-
Myo-inositol	300	250
Proline	-	690
Glutamine	1000	-
Casein	-	1000
Maltose	63 g	30 g
2,4-D	0.5	-
Benzylamino-purine	-	1
pH	5.4	5.8

* - not used in media.

2.3 Results

Embryos did not regenerate from the liquid drop culture method without ovary co-culture (Table 2.2). Only AC Ultima exhibited embryogenesis from the liquid drop culture method with ovary co-culture. Pronghorn and the breeding line 94S001008 did not exhibit embryogenesis at any treatment level (Table 2.2). AC Alta exhibited embryogenesis from the filter paper culture method with ovary co-culture, while both AC Ultima and Sandro were embryogenic with and without ovary co-culture employing ovary co-culture (Table 2.2). Green plant regeneration was very low. Six green plants (total) developed from AC Ultima (4 with the filter paper method and ovary co-culture).

Figure 2.1. A regenerated androgenic embryo from isolated microspores of the spring triticale cultivar 'AC Ultima'

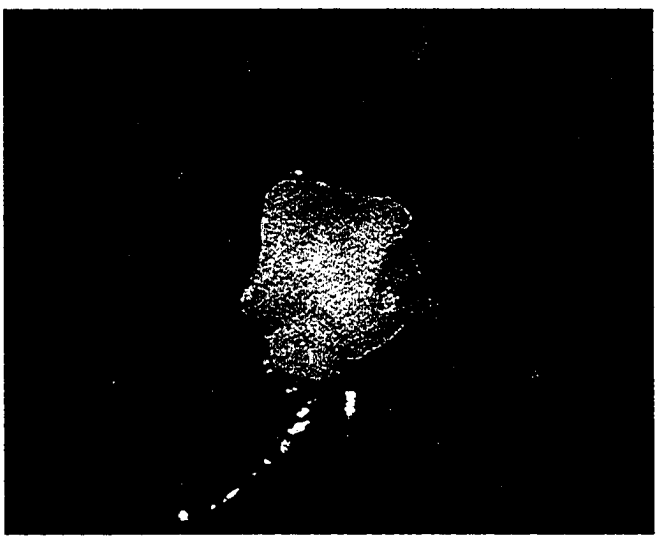

Figure 2.2. A green plant regenerated from an androgenic embryo of the spring triticale cultivar 'AC Ultima'

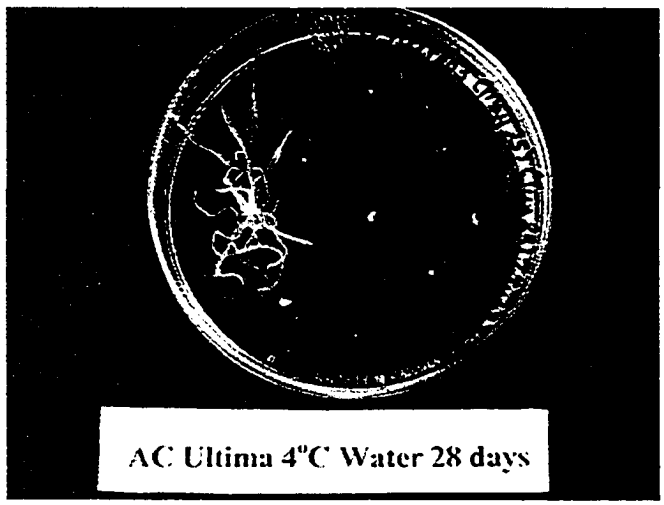

Table 2.2. Average number of microspore-derived embryoids (± 95 % confidence interval) for five spring triticale genotypes, employing either the filter paper or liquid drop method, with or without ovary co-culture.

	Filter Paper Method		Liquid Drop Method	
	Ovaries absent	Ovary co-culture	Ovaries absent	Ovary co-culture
AC Ultima	6 ± 3.13	12 ± 5.55	0	10 ± 6.09
Sandro	2 ± 2.99	25 ± 5.28	0	0
AC Alta	0	3 ± 3.20	0	0
Pronghorn	0	0	0	0
94S001008	0	0	0	0

$\alpha = 0.05$

2.4 Discussion and conclusions

Androgenic embryo (AE) yields differed among the five spring triticale genotypes evaluated in this study. Genotypic differences are a common phenomenon in many crop species (Li and Devaux, 2001; Hu, 1997; Guo and Pulli, 2000; Sopory and Munshi, 1996; Immonen and Robinson, 2000). Immonen and Robinson, (2000) reported genotypic differences between 10 winter triticale cultivars. They reported 'OAC Wintri' as highly responsive and 'Pika' as recalcitrant. Genotypic variance has been reported as being greater than treatment effect in some studies (Ryöppy, 1997). Genotypic differences are attributed to many factors including genetic

constitution, the nature of the plant (winter and spring types), and culturing techniques (Hu, 1997; Ryöppy, 1997; Kasha *et al*, 2000). Induction and regeneration of wheat isolated microspores are controlled by many genes including those reportedly on chromosomes 7A, 1B, 3A, and 2D (Hu, 1997).

Microspore embryo development and regeneration of microspore plantlets are also influenced by the nature (winter or spring types) of the plant. Spring wheat cultivars are more responsive to *in vitro* techniques like anther or microspore culture than winter types (Hu, 1997). The opposite may be true for triticale, with winter types being more responsive than the spring types (Ryöppy, 1997). The responsiveness of triticale is also complicated by the incorporation of more than one genome from the gramineae family (Ryöppy, 1997). Triticale is a 'young' species compared to the parental genomes wheat and rye. The synthetic allopolyploid is divided into three group: tetraploid (2n=4x=28), hexaploid (2n=6x=42) and octoploid (2n=8x=56). The complexity of the genomes created unstable plants, especially at the octoploid level (Ryöppy, 1997). Cytological unstable triticale aneuploids are often regenerated, with approximately 70 % haploid and 15 % aneuploid (Ryöppy, 1997).

Until recently, isolated microspore culture has been unsuccessful for triticale. It was previously thought that the anther provided a nurse effect, thereby resulting in a preference for anther culture (Ryöppy, 1997; Sayed-Tabatabaei *et al*, 1998). Research of culture techniques, media formulations and additions for wheat and barley suggested all contribute to culture effectiveness across all genotypes (Kasha *et al*, 2000; Finnie *et al*, 1989; Scott and Lyne, 1994). In the present, study microspore placement on solid media served to increase AE yield, while the liquid drop technique was not effective. Ziauddin *et al* (1992) reported that such a technique was successful with isolated barley microspores.

Ovary co-culture increased AE yields 2 to 12-fold for 3 genotypes in the present study. Li and Devaux (2001) suggested that ovary co-culture improved barley microspore embryogenesis by 2.1 fold. Zheng *et al* (2001) suggested that ovary co-culture increased wheat microspore embryogenesis by 4.5-fold for cultivars previously responsive. They also suggested ovary-conditioned media increased recalcitrant cultivar AE yields 100-fold (Zheng *et al*, 2001). If the addition of ovary-conditioned media could increase triticale embryogenesis, perhaps the nurse effect of the ovary could be identified and incorporated into the media. Genotypic effects are a response created by more than one factor (Kasha *et al*, 2000), therefore more research is required to isolated all factors involved that will increase responsiveness across all genotypes.

Using the results derived in this study, I decided to concentrate my efforts in the following section of this thesis research (Chapter 3) using the responsive cultivars Sandro and AC Ultima, employing the filter paper method. I wished to employ these cultivars and techniques to further explore the possibility of decreasing pretreatment duration.

Chapter 3
Pretreatment effects on embryo and green plant regeneration in triticale

3.0 Abstract

Reducing the time required to pretreat triticale microspores could increase the efficiency of regeneration of triticale microspore derived embryos to a level similar to wheat or barley. Nine microspore pretreatments were evaluated, including temperature of 4, 32 and 35°C in either 0.4 M mannitol, 25% w/v polyethylene glycol (PEG) and water. The duration of the 0.4 M mannitol, 25% PEG, and water treatments at 32°C and 35°C was 5 days, the 0.4 M mannitol and 25% PEG at 4°C treatment was 7 days, the second 0.4 M mannitol treatment was at 4°C for 14 days, and the water treatments were in 4°C for 28 days. Microspore donor parents 'AC Ultima' and 'Sandro' were grown in a soil free-mix at 350 – 450 μmol m^{-2} s^{-1} with a16.5 hour photoperiod and 15/10°C day/night temperature. Mid-late uninucleate microspores were isolated and incubated in the dark at 25°C for 21-28 days. The 4°C humid 28 day pretreatment successfully led to embryogenesis and green plant regeneration but the pretreatments evaluated did not.

3.1 Introduction

An important direct application of doubled haploidy (DH) as a breeding tool is homozygosity in one generation, reducing the time and cost of cultivar development (Hu, 1997; Liu *et al*, 2002b). *In vivo* and *in vitro* doubled haploid breeding techniques are used on many cereal crops (Hu, 1997; Kasha *et al*, 1995). *In vivo* techniques require the stimulation of incomplete fertilization, while *in vitro* stimulates plant cells or organs such as microspores, anthers or ovules (Kasha *et al*,

1995). The success of *in vitro* techniques is influenced by five factors, including donor plant vigor, microspore stage, media, genotype and pretreatment.

As donor plant vigor declines so does microspore viability (Kasha *et al*, 2000; Hess and Carman, 1998; Guo and Pulli, 2000). Microspore donors should be grown in a stress free environment free of moisture-, nutrient-, and pest-related stresses (Hess and Carman, 1998; Kasha *et al*, 2000).

Microspore stage affects androgenic embryo (AE) yield and green plant regeneration (Kasha *et al*, 1995; Logue, 1996). The mid-late uninucleate triticale microspore is an appropriate stage to induce the microspore's switch from gametophytic to sporophytic development (Sayed-Tabatabaei *et al*, 1998).

Selection of media type is based on AE yields and green plant regeneration. Solidified CHB culture media (Chu *et al*, 1990) regenerated more green triticale plants than MC17 liquid or solidified-culture media (Luckett and Darvey, 1992; Sayed-Tabatabaei *et al*, 1998).

Androgenic embryo yield and green plant regeneration may not be consistent between cultivars, due to genotypic differences (Immonen and Robinson, 2000; Guo and Pulli, 2000; Kasha *et al*, 1995). Genotypic differences occur in many crop species such as barley, wheat, rye, and triticale (Kasha *et al*, 1995; Immonen and Robinson, 2000). The differences in *in vitro* response between cultivars is difficult to control since it is suspected to be related to more than one factor, but media modifications have decreased genotypic differences between barley cultivars (Kasha *et al*, 2000). Genotypic differences are important for *in vitro* research, because this effect can often be greater than the treatment effect itself (Guo and Pulli, 2000).

Pretreatments are applied after harvesting spikes and prior to isolation of microspores (Hu and Kasha, 1999; Luckett and Darvey, 1992). The pretreatment usually involves a variation

of temperature, humidity, osmoticum, nutrient media, and/or chemical inducers (Sayed-Tabatabaei *et al*, 1998; Kasha *et al*, 2000; Hu *et al*, 1995; Zheng *et al*, 2001). The function of pretreatment is involved with the switch from gametophytic to sporophytic development, although how the pretreatment causes the switch is not completely understood (Sangwan and Sangwan-Norreel, 1996). Pretreatments vary between crop species, and cultivars within crop species (Hu, 1997). A 4°C humid 21-day pretreatment was successful in previous triticale isolated microspore research using cultivars with inherently high anther culture AE response (Sayed-Tabatabaei *et al*, 1998).

Decreasing pretreatment time could increase efficiency similar to barley isolated microspore culture (Kasha *et al*, 2000). Thus the objective of the present study was to evaluate various pretreatment combinations with the ultimate goal of decreasing pretreatment time and increasing the efficiency of the microspore culture process in triticale.

3.2 Materials and methods

3.2.1 Growth of donor plants

Two spring triticale cultivars, 'AC Ultima' and 'Sandro' (appendix 5.1-5.3), were selected to evaluate nine treatments for androgenic embryo (AE) yield and green plant regeneration (Table 3.1). The treatments varied number of days, temperature and pretreatment solutions (Table 3.1).

Table 3.1. Pretreatment combinations of cultivar, osmoticum and temperature.

	Pretreatment combinations		
Genotypes	Days	Temperature (°C)	Osmoticum
AC Ultima and Sandro	7	4	0.4 M mannitol
	*14	4	0.4 M mannitol
	7	4	25% w/v polyethylene glycol
	28	4	water
	5	32	0.4 M mannitol
	5	32	25% w/v polyethylene glycol
	5	32	water
	5	35	0.4 M mannitol
	5	35	25% w/v polyethylene glycol
	5	35	water

* Pretreatment combination only applied within growth cabinet b.

There were three plantings of five pots, with four adult plants per pot, grown for each of the 20 pretreatment x cultivar combinations (Table 3). The entire experiment was repeated three times (three blocks in time) for a total of 9 replicates. Six replications were grown in 2000 cm^3 pots containing soil-free mix (Stringam, 1971). The soil-free medium was mixed as follows; 36 L contained 1 part peat moss, 1 part fine vermiculite, ½ part medium grade sand, 135 g ground limestone, 70 g superphosphate, 120 g 18-6-12 or 21-5-12 (N-P_2O_5-K_2O) control release fertilizer and 1 g fritted trace elements (Stringam, 1971). Three replications of the 18 treatments were grown with a mixture of incandescence and cool white florescence lights (cabinet a). The other three replications of the 18 treatments were grown with a mixture of incandescence and metal halide lights (cabinet b). The 14-day 4°C 0.4 M mannitol treatment was only grown in cabinet b.

The light intensity of each cabinet was set within 350-450 μmol $m^{-2}s^{-1}$ with a photoperiod of 16.5 hours and day/night temperatures of 15/10°C.

The remaining three replications of 'AC Ultima' and 'Sandro' were grown in the pre-mixed soil-less mix, but donor plants exhibited stress symptoms, such as chlorosis and sterility, with no AE development. The ingredients of that pre-mix were; Sphagnum peatmoss, composted bark, perlite, vermiculite, starter nutrients, limestone and wetting agent.

Donor plants for each replication were planted one week apart and each pot thinned to four plants. To examine the effect of a 0.4 M mannitol at 4°C for 14 days, an additional pretreatment was added to six replications in cabinet b and grown in both soil free mixtures. All pots were watered as required until the six leaf stage, then at ZGS 16 the plants were watered three times a week with a weekly application of 20-20-20 200 ppm N. Watering was increased during and after spike emergence (ZGS 54-57).

3.2.2 Staging and pretreatment

Staging began as the spikes emerged from the sheath (ZGS 50-51). Each planting was harvested separately, and discarded after harvest. Anthers from the middle section of the spike were staged using Alexander stain. Spikes were selected with mid-late uninucleate microspores. To have a sufficient yield of microspores at isolation it was necessary to have 4 spikes for each pretreatment. Surface sterilization began by trimming the awns to make grinding easier, and the top three florets were discarded to fit the spikes into a 150 mm x 75 mm crystallizing dish. A 7% calcium hypochlorite bleach solution, and 4-6 drops of polyoxyethylenesorbitan monooleate (Tween 80) in double reverse osmosis (DRO) water was filtered through a Whatman grade 4 filter paper. The filtered calcium hypochlorite was added to the spikes for 10 minutes, ensuring the spikes were immersed. Each temperature treatment was surface-sterilized separately.

Surface sterilization was followed by three 5-minute rinses in sterilized DRO water, removing the calcium hypochlorite prior to adding pretreatment solutions. Pretreatment temperatures were 4, 32 and 35°C in water, 0.4 M mannitol solution and 25% w/v polyethylene glycol (PEG) solution. The surface-sterilized triticale spikes were placed in 150 x 15 mm Petri dishes with 25-30 ml of 0.4 M mannitol, PEG 25% w/v or 2-3 ml of water. To avoid water in contact with the triticale spikes, 2-3 ml of sterilized DRO water was added to a small Petri plate, then placed within the larger 150 x 15mm Petri plate containing the spikes, or a couple of drops were added opposite the spikes in the 150 x 15 mm Petri dishes. All Petri dishes were sealed with Parafilm and placed in separate incubators set at 4, 32 or 35°C.

3.2.3 Microspore Isolation

The isolation of the pre-treated microspores was conducted in a flow bench under sterile conditions. This procedure was done at; (1) 5 days for all 32 and 35°C pretreatments; (2) 7 days for 4°C mannitol and PEG pretreatments; (3) 28-days for the 4°C water pretreatments; (4) 14 days for the 4°C mannitol pretreatments. To simplify manual grinding, the spikelets were removed from the spike, and added to a mortar with a few ml of respective osmoticum. All water pretreatments were ground in 0.4 M mannitol. After grinding the tissue into slurry, the contents were poured through a 500-micron coarse nylon mesh and washed with respective grinding osmoticum. The filtrate was poured through a fine 100-micron nylon mesh to decrease green spike tissue, and then decanted into a 50 ml centrifuge tube. The samples were spun at 500-900 rpm for 5 minutes. Any samples with large amounts of green material were washed twice in 50 ml of osmoticum to decrease the amount of other spike tissue in the sample. The liquid was decanted and the pellet in the bottom lightly vortexed, washed with more osmoticum, transferred to a 15 ml centrifuge tube and re-spun. The microspores were washed once or twice in the 15 ml centrifuge tube, depending on the amount of green tissue remaining in the sample. Centrifugation

was limited to four times, and a slower speed of 600-700 rpm was used for the fourth spin, to ensure microspore survival.

After the last wash the microspores formed a yellow pellet, and were re-suspended in 2 ml of modified CHB (Chu *et al*, 1990) liquid induction media (Table 2.1). One Litre of solid media was prepared by first autoclaving 3.5 g of phytagel in 500 ml of DRO water (20-25 minutes at 121°C), and then combining with 500 ml of the filter-sterilized ingredients (Chu *et al*, 1990). Cell counts were performed with an inverted compound microscope by adding a drop of re-suspended microspores to a hemacytometer. A small sample of approximately 8-10 drops from one replication was inserted into Transwell plates for digital imaging. Drop-by-drop, the remaining washed and re-suspended microspores were placed on a piece of sterile 42.5 mm filter paper in a büchner filter funnel under vacuum. After a mound of microspores was gathered, the filter paper with microspores was placed on solid induction media in a 100 x 15 mm Petri dish. Each Petri dish was sealed with Parafilm and placed in the dark at 25°C for 21-28 days.

Embryos, 1 mm in diameter, were transferred from the modified CHB (Chapter 2, Table 2.1) media to a modified MS regeneration media (Kasha *et al*, 2000) (Chapter 2, Table 2.1). The AE were sealed with Parafilm, placed back in the dark at 25°C for 3 days, then moved to 22°C with an 8 photoperiod at 120 – 180 μmol m^{-2} s^{-1} until roots and shoots developed. Once the embryos formed a shoot approximately 2.5 cm long with a healthy root system they were transferred to a soil-free mixture. The plants were thereafter grown to maturity to determine ploidy level.

3.3 Results

'AC Ultima' had a more erect growth habit and matured earlier than 'Sandro'. The florets of 'Sandro' were consistently easier to remove from the rachis than 'AC Ultima'.

Nine pretreatments were applied to donor spikes of cabinet a, and ten within cabinet b. Androgenic embryo (AE) data included averages of three replications within cabinet a, averages of three replications within cabinet b and the average yield of both cabinets (Table 3.2). Microspores were counted from spikes grown in cabinet b, which are an average of actual counts from three replications of nine treatments. The 4°C water pretreatment induced the highest AE yields (Table 3.2). Even though other pretreatments appear to have AE yields, these yields were not significantly different from 0 at the 95% confidence level. Even though the mannitol, PEG, and high temperature pretreatments did not have significant AE yields, it is interesting to note the 14 day 4°C mannitol, 32 and 35°C mannitol and 32°C PEG pretreatments did produce some AE.

'AC Ultima' had a consistent and replicable AE embryo yield, unlike the inconsistent yield of 'Sandro'. The traditional cold pretreatment was the only effective pretreatment, and appeared to be more efficient for 'AC Ultima' than 'Sandro'. Therefore genotypic dependence was apparent in this triticale isolated microspore culture (IMC) study.

A pre-mixed soil-less mix was used for one replication of the nine treatments with no AE development, possibly related to stress expressed as chlorosis and sterility.

Microspores clearly switched from gametophytic to a multicellular sporophytic development (Figure 3.1). Androgenic embryos (AE) developed on the mound of microspores from individual microspores rather than callus (Figure 3.2). The androgenic embryos were white or cream colored when transferred to regeneration media (Figure 3.3). The AE germinated approximately 14 days after transferring to regeneration media (Figure 3.4). Callus development was rare for germinated AE, but apparent on some that never germinated. The results of regeneration revealed 65 plants, of which 34 were albinos and 31 green (Table 3.3). Of the 31 green plants 4 were fertile.

Table 3.2. Average microspore derived embryo (MDE) yield of AC Ultima and Sandro with 10 pretreatment combinations when grown in growth cabinets with different light conditions (a and b).

Genotype	Pretreatment	MDE (cab. a) MDE/dish	Microspore (cab. b) cell/ml x10^5	MDE (cab. b) MDE/ dish	MDE (cab. a and b) MDE/dish
AC Ultima	4°C 0.4M man. 7d	0	5.01 ± 0.0001	0	0
	4°C 0.4M man. 14d	- *	12.31 ± 0.0083	1 ± 2.3	- *
	4°C 25% w/v PEG 7d	0.3 ± 1.1	2.67 ± 0.003	0	0.2 ± 0.8
	4°C water 28d	45 ± 9.8	8.50 ± 0.0049	839 ± 5.1	442 ± 16.9
	32°C man. 5d	0.3 ± 1.1	6.10 ± 0.0083	0.3 ± 1.1	0.3 ± 0.72
	32°C 25% w/v PEG 5d	0	3.37 ± 0.0085	0	0
	32°C water 5d	0	2.25 ± 0.0046	0	0
	35°C man. 5d	1 ± 2.0	6.94 ± 0.0064	0	0.5 ± 1.39
	35°C 25% w/v PEG 5d	0	3.35 ± 0.002	0	0
	35°C water 5d	0	3.71 ± 0.0051	0	0
Sandro					
	4°C 0.4M man. 7d	0	5.47 ± 0.0037	0	0
	4°C 0.4M man. 14d	- *	9.75 ± 0.009	0	0
	4°C 25% w/v PEG 7d	0	1.79 ± 0.0021	0	0
	4°C water 28d	110 ± 20.6	4.77 ± 0.0067	0.7± 1.6	56 ± 14.5
	32°C man. 5d	0	6.61 ± 0.0021	0	0
	32°C 25% w/v PEG 5d	0	3.18 ± 0.0054	0	0
	32°C water 5d	0	5.21 ± 0.0045	0	0
	35°C man. 5d	0	6.87 ± 0.0057	0	0
	35°C 25% w/v PEG 5d	0	2.23 ± 0.0012	0	0
	35°C water 5d	0	9.06 ± 0.0048	0	0

* - pretreatment combination only applied within cabinet b. man. = mannitol, PEG = polyethylene glycol, d = days, cab. = cabinet. Confidence intervals were calculated as 95%.

Table 3.3. Androgenic embryo yield, albino and green plant regeneration of 'AC Ultima' and 'Sandro' with a 4°C water 28 day pretreatment.

Cultivar	Pretreatment	Embryo/dish	Albino plants (total)	Green plants (total)
AC Ultima	4°C water	442.3 ± 16.9	33	18
Sandro	4°C water	55.5 ± 14.5	1	13

Figure 3.1. AC Ultima microspore development from gametophytic to sporophytic development in the 4°C water pretreatment (400x magnification).

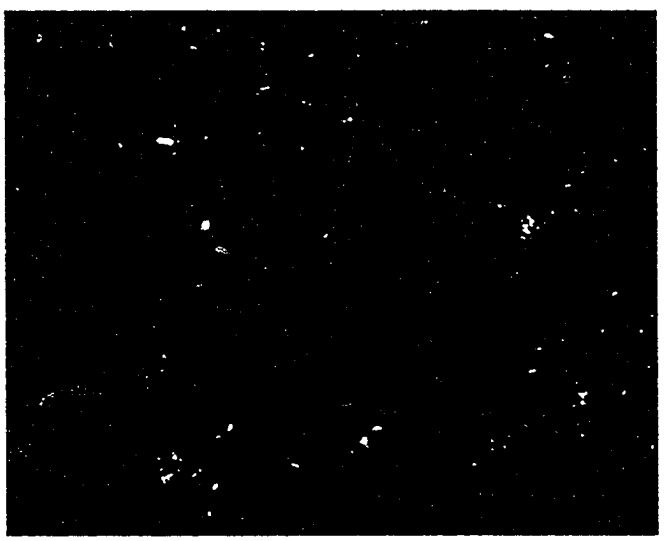

Figure 3.2. AC Ultima microspore derived embryoids within the 4°C water pretreatment (200x magnification).

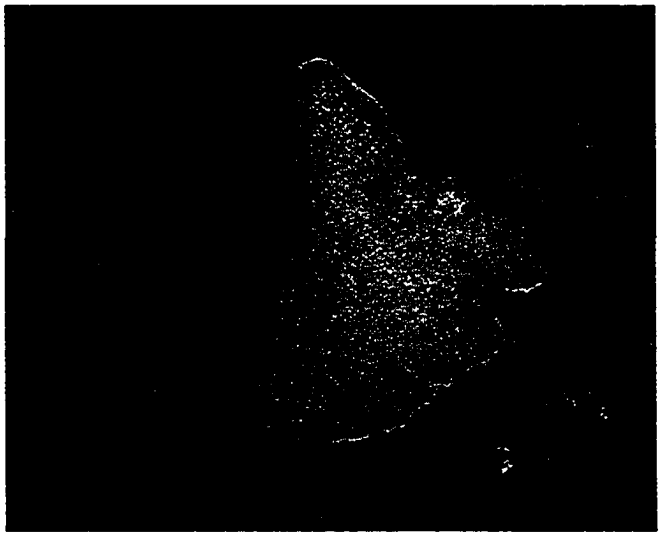

Figure 3.3. AC Ultima embryoid on regeneration media from the 4°C water pretreatment (200x magnification).

Figure 3.4. Green and albino plantlets regenerated from AC Ultima MDE.

3.4 Discussion and Conclusions

Triticale is a relatively new cereal crop compared to wheat and barley. A.S. Wilson reported the first successful hybridization of wheat (2n=6x=42) and rye (2n=2x=14) to the botanical society of Edinburgh in 1876 (Müntzing, 1979). There are three main groups of triticale: the tetraploid (2n=4x=28, AARR), hexaploid (2n=6x=42, AABBRR), and octoploid (2n=8x=56, AABBDDRR) (Ryöppy, 1997). Development of winter triticale cultivars can take 14 years with conventional breeding tools such as the pedigree system, but this can be reduced by almost half using doubled haploid techniques. The use of double haploidy for triticale began in the 1980's with anther culture, then chromosome elimination, and finally isolated microspore culture (IMC) in the 1990's (Ryöppy, 1997). The success of *in vitro* techniques, like anther culture or IMC depends on five factors, including donor plant vigor, microspore stage, media formulation, genotypes and pretreatment (Kasha *et al*, 1995; Luckett and Darvey, 1992). The function of pretreatment, including all four factors critical for *in vitro* techniques, is to arrest pollen development, thereby stimulating androgenic embryo (AE) development and plant regeneration (Hu, 1997; Luckett and Darvey, 1992). The role of pretreatment alone is not fully understood. Five possible theories exist: pretreatment 1) delays first mitosis; 2) increases embryogenic microspore viability; 3) arrests pollen development; 4) induces formation of two equal nuclei; and/or 5) creates modifications to the microspore wall and disorganizes the tapetum (Sangwan and Sangwan-Norreel, 1996). Pretreatment is often referred to as 'starvation', but some research suggests the addition of nutrients to the pretreatment solution does not consistently increase wheat AE development, although it appears to increase green plant regeneration (Liu *et al*, 2002a). Other research suggested the addition of macronutrients to a mannitol wheat pretreatment solution was

necessary for divisions within the microspore (Hu *et al*, 1995). Yet another suggested that, unlike wheat, triticale AE yield declines with the addition of nutrients to a pretreatment solution (Ryöppy, 1997).

The higher AE yields of 'AC Ultima' in the present study suggested the existence of genotypic difference. Genotypic differences are common for barley, wheat, rye and triticale (Kasha *et al* 1995: Guo and Pulli, 2000; Immonen and Robinson, 2000). Genotypic dependence has been reported in triticale anther and isolated microspore culture (Davies *et al*, 1998; Sayed-Tabatabaei *et al*, 1998; Immonen and Robinson, 2000). Such studies even revealed recalcitrant varieties such as 'Pika' with very low inherent AE yields. Rye is an obligate out crosser, thus genotypic dependence is a serious problem limiting the number of cultivars that can be tested (Guo and Pulli, 2000).

Currently triticale relies on a cold pretreatment of 4°C for 21 – 28 days (Sayed-Tabatabaei *et al*, 1998; Davies *et al*, 1998). The long 4°C pretreatment is a traditional pretreatment for many cereal crops. Therefore, as expected, it was effective in this study.

The humid 32°C and 35°C pretreatments yielded no AE. It was expected that the warm humid pretreatment would provide better results considering the success of similar pretreatments for wheat (Touraev *et al*, 1996). Touraev *et al* (1996) used a 33°C 4-day humid pretreatment on excised wheat spikes. The results suggested the 33°C pretreatment could induce MDE more efficiently than humid 25°C 8 day or 4°C 4-day pretreatments (Touraev *et al*, 1996). Although the temperatures and durations appear similar to the research of Touraev *et al* (1996), perhaps the extra day applied within this study was detrimental. Perhaps the unsuccessful 32°C and 35°C humid treatments are related to the rye (*Secale cereale* L.) component of triticale. Flehinghaus *et al* (1991) reported that direct rye anther culture at 27°C had similar AE yields, but regenerated

less green plants than 14°C or the 18°C pretreatment temperatures. Flehinghaus *et al* (1991) also suggested that an 8°C pretreatment was detrimental, inducing less AE and regenerating less green plants than 27°C, 14°C or the 18°C treatments.

Mannitol pretreatments in this study consisted of placing excised spikes in 0.4 M mannitol at 4°C 7 days, 4°C 14 days, 32°C 5 days and 35°C 5days. The 4°C 14 day, 32 and 35°C 5-day mannitol were the only pretreatments (other than the control) to induce AE development. It was expected that the mannitol pretreatments of 0.4 M would be more successful, as Guo and Pulli (2000) suggested a 0.3M mannitol was sufficient for rye IMC, and Kasha *et al* (1995) suggested a 0.4 M mannitol solution for wheat IMC. The 0.4 M pretreatment used by Kasha *et al* 1995 and Hu *et al* 1995 decreased wheat pretreatment time by approximately 20 days. Kasha *et al* (2000) also suggested that 0.3M mannitol was more effective for regenerating green barley plants than other pretreatment combinations (Kasha *et al*, 2000). Thus a 4°C 7 and 14 day 0.4 M mannitol pretreatment was added to the study. The duration was increased to 14 days to determine if the size of the triticale spike was limiting the effect of mannitol. Flehinghaus *et al* (1991) reported that rye AC increased MDE yield and green plant regeneration at 14 to 18°C. Guo and Pulli (2000) successfully applied a 0.3 M mannitol 10 to 12°C for 4 to 7 days pretreatment for rye IMC. Perhaps the pretreatment temperature for triticale could be raised to 10°C or more to accommodate the rye genome within triticale.

High temperature mannitol pretreatments were also tested in this study. The 32°C and 35°C mannitol pretreatments induced AE, but yields were not significantly different than zero. Hu *et al* (1995) examined the effects of several pretreatments to wheat (*Triticum aestivum* L.) microspores. Increased MDE yields were achieved with a 28°C pretreatment containing a solution of 0.4 M mannitol with macronutrients from FHG media as described by Kasha *et al*, 1990. Liu *et al* (2002a) suggested that chemical inducers assist with wheat embryo induction and

nutrient solutions assist with green plant regeneration. Liu *et al* (2002a) exposed wheat spikes to a nutrient solution containing chemical inducers for 69 hours, shorter than the high temperature pretreatments of this study. This suggests that the addition of chemical inducers or decreasing the duration of high temperature pretreatments may be considered for future studies.

Pretreatments containing polyethylene glycol (PEG) assist with AE development in high temperature canola pretreatments (Telmer *et al*, 1995; Ilić-Grubor *et al*, 1998). Pretreatment containing PEG was incorporated in the study to determine if it assisted with triticale MDE development. Nevertheless there were no significant MDE yields for any PEG treatment.

Research of maltose suggests it can increase green plant regeneration (Finnie *et al*, 1989). Perhaps finding the optimum maltose concentration could decrease the number of albino plants regenerated. Sayed-Tabatabaei (1998) used 63g of sucrose within CHB media with no albinos from select lines well adapted for AC, while Guo and Pulli (2000) suggested rye IMC at 60 g/L of maltose, and others suggest 90 g/L of maltose is needed to regenerate wheat isolated microspores (Zheng *et al*, 2001; Liu *et al* 2002b). In general it is thought that the induction of AE is directly related to genotypic dependence, while green plant regeneration is linked to culture conditions.

Under the conditions of this study, the 4°C humid 28 day pretreatment was successful, but the shorter pretreatments were not. Thus the objective of decreasing pretreatment time so as to increase the efficiency microspore culture technique similar to wheat and barley IMC was not successful. Rather, I have reaffirmed the previous success of a long humid cool pretreatment period.

Chapter 4
General discussion and conclusions

Doubled haploidy (DH) of cereal crops like barley, wheat, rye, or triticale use *in vivo* and *in vitro* techniques (Hu, 1997; Kasha *et al*, 1995). *In vivo* techniques stimulate the entire plant, while *in vitro* stimulate explants like anthers, microspores, or ovules (Kasha *et al*, 1995). Triticale is a relatively new crop compared to barley or wheat. Anther culture (AC) has been applied to hexaploid and octoploid triticale for approximately 26 years, and tetraploid triticale for approximately 16 years (Ryöppy, 1997). Until recently, a suitable pollen donor was not available for triticale *in vivo* techniques, and isolated microspore culture (IMC) was unsuccessful (Ryöppy, 1997). Corn (*Zea mays*) pollen was found suitable for wheat and triticale, but research continues to increase the efficiency of triticale *in vivo* DH similar to wheat (Wędzony *et al*, 1998).

Wheat and barley DH research switched from *in vivo* to *in vitro* techniques (Kasha *et al*, 1995; Kasha *et al*, 2000). *In vitro* techniques are possibly more efficient, because there are more microspores than ovaries in a spike (Kasha *et al*, 1995; Kasha *et al*, 2000). Donor plant vigor, microspore stage, media, genotype and pretreatment influence the success of *in vitro* techniques (Hess and Carman, 1998; Kasha *et al*, 2000; Hu *et al*, 1995). Triticale IMC research is limited, but the five factors influencing IMC have been examined by Sayed-Tabatabaei *et al* (1998). They suggested growing microspore donor plants in a low stress environment. They selected microspores at the mid-late uninucleate stage, and used CHB (Chu *et al*, 1990) solidified media rather than MC17 (Luckett and Darvey, 1992) liquid or solidified (Sayed-Tabatabaei *et al*, 1998). Sayed-Tabatabaei *et al* (1998) used a 4°C humid 21 day pretreatment to assist the microspore switch from gametophytic to sporophytic development. To decrease genotypic differences they selected cultivars that were responsive to AC techniques (Sayed-Tabatabaei *et al*, 1998).

Androgenic embryo and green plant yield can vary between cultivars (Immonen and Robinson, 2000; Guo and Pulli, 2000; Kasha *et al*, 1995). The difference between cultivars can be extreme (Immonen and Robinson, 2000). Immonen *et al* (2000) suggested that winter triticale cultivars 'Pika', 'OAC Trillium', and 'Ulrika' are recalcitrant and that 'OAC Wintri' had the best induction frequency. Genotypic difference can influence research when the effect of the genotype is greater than the effect of the treatment applied (Guo and Pulli, 2000). Since genotypic differences are possibly controlled by more than one factor, such effects are difficult to control (Kasha *et al*, 2000).

Wheat and barley IMC research has switched from the traditional 4°C humid 21-28 day pretreatment to a 3 – 4 day pretreatment (Kasha *et al*, 1995; Kasha *et al*, 2000; Hu *et al*, 1995). Thus decreasing pretreatment time for triticale IMC, while examining the effect of genotypic difference was the basis of this thesis.

4.0 Conclusions:

Assessment of genotypic effects, culture technique and embryo co-culture for triticale isolated microspore culture.

- Androgenic embryo yield differences between the spring triticale cultivars 'AC Ultima', 'Sandro', 'AC Alta', 'Pronghorn', and '94S001008' revealed genotypic differences.
- Androgenic embryo yield was increased with ovary co-culture, but only for cultivars responsive without co-culture.
- The filter paper media technique significantly increased AE yields over the liquid drop method.

The effect of selected pretreatment conditions to microspore derived embryo and green plant yield.

- As expected, the 4°C humid 28 day pretreatment was successful, reaffirming previous triticale isolated microspore and anther culture research (Sayed-Tabatabaei *et al*, 1998; Immonen and Robinson, 2000)
- Androgenic embryo yields were not significant in the shorter pretreatments, thus the objective to reduce pretreatment time was not achieved.
- The AE yield difference between 'AC Ultima' and 'Sandro' suggested the existence of genotypic differences. 'AC Ultima' had consistent AE yields while Sandro yields were sporadic, suggesting this technique requires more research before implementation as a breeding tool.

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Appendix A

Illustrations and poster presentation

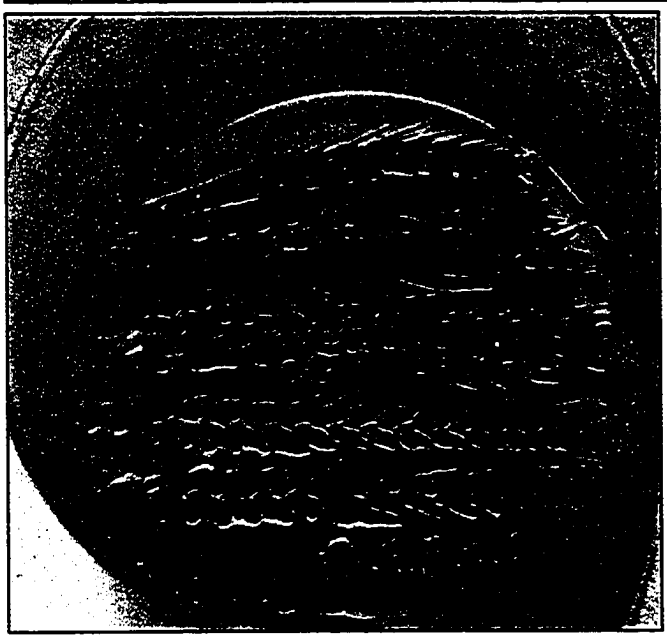
5.0 Illustrations

Appendix 5.1
One planting each week of AC Ultima for three weeks at the University of Alberta.

Appendix 5.2
One planting each week of Sandro for three weeks at Alberta Agriculture Food and Rural Development Field Crop Development Centre.

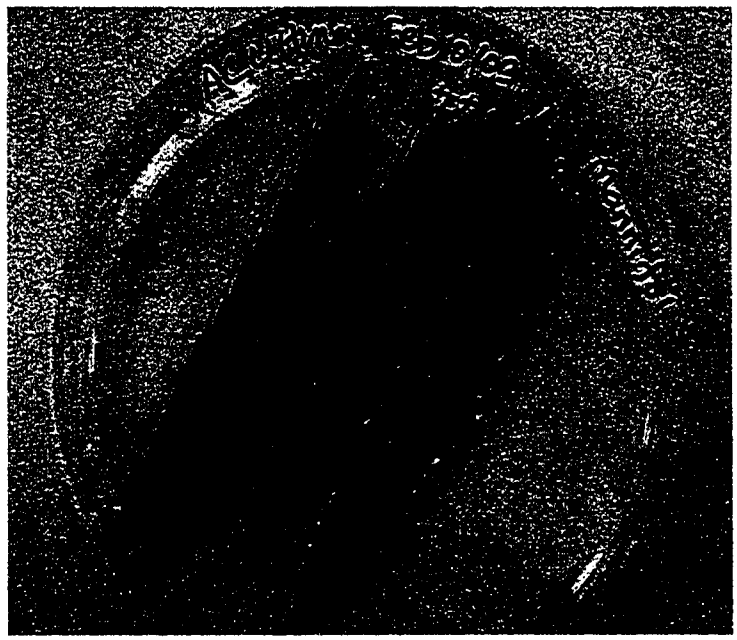
Appendix 5.3

One planting each week of AC Ultima for three weeks at Alberta Agriculture Food and Rural Development Field Crop Development Centre.

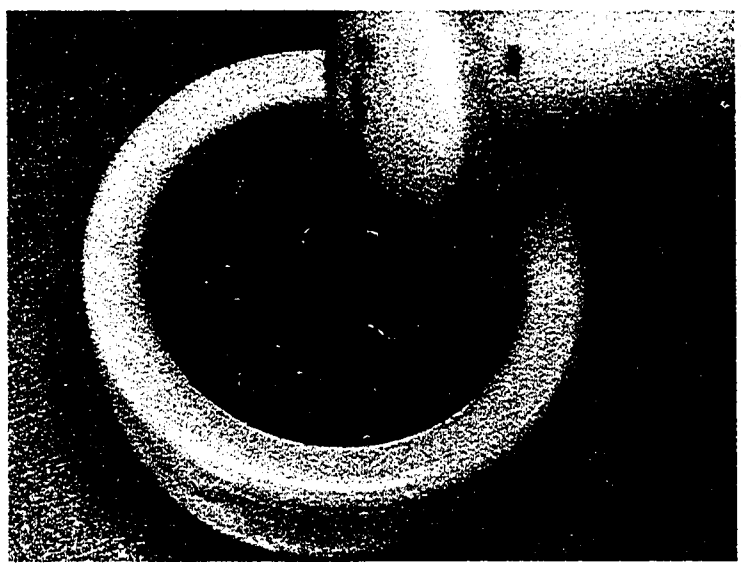


Appendix 5.4

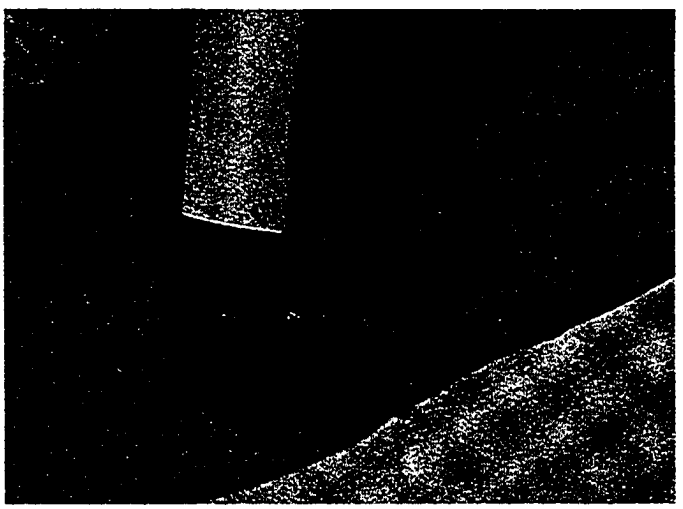
Surface sterilization of AC Ultima spikes in 7 % calcium hypochlorite.



Appendix 5.5
Spikes of AC Ultima in pretreatment solution after surface sterilization.

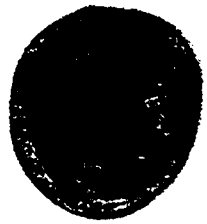


Appendix 5.6
Florets of AC Ultima during the grinding process prior to microspore isolation.



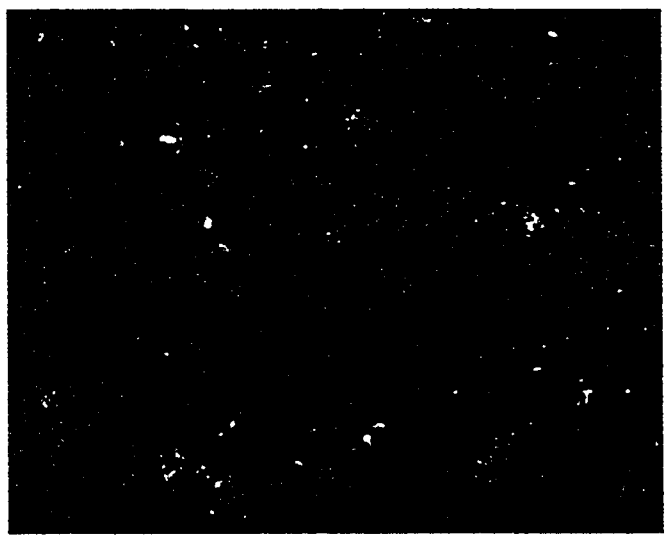
Appendix 5.7
Pellet of A pellet of AC Ultima microspores in a 50 ml centrifuge tube after centrifugation

Appendix 5.8
Isolated microspores of AC Ultima collected on filter paper prior to placement on solid induction media.



Appendix 5.9
Sandro microspore switched from sporophytic to gametic development, in the 4°C water 28-day pretreatment (400X magnification).

Appendix 5.10
AC Ultima microspore switched from sporophytic to gametophytic development, in a 4°C water 28-day pretreatment (400X magnification).



Appendix 5.11
Androgenic embryos of AC Ultima from the 4°C water pretreatment (200X magnification)

Appendix 5.12 AC Ultima fertile (A) and sterile (B) microspore derived haploids.