Quantitative trait loci (QTL) mapping and doubled haploid technology for spring wheat improvement

by

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Abstract

Efficiency is important in wheat breeding programs. A series of studies were conducted to determine genetic differentials, map genomic regions affecting various agronomic and quality traits in conventional and organic management systems and to improve doubled haploid (DH) technology in spring wheat. A mapping population developed from a cross between Attila x CDC Go performed differently in the two management systems. Heritability estimates differed between systems for five of the nine traits including grain yield, tillers, plant height, kernel weight and grain protein content. Direct selection in each management system resulted in 50% or less lines selected in common for eight of the nine (except for flowering time) traits. Overall, we mapped 46 QTL for various agronomic and quality traits across organic and conventional management systems for three years. Most of these QTL were specific to the management systems; however, consistent QTL for grain yield, test weight, kernel weight and days to flowering were mapped in both systems on chromosomes 6A, 1B, 3A and 5B, respectively. These consistent QTL also behaved differently in both systems with respect to their phenotypic variation and additive effects leading to the conclusion that selection and testing of genotypes for production and quality traits should be done within environmental conditions which are comparable to where candidate lines are intended to perform.

The technique of isolated microspore culture (IMC) is being used in many species to produce DH plants; however, low frequency of green plant production, microbial contamination and albinism limit its use in wheat breeding programs. A series of experiments were conducted to improve microspore embryogenesis. In four different

experiments, induction medium (NPB-99+10% Ficoll) was supplemented with various organelle antioxidants, antibiotics and phytosulfokine alpha (PSK-α) along with optimization of induction medium osmolality. Glutathione (2 µM), PSK-α (10^{-7} M) and osmolality of induction medium at 350 mOsm kg^{-1} enhanced the efficiency of microspore embryogenesis in wheat and triticale. Cefotaxime at 100 and 50 mg L^{-1} also helped to control recurrent bacterial contamination in our laboratory, reduce the occurrence of albinism and substantially increase the formation of embryo/embryo like structures and green plant production in wheat and triticale, respectively. The systematic use of these treatments in IMC of wheat and triticale is recommended.

Preface

Quantitative trait loci (QTL) mapping, marker assisted breeding (MAB) and doubled haploid (DH) production technology are important tools in plant breeding. These technologies can be used together to enhance our understanding of the genetics of various traits, improve selection efficiency and facilitate the production of complete homozygous lines only in one generation that can lead to rapid cultivar development. The thesis consists of various studies and literature review related to these technologies.

Second chapter of the thesis has been published as a review article "Randhawa HS, Asif M, Pozniak C, Clarke JM, Graf RJ, Fox SL, Humphreys DG, Knox RE, DePauw RM, Singh AK, Cuthbert RD, Hucl P, Spaner D (2013) Application of molecular markers to wheat breeding in Canada. Plant Breeding. 132: 458-471". I wrote first two drafts of the manuscript that was shared with other authors for further improvement/suggestions. I extracted genomic DNA of 171 recombinant inbred lines (RILs) and send to Diversity Array Technology (DArT), Yarralumla, Australia for genotyping (Chapter 3). I also recorded phenotypic data for various agronomic, quality and diseases resistant traits in Attila x CDC Go population during 2011. I used/analyzed phenotypic data of 2008, 2009 and 2010 to identify differences in heritability, correlations and selection response and to identify and map genomic regions for various agronomic and quality traits between organic and conventional management systems under the guidance of Dr. Rong-Cai Yang. The population was screened for *Rht-B1* gene by Enid Perez. The genetic map was provided by DArT, Yarralumla, Australia.

The research work presented in Chapter 5-8 has been published as follows:

Asif M, Eudes F, Goyal A, Amundsen E, Randhawa HS, Spaner D (2013) Organelle antioxidants improve microspore embryogenesis in wheat and triticale. In Vitro Cellular and Developmental Biology - Plant. 49:489-497 (Chapter 5)

Asif M, Eudes F, Randhawa HS, Amundsen E, Spaner D (2014) Induction medium osmolality improves microspore embryogenesis in wheat and triticale. In Vitro Cellular and Developmental Biology - Plant. 50:121-126 (Chapter 6)

Asif M, Eudes F, Randhawa HS, Amundsen E, Yanke J, Spaner D (2013) Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale. Plant Cell Reports. 32:1637-1646 (Chapter 7)

Asif M, Eudes F, Randhawa HS, Amundsen E, Spaner D (2014) Phytosulfokine alpha enhances microspore embryogenesis in both triticale and wheat. Plant Cell Tissue and Organ Culture. 116:125-130 (Chapter 8).

In Chapter 5, I along with Goyal A and Amundsen E conducted experiments and recorded data. I analyzed and wrote manuscript. Eudes F, Randhawa HS and Dean S helped in the improvement of manuscript. I layout and conducted experiments related to wheat that are presented in Chapter 6, 7 and 8. Amundsen E did experiment on triticale. I analyzed data and wrote manuscripts. Eudes F, Randhawa HS and Dean S helped in the improvement of manuscripts. Yanke J assisted in the identification of bacterial isolates presented in Chapter 7.

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I would like to express my sincere gratitude to my academic supervisor, Dean Spaner who generously provided me guidance, constructive suggestions and time throughout this project. The research work presented in this thesis would not have been accomplished without his continued help and encouragement. I feel great pleasure and honor to work with him. I am also grateful to the members of my supervisory committee, Rong-Cai Yang, Habibur Rahman and Harpinder Randhawa for their time and technical expertise to my research work. I am also indebted to Francois Eudes who provided me help in laboratory experimentation.

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Last but not least, I must express my profound gratitude to my wife, Sabeen Asif for her love, moral support and belief in me. Special thanks to my sons, Mueez and Shahzaib for being there every day with lot of laughter and fun. This dissertation is dedicated to my grandmother and parents who always encouraged my educational career and professional development.

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List of Abbreviations

ABA	Abscisic Acid
$AgNO_3$	Silver Nitrate
AMs	Arbuscular mycorrhizas
ANOVA	Analysis of Variance
APM	Amiprophos-methyl
ASC	Ascorbate
ATP	Adenosine Triphosphate
BA	Benzyl Adenine
BAP	Benzylaminopurine
BX	Benzoxazinoids
Cef	Cefotaxime
CIM	Composite Interval Mapping
CPSR	Canada Prairie Spring Red
CPSW	Canada Prairie Spring Wheat
$CuSO_4$	Copper Sulphate
CWAD	Canada Western Amber Durum
CWES	Canada Western Extra Strong
CWGP	Canada Western General Purpose
CWHWS	Canada Western Hard White Spring
CWRS	Canada Western Red Spring
CWRW	Canada Western Red Winter
CWSWS	Canada Western Soft White Spring class
DArT	Diversity Array Technology
DH	Doubled Haploid
DHA	Dehydroascorbate
DMAP	4-(dimethylamino) pyridine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
ELS	Embryo Like Structure

ERS	Edmonton Research Station
FAO	Food and Agriculture Organization
FDK	Fusarium Damaged Kernels
FHB	Fusarium Head Blight
GPC	Grain Protein Content
GSH	Glutathione
GSSG	Glutathione Disulfide
HSP	Heat Shock Proteins
IAA	Indole Acetic Acid
IMC	Isolated Microspore Culture
IWM	Integrated Weed Management
IWMS	Integrated Weed Management Strategy
LAI	Leaf Area Index
LOX	Liposygenase
MAB	Marker Assisted Breeding
MAS	Marker Assisted Selection
MB	Methylene Blue
MCS	Multi Cellular Structures
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NH_4NO_3	Ammonium Nitrate
NIR	Near Infrared Reflectance
NO	Nitric Oxide
NtBHA	N-t-butyl Hydroxylamine
NUE	Nutrient Use efficiency
PAA	Phenyl Acetic Acid
PAR	Photosynthatically Active Radiation
PCD	Programme Cell Death
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PGPR	Plant Growth Promoting Rhizosphere

PSK-α	Phytosulfokine Alpha
QTL	Quantitative Trait Loci
RIL	Recombinant Inbred Line
ROS	Reactive Oxygen Species
RUE	Radiation Use Efficiency
SA	Salicylic Acid
STS	Sequence Tagged Site
SSD	Single Seed Descent
TDZ	Thidiazuron
Van	Vancomycin
WHO	World Health Organization
$ZnSO_4$	Zinc Sulfate

1.0 Introduction to the thesis, objectives and hypothesis

I was employed with Pakistan Agricultural Research Council, Islamabad, Pakistan as a Research Scientist since 1996 where I was involved in the development of high yielding, drought tolerant and disease resistant wheat cultivars through conventional breeding. In 2010, I contacted my supervisor, Dr. Dean Spaner to improve my education and learn new breeding techniques like quantitative trait loci (QTL) mapping, marker assisted breeding and doubled haploid (DH) technology, and he agreed to assist and supervise me during the period of my PhD studies.

During first year of my stay at University of Alberta, Edmonton, Alberta, I completed my course work, recorded phenotypic data for various agronomic, quality and disease resistant traits in Attila x CDC Go population and sent genomic DNA of 180 recombinant inbred lines (RILs) to Diversity Array Technology, Yarralumla, Australia for genotyping. I also wrote one comprehensive review manuscript and two books on three different aspects that helped me to prepare for oral exam and to identify null hypotheses/objectives associated with research work presented in the thesis.

1. Randhawa HS, Asif M, Pozniak C, Clarke JM, Graf RJ, Fox SL, Humphreys DG, Knox RE, DePauw RM, Singh AK, Cuthbert RD, Hucl P, Spaner D (2013) Application of molecular markers to wheat breeding in Canada. Plant Breeding. 132:458-471 (Thesis: Chapter 1)
2. Asif M (2013) Progress and opportunities of doubled haploid production. SpringerBriefs in Plant Science. Volume 6. Springer, USA (Appendix in the Thesis)
3. Asif M, Iqbal M, Randhawa HS, Spaner D (2014) Managing wheat breeding for organic systems: enhancing competitive ability against weeds. SpringerBriefs in Agriculture. Volume 5. Springer, USA (Not included in thesis)

In second year of my PhD, I moved to Agriculture and Agri-Food Canada, Lethbridge Research Centre where I conducted four experiments on the improvement of doubled haploid (DH) production in wheat via isolated microspore culture (IMC). I assisted in the collection of stripe rust data for various populations and in making desirable recombinants through various crossing methods and marker assisted selection. I also helped in the development of three doubled haploid mapping populations in wheat through corn based pollination method. After completing my experiments on IMC, I

came back to the University of Alberta, Edmonton, Alberta during third year where I started analyzing data and writing manuscripts on QTL mapping and IMC experiments. I assisted my supervisor in his wheat breeding programs crossing and selection activities. During the field season, I collected phenotypic data for various agronomic traits, scored various generations and mapping populations for leaf and stripe rusts and selected superior genotypes based on their phenotypic performance in the field conditions.

The specific objectives of the thesis research work were to:

1. Uncover differences in heritability, selection response and genetic gains between conventional and organic management systems.
2. Identify and map genomic regions (QTL) associated with various agronomic and quality traits.
3. Compare and uncover QTL behaving differently in conventional and organic management systems.
4. Examine the effect of *Rht-B1* on various traits in conventional and organic management systems.
5. Increase the production of embryo/embryo likes structure and green plants, and to minimize albinism during DH production via IMC in wheat by optimizing induction medium.
6. Identify microbial contaminants of isolated microspore cultures and devise a strategy to control them.

These objectives were tested under the following null hypotheses:

1. Agronomic and quality traits do not differ for heritability, correlation and selection response between very weedy organic and weed free conventional management systems.
2. Genomic regions associated with various agronomic and quality traits behave in a similar manner in conventional and organic management systems.
3. The effect of *Rht-B1* on various traits is the same in conventional and organic management systems.
4. Osmolality, organelle antioxidants and phytosulfokine alpha in induction media do not alter/influence the production embryo/embryo like structures, green and albino plants.
5. Microbes do not play a role in contaminating microspore cultures.
6. Antibiotics (Cefotaxime and Vancomycin) do not control bacterial contamination of isolated microspore cultures and have an adverse effect on embryos/embryo like structures and green plant production.

2.0 Application of molecular markers to wheat breeding in Canada[1]

Wheat is the most widely-grown cereal crop globally (217 M ha). In 2012, world wheat production was 671 m tons, making it the third most produced cereal after maize and rice (FAOSTAT 2012). The most common species grown are *Triticum aestivum* L. (common wheat) and *Triticum turgidum* var *durum* L. (durum wheat). Common wheat accounts for 95% of the total wheat consumed world-wide. Both winter habit wheat, sown in the fall and harvested in summer (10 month cycle); and spring habit wheat, planted in April or May and harvested in August to October (4-5 months cycle) are grown in Canada. In Canada, common wheat is comprised of various classes based on growth habit (winter or spring) and quality factors such as protein concentration, gluten strength, kernel hardness and color (hard and soft, red and white) (McCallum and DePauw 2008). Each class has specific characteristics related to end-use functionality for bread, noodles, pastries, confections, and other food uses. Durum is used mainly to make semolina products including pasta and couscous.

Canada is the seventh largest wheat producer in the world with production of 23.1 m tons in 2010 (FAOSTAT 2012). Of the total wheat production in Canada, spring hexaploid wheat accounts for 69%, durum wheat accounts for 23%, and winter wheat accounts for 8% (DePauw et al. 2011a). About 96% of wheat is grown in the western prairie provinces of Alberta, Saskatchewan and Manitoba and 4% is grown in eastern Canada. Canada is recognized globally for high end-use quality wheat and is the second largest exporter after the United States of America, with 19.3 million of the 26.8 m tons production in 2009 being exported. Canadian wheat production has increased substantially since 1961 and the average grain yield per hectare has increased from 1512 kg ha^{-1} during 1961-70 to 2478 kg ha^{-1} during 2000-10 (Fig. 2-1). This increase in production represents a growth rate of 1.3% per annum and can be attributed to the development of high yielding, disease and insect resistant cultivars, and better

[1] This chapter has been published as a review article: Randhawa HS, Asif M, Pozniak C, Clarke JM, Graf RJ, Fox SL, Humphreys DG, Knox RE, DePauw RM, Singh AK, Cuthbert RD, Hucl P, Spaner D (2013) Application of molecular markers to wheat breeding in Canada. Plant Breeding. DOI: 10.1111/pbr.12057

agronomic practices because the area sown to wheat has declined from 10.2 m ha in 1961 to 8.3 m ha in 2010 (FAOSTAT 2012).

The wheat grown in Alberta, Saskatchewan, and Manitoba consists of nine classes (Fig. 2-2), including Canada Prairie Spring Red (CPSR), Canada Prairie Spring White (CPSW), Canada Western Amber Durum (CWAD), Canada Western Extra Strong (CWES), Canada Western Hard White Spring (CWHWS), Canada Western Red Spring (CWRS), Canada Western Red Winter (CWRW), Canada Western Soft White Spring (CWSWS) and Canada Western General Purpose (CWGP). Canada Western Red Spring is the largest class of wheat grown in the prairie region, followed by Canada Western Amber Durum (McCallum and DePauw 2008).

During the last 15 years, marker-assisted breeding (MAB) has gained importance among wheat breeders in Canada. The application of molecular markers has enabled breeders to select superior genotypes for traits that are difficult to select based solely on phenotype or to pyramid desirable combinations of genes into a single genetic background. Marker-assisted breeding also offers the opportunity to improve response from selection because molecular markers can be applied earlier in the life cycle (for example gametic selection in the F_1 seedling stage). Marker-assisted breeding not only contributes improved precision for selection of specific traits but is also cost effective compared to conventional plant breeding procedures. Marker assisted breeding also offers the opportunity to hasten transfer of desirable alleles from unadapted genetic backgrounds into a desirable germplasm through cross breeding. To date, 30 different loci responsible for traits like resistance to various diseases, quality, and agronomy (plant height, photoperiod response, grain weight, tolerance to abiotic stress, *etc.*) have been cloned and 97 functional markers have been developed to categorize 93 alleles based on gene sequences (Liu et al. 2012). Within traditional breeding systems, although marker-assisted breeding can be applied to all segregating generations, it is most commonly applied to early generations to enrich populations with favorable genes. The application of MAB in plant breeding programs depends on several critical factors including the following: (i) the molecular marker and gene of interest should be very closely linked, (ii) the marker needs to be validated to show trait association in the desired genetic backgrounds grown under target environments (Sharp et al. 2001) and (iii) the screening methodology should be cost effective, time saving, and highly

reproducible across laboratories. In Canada, wheat breeders, agronomists, pathologists and physiologists have given special emphasis to improving adaptation to biotic and abiotic stresses (ability to produce stable grain yield over locally variable environmental conditions), earliness, and end-use quality of wheat. Breeding for disease resistance, particularly against the rusts; leaf rust (*Puccinia triticina*), stem rust (*Puccinia gramminis* f. sp. *tritici*) and stripe rust (*Puccinia striiformis*); Fusarium head blight (FHB) (*Fusarium graminearum*); and insects including wheat midge (*Sitodiplosis mosellana* Géhin) and wheat stem sawfly (*Cephus cinctus* Nort.) has been practiced routinely in wheat breeding programs (Table 2-1). A comprehensive list of various genes for different traits known to be present in CWRS cultivars and germplasm lines is presented in Table 2-2.

The application of doubled haploid (DH) technology in wheat breeding programs has increased the speed of cultivar development, particularly in winter wheat, where use of contra-season nurseries to achieve two breeding cycles per year is not practical. Wheat breeders screen parental plants for various alleles before DH production and haploid plants are subjected to marker-assisted selection prior to chromosome doubling to ensure the retention of gene(s) of interest and to discard undesirable genotypes. In this chapter, we will focus on the current practical applications of marker-assisted breeding for various traits in Canadian wheat breeding programs.

2.1 Biotic stresses

2.1.1 Rust resistance

Rusts are considered to be the most devastating diseases of wheat, causing yield and quality losses. Three types of rusts: leaf, stem, and stripe, occur in Canada with varying degrees of intensity. Stem rust, caused by *P. graminis,* resulted in severe epidemics in Canada during the early and mid-1900s. A major stem rust epidemic caused huge losses in the 1950s as a result of a pathogen race change (15B-1) and due to the prevalence of susceptible cultivars (Peturson 1958). Since 1950, durable rust resistance has been achieved by pyramiding numerous effective stem rust resistance genes into modern Canadian wheat cultivars, along with breaking the sexual cycle through the elimination of barberry (*Berberis vulgaris*), the alternate host. A new race of stem rust known as Ug99 (TTKS), was originally detected in Uganda in 1999 (Pretorius et

al. 2000) and later detected in eastern and southern Africa. Since then, several epidemics in Kenya and Ethiopia have been reported along with the occurrence of numerous Ug99 variants in South Africa (Visser et al. 2011). Marker technology for stem rust resistance focused on *Sr2, SrCad* and *Sr57*. *Sr2* is effective against stem rust races found in North America. Molecular markers including *csSr2* and *Xgwm533* linked to *Sr2* and *FSD_RSA* and *cfd49* linked to *SrCad* are the recommended markers for selection of these genes (Mago et al. 2011; Spielmeyer et al. 2003). Two Canadian cultivars, [AC Cadillac (DePauw et al. 1998)] and Peace, have shown resistance to Ug99 at the seedling and adult growth stages and to all prevalent stem rust races in North America. Genetic mapping in two different DH populations has uncovered the presence of the stem rust resistance gene (*SrCad* now designated as *Sr42*) on chromosome 6DS, which is linked to the bunt resistance gene *Bt10* (Hiebert et al. 2011). Molecular characterization of these populations also revealed the presence of *Lr34/Yr18*. Hiebert et al. (2011) reported that the presence of *SrCad* along with *Lr34* provides a high level of resistance against Ug99, whereas, moderate resistance was observed when only *SrCad* was present. The identification of *SrCad* is a valuable breeding resource to help combat stem rust, especially Ug99 and its variants.

Leaf rust infection is also an annual occurrence in western Canada. Disease severity differs considerably from year to year, but usually ranges from trace amounts to 25% flag leaf infection. Breeding for resistance to leaf rust started in the late 1930s, when the susceptibility of Thatcher, which was grown extensively in the western prairie provinces from 1939 to 1960, resulted in severe yield and economic losses (McCallum and DePauw 2008; McCallum et al. 2012). Since then, many resistance genes have been deployed. The most common leaf rust resistance genes used in Canadian wheat cultivars include *Lr1, Lr10, Lr13, Lr14a, Lr16, Lr21,* and *Lr34* (McCallum and DePauw 2008; McCallum et al. 2007), of which *Lr1, Lr10, Lr13*, and *Lr14a* are no longer effective to Canadian races (Fetch et al. 2011). Recently, virulence on the widely used gene *Lr21* was detected in western Canada (McCallum unpublished). *Lr34* remains effective and *Lr16, Lr21,* and *Lr22a* in combination with *Lr34* are still effective and offer partial to complete resistance against the prevalent leaf rust races in western Canada (Fetch et al. 2011). Therefore MAB for leaf rust resistance in Canada has focused on *Lr34*, *Lr16, Lr21,* and *Lr22a.*

The resistance conferred by *Lr34* has never been defeated by race changes in *P. triticina,* and its co-segregation with resistance to stripe rust [*Yr18,*(Singh 1992)], powdery mildew [*Pm38,* (Spielmeyer et al. 2005)], Stem rust [*Sr57*, (Keller et al. 2012)] and Barley Yellow Dwarf Virus [*Bdv1*, (Singh 1993)] has provided broad based resistance. McCallum et al. (2012) reported that more than half of western Canadian cultivars carry *Lr34*. Molecular assisted selection of *Lr34* is routine in nearly all Canadian wheat breeding programs. The tightly linked *csLV34* marker {Lagudah et al. (2009); Lagudah et al. (2006)} and more recently the *caIND11* marker (Dakouri et al. (2010), are being used to pyramid *Lr34* along with other rust genes using MAB. The *caIND11* marker is used as a diagnostic marker to characterize parents for the presence or absence of the *Lr34* resistance allele.

In recent years, no leaf rust epidemics have been reported in Canadian durum wheat due to past breeding efforts involving incorporation of effective resistance genes in cultivars. However, emergence of new races requires continued efforts to deploy new leaf rust resistance genes. For example, a virulent race BBG/BN and its variant BBG/BP have overcome the resistance of widely-adapted durum cultivars evaluated in North-western Mexico. These races pose a serious threat to durum production in Canada because they may spread across the continent through the North American rust corridor. Predominant Canadian durum cultivars are susceptible to BBG/BN and its variants (Singh et al. 2013) necessitating identification of effective sources of leaf rust resistance that can be bred into Canadian durum wheat. *Lr14a* is effective against BBG/BN and BBG/BP and the SSR marker *Xgwm146* linked to *Lr14a* is currently used in the Canadian durum breeding programs to select for resistance in the absence of the race in Canada.

Stripe rust is an emerging threat to wheat production in western Canada. Although it has been a problem in the CWSWS class in southern Alberta (Sadasivaiah et al. 1993), recently other classes (including CWRS, CWRW, and CPSR) have experienced infection. Stripe rust has been detected every year since 2000, and serious epidemics were reported in parts of western Canada in 2005, 2006 and 2011 (McCallum et al. 2006; Randhawa et al. 2012b). Randhawa et al. (2012a) characterized stripe rust resistance in 104 Canadian wheat cultivars and reported the presence of four stripe rust

resistance genes (*Yr10, Yr17, Yr18* and *Yr36*). From that study, most common wheat cultivars carried *Yr18* and exhibited intermediate to moderate resistance to stripe rust.

The *Yr36* gene is linked to *GpcB1* and is being introduced in breeding populations through MAB for elevated protein content. Marker assisted selection was utilized in cultivars, which carry *Yr18* and *Yr36.* As a result Lillian (DePauw et al. 2005) and Burnside (Humphreys et al. 2010b) exhibited high levels of resistance to stripe rust. The *Yr17* gene that is closely linked with *Lr37* (leaf rust) and *Sr38* (stem rust) was detected in CDC Stanley, which has shown moderate resistance, suggesting that *Yr17* still provides some resistance against stripe rust in western Canada, but new stripe rust races have overcome this gene in the United States (Chen et al. 2010b; Chen et al. 2002). An opportunity still exists for future MAB with *Yr17* but the deployment of *Yr29/Lr46, Yr46/Lr67* and *Yr47/Lr52* using MAB would further improve stripe rust resistance in Canadian bread wheat cultivars.

Gene pyramiding for durable resistance to leaf, stem and stripe rusts entails stacking multiple genes into a cultivar for simultaneous expression. Rust gene pyramiding has been considerably facilitated by the use of DNA markers closely linked to genes of interest and thus, has increased the speed of the pyramiding process. Examples of genes being used for pyramiding to improve rust resistance in Canadian wheat breeding programs include *Lr14a* (for durum only), *Lr18, Lr19, Lr21, Lr22a, Lr24, Lr32, Lr34, Lr37, Lr46, Lr57, Lr58, Lr67* for leaf rust, *Sr2, Sr12, Sr22, Sr24, Sr26, Sr36, Sr31, Sr32, Sr29, Sr38, Sr39, Sr40, SrWeb* for stem rust including race Ug99 and variants, and *Yr5, Yr10, Yr15, Yr17, Yr18, Yr29, Yr36, Yr40, and Yr46* for stripe rust (Table 2-3).

2.1.2 Fusarium head blight resistance

Fusarium head blight (FHB) is a major disease of wheat, reducing yield and causing quality losses that negatively affect milling, baking and pasta-making properties. In the 1990s, FHB caused severe losses to the Canadian grain industry totaling approximately US$300 million in Manitoba (Windels 2000). The most serious problem associated with FHB is the contamination of grains with mycotoxins, especially deoxynivalenol (DON), which can render the grain unsuitable for human and livestock consumption. A number of *Fusarium* spp. can cause FHB, however, the principal causal organisms are *Fusarium graminearum* Schwabe teleomorph *Gibberella zeae* (Schwein.

Petch), *F. avenaceum* (Corda ex Fr.) Sacc., and *F. culmorum* (Smith) Sacc. (Gilbert and Tekauz 2000). Infection at early grain developmental stages result in DON accumulation and large yield losses due to physical damage (McMullen et al. 1997). Wheat grains damaged by FHB are called Fusarium damaged kernels (FDK), which are distinguished as thin or shrunken chalk-like grains often with a white to pinkish fibrous-mould appearance. In Canadian wheat, tolerance levels of FDK are extremely low and more than 0.25% FDK by weight will result in the downgrading of a CWRS #1 grade wheat to CWRS#2. If the presence of FDK is greater than 1%, downgrading from CWRS#1 to CWRS#3 will occur, and greater than 2% FDK will result in a CWRS#4 grade (Fernandez et al. 2009), thereby causing significant economic losses to wheat growers in Canada.

Breeding wheat for resistance against FHB is one of the most effective methods to reduce the risk associated with this disease (Anderson 2007). Resistance against FHB is multigenic and its expression is highly dependent on the disease triangle, *i.e*. the interaction of pathogen, environment and host. Various quantitative trait loci (QTL) have been identified that confer resistance to FHB; however, the proportion of variation explained by these QTL is relatively small. Different types of FHB resistance have been identified, including resistance to initial infection (type I), resistance to spread (type II) and resistance to DON accumulation (type III) (Mesterhazy 1995; Schroeder and Christensen 1963). Selection for all three types of resistance using MAB is a priority in Canadian breeding programs as each is governed by multiple, independent genes. The first QTL (*Qfhs.ndsu-3BS*) for FHB resistance (type II) was identified by Waldron et al. (1999) from Sumai 3 on 3 BS along with two other QTL on chromosome 6BS. The region of 3BS was characterized using various molecular markers and named *Fhb1* {(Liu and Anderson (2003b, a)}and Guo et al. (2003). Flanking STS and PCR markers for *Fhb1* are now available (Cuthbert et al. 2006; Liu et al. 2008; Liu et al. 2006) to help wheat breeders deploy this gene/QTL into their breeding lines. Another major QTL (*Qfhs.lfl-6BS*) conferring type II resistance derived from Sumai 3 and its relatives was named *Fhb2* and mapped on chromosome 6BS, 2 cM from SSR locus *Xgwm644* (Cuthbert et al. 2007; Haeberle et al. 2009; Lin et al. 2004; Shen et al. 2003; Waldron et al. 1999; Yang et al. 2005). The Canadian cultivar Waskada (Fox et al. 2009) may contain *Fhb2,* but a recombination event near the location of the gene precludes confirming its presence. Lin et al. (2004) and Lin et al. (2006) found four QTL for FHB on chromosomes 2B, 3B, 4B

and 5A using Wangshuibai and Nanda2419 as parents. The 4B QTL were fine-mapped later by Xue et al. (2010) and designated *Fhb4. Fhb4* is flanked by the markers *Xhbg226* and *Xgwm149*. McCartney et al. (2007) assessed the expression and degree of additivity of FHB QTL's in elite Canadian spring wheat germplasm. They reported marginal additivity among the particular FHB QTL studied in the particular environments of the experiments. They also reported significant linkage drag, such as a negative association with plant height, and association of the Sumai 3 5AS resistant allele with reduced grain protein content. *Fhb1* and *Fhb5AS* have been combined in the recently released cultivar Cardale (Fox et al. 2013).

In general, Canadian wheat cultivars of the CPS, CWSWS, and CWAD classes are susceptible to FHB and breeders are working to pyramid *Fhb1, 2, 4* and *Fhb5AS* into their lines using MAB. The CWRS cultivars range in FHB resistance from moderately resistant to susceptible (http://www.gov.mb.ca/agriculture/crops/diseases/fac12s01.html). The older cultivar Neepawa exhibits intermediate resistance to FHB, which may be due the presence of Brazilian cultivar Frontana in its pedigree (Gilbert and Tekauz 2000). Several cultivars that have Neepawa in their pedigree/background, including Katepwa (Campbell and Czarnecki 1987), AC Barrie (McCaig et al. 1996), and AC Cora (Townley-Smith and Czarnecki 2008), also exhibit intermediate resistance to FHB. Newer CWRS cultivars like Waskada (Fox et al. 2009), Carberry (DePauw et al. 2011b) and Cardale (Fox et al. 2013) with better FHB resistance than AC Barrie have been released for commercial cultivation. Some of these cultivars carry the *Fhb1* gene which will form the basis for further improvements through pyramiding with additional genes, using MAB. In the CWAD class, the expression of resistance in lines carrying *Fhb1* and *Fhb2* is not as good as in common wheat. However, several QTL for FHB resistance have been reported in wild relatives of durum wheat (Ruan et al. 2012; Somers et al. 2006), and DNA markers associated with these QTL are currently being applied to stack the QTL into adapted Canadian durum wheat lines.

2.1.3 Tan spot

Tan spot caused by *Pyrenophora tritici-repentis* is a commonly occurring insidious disease on the Canadian prairies that regularly causes considerable losses.

Because of its endemic nature, tan spot has received little attention in breeding, with other diseases that are epidemic in nature or that impart toxins on the grain such as FHB which is receiving most of the attention. Markers have been developed for the *Tsn1* locus (Singh et al. 2010b). Canadian durum breeding has focused on the incorporation of *Tsn1* resistance using flanking markers *Xfcp620* and *Xfcp394*.

2.1.4 Common bunt and loose smut

Common bunt caused by *Tilletia tritici* (Bjerk.) G. Wint. in Rabenh and *T. laevis* Kühn in Rabenh is a threat to wheat production, particularly because the spores of this fungus contaminate grain and impart a foul odor. However, if left uncontrolled, the disease can also cause substantial yield loss through the replacement of grain with fungal reproductive structures called bunt balls. In Canada, common bunt has been controlled effectively over the vast wheat growing acreages by field-type genetic resistance and, where genetic resistance was lacking, by seed treatment fungicides. The field-type resistance has largely been derived from Canadian cultivars such as Neepawa, Katepwa and Columbus. Markers for this field-type of resistance were identified in the cultivar AC Domain (Fofana et al. 2008) and McKenzie (Knox et al. 2013). The field resistance is supplemented with the major resistance gene, *Bt10* (Laroche et al. 2000) and a source from Blizzard (Wang et al. 2009). These genes are common in Canadian wheat germplasm and the markers for these genes are at different stages of implementation and use, but are mainly being used to characterize material to understand the genes contributing to resistance in potential crossing parents.

Loose smut is caused by *Ustilago tritici* and although typically causing only minor losses, it can cause significant loss if left uncontrolled. Good resistance is available for the genetic control of loose smut, but the biggest difficulty in incorporation of resistance is the labor intensive nature of disease evaluation for selection purposes. A series of markers have been identified from the cultivar Glenlea for resistance to loose smut. The genes that these markers relate to are found on chromosomes 3A, 7A, 7B and 5B. The Glenlea resistance localized to 5B is found at the distal end of the long arm of the chromosome. The *Utd1* resistance gene, present at the distal end of the short arm of chromosome 5B, has also been identified in durum wheat with markers *Xgwm234*

and *Xgwm443* (Randhawa et al. 2009). These markers are currently being validated and are in the initial stages of introgression.

2.1.5 Ergot

Ergot is a disease of wheat caused by the fungal pathogen *Claviceps purpurea*. The disease is manifested through the development of sclerotia in florets of the wheat spike in place of the seed. The ergot bodies contain compounds toxic to humans and animals requiring cleaning and blending of the grain, and in sufficient numbers the ergot bodies can render the grain unusable. Resistance has been identified in the CIMMYT durum line Green 27 (Menzies 2004). Markers have been developed for a major gene for honeydew stage ergot resistance found in Green 27 and are being used to characterize parental lines for breeding Canadian durum wheat and to track resistance in lines that derive from Green 27.

2.1.6 Insect resistance

Wheat growers in western Canada face losses due to insect damage. Among these insects, the wheat stem sawfly, *Cephus cinctus* (Norton), is one of the most important species causing significant yield losses {reviewed by Beres et al. (2011a)}. The larvae of wheat stem sawfly cause damage by girdling the inside of the wheat stem, thereby weakening the stem and resulting in breakage. The genetics of solid stem resistance have been studied extensively in hexaploid and durum wheat and microsatellite markers (e.g. *Xgwm247, Xgwm340, Xgwm547, Xbarc77, Xgwm181 and Xgwm114)* have been identified and deployed in both hexaploid and durum wheat breeding programs (Clarke et al. 2002; Cook et al. 2004; Houshmand et al. 2007). Marker assisted breeding is particularly important in hexaploid wheat, where the expression of stem solidness varies with light intensity, temperature, seeding density and moisture supply (reviewed by Beres et al. (2011b). Lillian is currently a very widely grown cultivar, which confers stem solidness and tolerance to the wheat stem sawfly (DePauw et al. 2005). DT818 was the first solid stem durum cultivar in Canada and was supported for registration in 2012 (Singh et al., personal communication). These

cultivars have been used extensively as parents, providing ample opportunity to apply the markers to enrich the allele frequency for stem solidness.

Another important insect pest of wheat in western Canada is *Sitodiplosis mosellana* (Géhin), commonly known as the orange wheat blossom midge (Lamb et al. 2000; Lamb et al. 1999). The first severe outbreak of orange wheat blossom midge was reported in 1983 on the border of Saskatchewan and Manitoba (Olfert et al. 1985). Canadian entomologists detected a source of antibiotic resistance in several US winter wheat cultivars from which the resistance gene *Sm1* was transferred into Canadian spring wheat backgrounds (Barker and McKenzie 1996). The *Sm1* gene is present on the sub-terminal region of chromosome 2BS in the cultivar Augusta (Thomas et al. 2005) and is genetically linked to the leaf rust gene *Lr16* (McCartney et al. 2005b). Unity (Fox et al. 2010), which incorporates *Sm1* was the first CWRS cultivar and was registered in 2007. Using MAB, the *Sm1* gene was incorporated into Goodeve (DePauw et al. 2009). Since then, a number of additional cultivars expressing *Sm1* resistance have been released using combinations of phenotypic and marker assisted selection; these cultivars include the following: Fieldstar, Shaw, CDC Utmost, Vesper (CWRS), Conquer, Enchant, (CPS), and Glencross (CWES) (www.midgetolerantwheat.ca). Several advanced durum lines that were developed using MAB under the Crop Development Centre, University of Saskatchewan and Agriculture and Agri-Food Canada durum breeding programs are in the registration testing stage of commercialization.

Two DNA markers have been used for selection of *Sm1* in Canadian wheat breeding programs. Of these, *XBarc35* has proven to be more useful than the alternative marker *XWM1* because it is a codominant. Most programs use *XBarc35* for selection of *Sm1*, but phenotypic selection is also used in conjunction with MAB, because phenotypic selection favors the retention of antixenotic resistance, where reduced egg laying results in fewer opportunities to detect midge damage and, therefore, allows a greater number of selections. The use of only markers (for *Sm1*) ignores these opportunities and does not differentiate any observed variation in the level of expression (allele variation or other genetic factors) that can be enhanced with additional field selection.

2.2 Grain quality

2.2.1 Protein content

To meet the requirements for cultivar release in Canada, grain protein concentration (GPC) has been maintained while concomitantly increasing grain yield (DePauw et al. 2007). This is challenging because GPC is a quantitatively inherited trait that is negatively correlated with grain yield (Steiger et al. 1996) and is greatly influenced by environmental factors, especially rate and time of nitrogen application and availability of moisture. However, there was sufficient genetic variation to increase both grain yield and GPC in CWRS (DePauw et al. 2007) and in CWAD, although maintenance of high GPC reduced yield potential by an estimated 8 to 15% in durum (Clarke et al. 2010). The negative correlation of yield and GPC has prompted research to identify other sources of high GPC.

A source of high GPC was identified in a wild population of tetraploid wheat *Triticum turgidum* L. spp. *dicoccoides* (Avivi 1978). The chromosomal region controlling high GPC from the Israeli accession FA15-3 was then successfully transferred to the hexaploid wheat cultivar Glupro (RL4352-1/ *Triticum turgidum* L. spp. *dicoccoides* accession FA15-3//Len). This cultivar exhibited high GPC but the trait was linked to low grain test weight. Later, the region responsible for elevated protein was identified on the 6BS chromosome of Glupro (Joppa et al. 1997; Mesfin et al. 1999; Olmos et al. 2003). A PCR based marker developed by Khan et al. (2000) was used to transfer *Gpc-B1* in the 6BS chromosomal region from the breeding line 90B07-AU2B (Pasqua*2/Glupro) to BW621 (DePauw et al. 2005). The DNA marker associated with the high protein content was then used to select BC_2F_1 plants from the cross BW621*2/90B07-AU2B. A line derived from this cross was eventually released as Lillian (CWRS) in 2003 (DePauw et al. 2005). DePauw et al. (2007) further reported that the chromosomal region of Lillian associated with *Gpc-B1* is smaller than its parent 90B07-AU2B and grandparent Glupro. The *Gpc-B1* gene is linked to *Yr36,* thereby also providing resistance to stripe rust (Uauy et al. 2005). Furthermore, Lillian also exhibited test weight and maturity equal to the check cultivars in the Western Bread Wheat Cooperative registration trials (DePauw et al. 2005). Lillian is one of the most widely grown common wheat cultivars in Canada and was seeded on 17.4% of CWRS area in the prairie provinces in 2011. It is

resistant to prevalent races of stripe rust in southern Alberta due to the presence of *Yr18/Lr34* and *Yr36/Gpc-B1* (DePauw et al. 2011a; Randhawa et al. 2012a). Four other cultivars that carry *Gpc-B1*, Burnside, Glencross, Somerset and Conquer, have also been released for commercial production in Canada. Burnside is a high yielding cultivar that exhibited 0.9% higher grain protein content than the check cultivars in Canadian cooperative registration trials. It matured 2 days earlier and the test weight was similar to the check cultivars (Humphreys et al. 2010b). A sequence-tagged site (STS) marker linked to *Gpc-B1* (Distelfeld et al. 2006) is now being used routinely to incorporate this gene into common wheat (spring and winter) and durum cultivars. Several common and durum wheat breeding lines are currently in pre-variety registration trials that have been selected to carry the functional *Gpc-B1* allele using available DNA markers (http://maswheat.ucdavis.edu).

2.2.2 Gluten strength

Developing cultivars of the Canadian hard white wheat class has required the selection for improved gluten strength. Overexpression of the Bx7 allele at the *Glu-B1* locus contributes to improved gluten strength properties (Ragupathy et al. 2008). Marker assisted breeding for the Bx7OE allele is underway to enhance the gene frequency of stronger gluten genotypes to improve the chances of meeting the standards (comparable dough strength and protein content with CWRS) for the Canadian hard white wheat class.

2.2.3 Cadmium content

International standards limit the concentration of the heavy metal cadmium in food products to prevent chronic toxicity in humans. North American durum has tradionally shown elevated cadmium relative to common wheat, so that low grain cadmium content has been a selection criterion in Canadian durum wheat breeding programs since the early 1990s (Clarke et al. 2010). Low grain cadmium content is regulated by a single dominant gene, *Cdu-B1,* present on the long arm of chromosome 5B (Knox et al. 2009; Penner et al. 1995) that reduces cadmium levels by 50% or more. A dominant random amplified polymorphic DNA marker (*OPC-20*) was linked with the high

cadmium allele (Penner et al. 1995). Wiebe et al. (2010) developed an EST-derived marker (*XBF474090)* that co-segregated with *Cdu1* which has since been converted to a co-dominant CAPS marker (*Usw47*) that can successfully differentiate between genotypes accumulating high and low cadmium. The durum cultivars that have low grain cadmium include the following: Strongfield, (Clarke et al. 2005), Brigade (Clarke et al. 2009a), Eurostar (Clarke et al. 2009b), CDC Verona (Pozniak et al. 2009), Napoleon (Humphreys et al. 2010a), Enterprise (Singh et al. 2010a), Transcend (Singh et al. 2012a) and CDC Vivid (Pozniak 2013). These cultivars carry a low cadmium null molecular variant for *OPC-20* cadmium marker (Penner et al. 1995). Marker-assisted selection for low grain cadmium was used in the development of Brigade, CDC Verona, and CDC Vivid. The *OPC-20* and *Usw47* markers are being employed in breeding programs to select genotypes with low grain cadmium content. Using a map-based cloning approach, several additional DNA markers have been developed, where no recombination has been detected between expression of phenotype and the marker.

2.2.4 Pasta color and lipoxygenase activity

The yellow color of pasta products is one of the main criteria used by consumers to assess pasta quality and is a desirable trait selected in Canadian breeding programs. Pasta color depends on several factors, including the semolina carotenoid (predominately lutein) content, carotenoid degradation by lipoxygenase (LOX), and pasta processing conditions. The inheritance of yellow color is complex and is controlled largely by additive gene action and is highly heritable (Clarke et al. 2006). Several QTL have been identified in both durum and hexaploid wheat on chromosomes 1A (Patil et al. 2008), 1B (He et al. 2008), 3A (Parker et al. 1998), 3B (Patil et al. 2008), 4A and 5A (Hessler et al. 2002), 2A, 4B and 6B (Pozniak et al. 2007), and 5B (Patil et al. 2008). Most of these QTL have been associated with yellow color/pigment using association mapping (Reimer et al. 2008). However, a majority of mapping studies are in agreement that the group 7 chromosomes largely influence the expression of grain pigment in wheat and durum (Pozniak et al. 2007; Singh et al. 2009). DNA markers developed from allelic variation in genes coding for two phytoene synthase genes, *Psy1-A1* (Reimer et al. 2008; Singh et al. 2009) and *Psy1-B1* (Pozniak et al. 2007; Reimer et al. 2008; Zhang and Dubcovsky 2008) have been associated with the QTL on the group 7 chromosomes, and

are being used as a selection tool for higher yellow pigment at the Crop Development Centre, University of Saskatchewan.

Lipoxygenase (LOX) activity is the major contributor of oxidative degradation of carotenoids in durum wheat (Borrelli et al. 1999) and elevated LOX is strongly associated with a reduction in yellow color of pasta (Fu et al. 2011). In durum wheat, two duplicated *Lpx1* genes (*Lpx-B1.1 and Lpx-B1.2*) have been identified on chromosome 4B (Carrera et al. 2007), and deletion of *Lpx-B1.1* is strongly associated with a strong reduction in LOX activity in semolina (Carrera et al. 2007; Verlotta et al. 2010). A DNA marker that detects the absence of *Lpx-B1.1* has been developed (Carrera et al. 2007) and is routinely used in Canadian durum breeding programs to select for low lipoxygenase activity. At the time of publication, nearly 60% of early generation breeding lines developed at the Crop Development Centre, University of Saskatchewan, lack *LpxB1.1*, and all were developed through MAB.

2.2.5 Pre-harvest sprouting

Preharvest sprouting resistance is a genetically complex and important quality trait. Preharvest sprouting, when it occurs, causes substantial losses through down grading of the grain. In durum, a number of preharvest sprouting resistance loci were identified, many of which overlap with loci found in hexaploid wheat (Knox et al. 2012). An important locus in red wheat resides on chromosome 4A (Singh et al. 2012b). One strategy with respect to selection of quantitative traits through MAB in Canadian breeding programs is to focus on those loci which appear consistently across environments in the target region of deployment and which contribute the greatest effect on the trait. These loci are considered foundational to the expression and further enhancement of the trait. The 4A locus near *Xbarc170* is one of these loci, but validation is also being performed for loci on chromosomes 1A, 1B, 5B and 7A.

2.3 Phenology

2.3.1 Earliness

Earliness or flowering time in Canadian spring wheat is important due to the very short 100 to 115 day growing season of the prairie provinces. Earliness may also protect spring wheat against various abiotic stresses including drought, frost and pre-harvest sprouting. Early maturity poses a serious challenge because it is negatively correlated with grain yield (DePauw et al. 1995; Reid et al. 2009b). Molecular characterization of 42 Canadian spring wheat genotypes demonstrated the presence of *Vrn-A1* and *Vrn-B1* in 83% and 50% of genotypes, respectively (Iqbal et al. 2007). Further studies illustrated that the *Vrn-A1a* allele is the most important for early flowering in Canadian spring wheat (Iqbal et al. 2007; Kamran et al. 2013). Molecular markers linked to alleles of *VrnA1* including *Vrn-A1a, Vrn-A1b, Vrn-A1c, vrn-A1, Vrn-A1c* and *vrn-A1* (Fu et al. 2005; Yan et al. 2004)*, Vrn-B1, vrn-B1* (Fu et al. 2005)*, Vrn-D1, vrn-D1* (Fu et al. 2005), *Vrn-B3* and *vrn-B3* (Yan et al. 2006) are being used by the University of Alberta wheat breeding program to develop early maturing cultivars and to quantify the effects of these loci.

2.4 Future directions

Wheat breeding has made significant progress during the last 50 years and it is critical that this progress continues so as to feed the ever increasing global population. In this regard, MAB offers promise to accelerate cultivar development and to produce cultivars with better pest resistance, agronomic traits and quality traits. DNA marker technology that supports MAB is progressing at a rapid pace. Many research institutes involved in wheat cultivar development and germplasm evaluation now possess essential tools/apparatus and expertise for marker genotyping and QTL analysis. Furthermore, the development of user-friendly databases like Gramene, GrainGenes and MAS wheat [(Matthews et al. 2003; Ware et al. 2002); maswheat.ucdavis.edu] will encourage the widespread use of MAB for wheat improvement.

Starting in the late 1990s, molecular markers became an important tool for Canadian wheat breeding programs. However, the lack of tightly-linked diagnostic markers, QTL x environmental interaction and prevalence of QTL background effects has

limited the application of MAB for some traits. In the future, gene based high-throughput genotyping will result in more effective genetic mapping/genome analysis and will open new avenues for its integration in wheat breeding programs globally. In particular, genomic selection (GS) is showing potential to reduce selection time and improve economic traits in crop breeding programs (Crossa et al. 2010; Heffner et al. 2009). The goal of genomic selection is to predict the breeding value of individuals, such that several cycles of selection can occur in a single year and prior to resource intensive yield testing experiments. To ensure accurate prediction of breeding value, a statistical model must first be developed on a well-genotyped training population of relevant germplasm that has been well-phenotyped in target environments. In Canadian durum wheat breeding programs, carefully selected germplasm for molecular training is comprised of locally-adapted lines that are either parents or recent ancestors of populations under selection (Pozniak et al. 2012). However, for GS to be effective, sufficient marker density to achieve genome-wide coverage is required, and is a function of linkage disequilibrium (LD) in the training population. Linkage disequilibrium decay is variable (Chao et al. 2010), and is a function of mutation rates, recombination frequency, population size and admixture. Chao et al. (2010) suggested that at least 17,500 markers would be required to cover the wheat genome at 0.2 cM intervals. Fortunately, genotype by sequencing strategies (Poland et al. 2012) and high density SNP detection platforms (Chao et al. 2010; Paux et al. 2010) have been developed for wheat with the ability to detect several thousand SNPs and is showing promise as a tool for genome wide selection strategies. However, genomic selection is not assumed to be a replacement for traditional field-based selection programs, and several strategies for implementation in current breeding programs have been well summarized (Nakaya and Isobe 2012). While genomic selection is currently being considered in Canada as a tool to assist breeders in improving selection response in wheat, it will likely not be implemented immediately until prediction models from existing data sets (Pozniak et al. 2012) are fully validated. Moreover, the application of genomic selection in wheat cultivar development may be restricted to groups that possess the germplasm and molecular resource base to implement this strategy on a large scale.

In other crops, access to a high quality reference sequence has provided a useful resource for genome-wide marker discovery. In particular, SNP markers and other

structural polymorphisms (copy number variation, presence-absence variation, insertion-deletions) can be identified from targeted re-sequencing activities and through comparative analysis with the available reference sequence (Paux et al. 2012). Currently, a reference sequence is being generated by the International Wheat Genome Sequencing Consortium (IWGS; http://www.wheatgenome.org) by generating individual physical maps and sequencing the minimum tiling path of each of the 21 hexaploid wheat chromosomes. Given the size of the hexaploid wheat genome (17 Gb), this strategy is seen as the most reasonable approach to reduce sequencing complexity and associated bioinformatics challenges, and to generate a sequence that is properly assembled and linked to existing genetic and phenotypic maps (Paux et al. 2012). This latter point may be of greatest interest to plant breeders because once relevant QTL are identified from genetic mapping and association mapping experiments, it will be possible to anchor to the corresponding physical region and the available sequence. The sequence could then be mined for useful diagnostic markers for MAB and high-resolution mapping, or to identify candidate genes for reverse genetic studies. In the context of the IWGSC, our group is contributing to the sequencing of wheat chromosomes 1AS and 6D (project Canadian Triticum Applied Genomics (CTAG); PIs C. Pozniak and P. Hucl; http://www.cantag.ca). Indeed, associating sequence variation with relevant phenotypes will still be a significant challenge in the future (Berkman et al. 2011), but access to a reference sequence is expected to provide useful tools that can be quickly applied to wheat breeding programs. A major challenge in breeding is identifying parents with known alleles that complement each other. Gene identification is taking advantage of current sequence information at an ever increasing pace. Perfect markers developed from the alleles of important genes will not only help in the selection of progeny, but more importantly will aid in the selection of parents in crosses with the greatest potential to generate progeny with favorable allele combinations. Throughput remains a challenge. The number of combinations of genes to be considered is exponential. With the potential to analyze genetically hundreds of loci, the limitation will be generating and sampling DNA from a sufficient number of individuals to identify optimum genetic recombinants. To deal with the myriad of genetic information, we will not only want to use our increased understanding of the wheat sequence to focus on recombination to generate variability, but also to understand

linkage blocks worth conserving. We will also need to develop improved approaches to non-destructive sampling of large numbers of individual genotypes.

2.5 Tables

Table 2-1. List of Canadian wheat cultivars developed using marker-assisted breeding.

Cultivar	Class	DNA marker/gene	Registration Year
Lillian	CWRS	*Yr36/Gpc-B1, Lr34/Yr18, Sst1*	2003
Burnside	CWRS	*Yr36/Gpc-B1, Lr34/Yr18*	2004
Somerset	CWRS	*Yr36/Gpc-B1*	2004
Goodeve	CWRS	*Sm1*	2007
Glencross	CWES	*Sm1, Yr36/Gpc-B1, Lr34/Yr18*	2008
Brigade	CWAD	*Cdu1*	2008
CDC Verona	CWAD	*Cdu1*	2008
CDC Vivid	CWAD	*Cdu1*	2012
CDC Desire	CWAD	*Cdu1*	2012

Table 2-2. List of CWRS class wheat cultivars and experimental lines along with their investigated gene composition.

Name/ID	Pedigree	Gene Composition
Marquis, BW1	Hard Red Calcutta/Red Fife	*LrCen, 22b, Sr7b, 18, 19, 20,*
Neepawa, BW2	CT257/CT249	*Lr13, 22b, Sr5, 7b, 9g, 12, 16, Yr7, Vrn-A1a, vrn-B1, vrn-D1*
Manitou, BW3	CT257//Thatcher*6/PI170925	*Lr13, 22b, Sr5, 6, 7a, 9g, 12, 16, Yr7,*
Canuck, BW4	Canthatch//(4351-331)CT-609/Rescue	*LrCen, Bt1,*
Sinton, BW5	Thatcher*6/Kenya Farmer/2/Lee*6/Kenya Farmer, CT262)/3/ Manitou	*Lr10, Sr5, 9g, 12, 16, Yr7,*
Napayo, BW7	Manitou*2/RL4124.1	*Lr13, 22b,*
Benito, BW20	Neepawa/3/CT433*4//Manitou/CI7090	*Lr1, 2a, 12, 13, 22b, 31,*
Katepwa, BW49	Neepawa*6/CT244/3/Neepawa*6//CI8154/2*Frocor	*Lr13, 22b, Sr5, 7a, 9b, 11, 12, 16, Vrn-A1a, vrn-B1, vrn-D1*
Columbus, BW55	Neepawa*6/RL4137	*Lr13, 16h, 22b, Sr23h,*
Pacific, BW90	BW15/BW38//RL4359/RL4353	*Lr34*, Yr18*,*
Roblin, BW92	BW15/BW38//BW40/RL4353	*Lr1, 10, 13, 34*, Sr5, 11, 12, Yr18*,*
Pasqua, BW114	BW63*2/Columbus	*Lr11, 13, 14b, 30, 34*, Sr5, 6, 7a, 9b, 12, 31, Yr9, 18*,*
AC Minto, BW120	Columbus/BW63//Katepwa/BW552	*Lr11, 13, 22a,*
AC Domain, BW148	BW83/ND585	*Lr10, 12, 16, Sr23,*
AC Cora, BW152	Katepwa/RL4509	*LrCen, 13, 21*,*
Invader, BW158	Sinton/Stoa	*Lr10, 16, Sr23,*
AC Majestic, BW173	Columbus*2//Saric70/Neepawa/ 3/Clms*5//Saric70/Neepawa	*Lr13, 16, Sr23,*
AC Splendor, BW191	Laura/RL4596//Roblin/BW107	*Lr13, 16, Sr23,*
McKenzie, BW205	Columbus/Amidon	*Lr10, 13, 16, 21*, Sr23,*
Prodigy, BW220	SWP2242/Stoa	*Lr16, Sr23,*

BW226	Sharp/BW134	*Lr16, Sr23,*
5600HR, BW238	N91-2071/AC Minto	*Lr13, 16, 22a, Sr23,*
Journey, BW243	CDC Teal//Grandin/PT819	*Lr13p, 16, Sr23,*
5500HR, BW245	N91-2381/AC Minto	*Lr16, 22a, Sr23,*
Superb, BW252	Grandin*2/AC Domain	*Lr2a, 10,*
5601HR, BW256	N93-2410/AC Majestic	*Lr16, Sr23,*
BW257	N93-2424/AC Domain	*Lr16, 23,*
Harvest, BW259	AC Domain*2/ND640	*Lr16, Sr23, Vrn-A1a, Vrn-B1, vrn-D1*
BW267	N94-2189/N92-2308	*Lr16, Sr23,*
BW270	BW165/RL4660	*Lr34, Yr18*,*
BW274	Pasqua*2/Ning 8331	*Lr34p,*
BW278	AC Domain*2/Sumai 3	*Lr16, Sr23, Fhb2?,*
BW293	Grandin*2/Caldwell	*Lr2a,*
5602HR, BW297	AC Barrie/Norpro	*Lr16, 34*, Sr23p, Yr18*, Ovp,*
CDC Alsask, BW301	AC Elsa/AC Cora	*Lr21*, 34*, Yr18*,*
Somerset, BW307	90B01-AD4D/Pasqua	*Yr36*, Gpc-B1*,*
BW310	RL4802/AC Majestic	*Lr16, Sr23,*
Alsen, BW316	ND674//ND2710/ND688	*Lr2a, 10, 13, 16, 34*, Sr23, Yr18*, Fhb1*, 5AS,*
BW317	AC Cadillac/8405-JC3C//AC Elsa	*Lr16, Sr23,*
BW330	BW 278/2*AC Superb	*Lr16, Sr23, Fhb5AS,*
BW334	9007-FB1C/AC Elsa//AC Barrie	*LrCen, 16, 34, Sr23, Yr18,*
Kane, BW342	AC Domain/McKenzie	*Lr10, 16, 21*, Sr23, pinA,*
BW343	93B42-V2A/Superb	*Lr16, Sr23, Fhb5AS,*
BW344	BW278/2*Superb	*Lr2a, Fhb5AS,*
BW345	BW230//BW174*2/Clark	*Lr16, 22a, Sr23, Sm1,*
BW346	RL 4802//(96MHN5295-1)BW 174*2/Clark	*Lr16, Sr23, pinB,*

BW353	McKenzie//(97NPI15-55)FHB5227/Lars	*Lr16, 21, 34, Sr23, Yr18,*
Waskada, BW357	BW278/2*Superb	*Lr16, Sr23, Fhb2?, Ovp*, pinA,*
BW360	McKenzie*3//BW174*2/Clark	*Lr21, Sm1,*
BW361	Augusta/HWAlpha//3*Superb	*pinB,*
Unity, BW362	McKenzie*3//BW174*2/Clark	*Lr21*, Sm1*, pinA,*
Fieldstar, BW365	McKenzie*3//BW174*2/Clark	*Lr16, 21*, Sr23, Sm1*, pinA,*
BW367	BW150*2//Tp/Tm/3/2*BW252/4/98A190/5/BW252	*Lr21, Sm1, pinA,*
BW379	95NPY-1253/Superb	*Lr16, 34, Sr23, Yr18, Fhb1, pinB,*
BW380	93B42-V2A/RL4851//BW252	*Lr2a, Fhb2,*
BW384	BW150*2//Tp/Tm/3/2*BW252/4/98A190/5/BW252	*Lr21, Sm1,*
5603HR, BW388	McKenzie//FHB5227/Lars	*Lr16, 21*, Sr23, Fhb2?,*
BW391	N95-2249/AC Domain(N99-2095)//BW763	*Lr16, 34, Sr23, Yr18,*
Shaw, BW394	Harvest/BW313 (RL4979)	*Lr34*, Yr18*,*
BW396	Augusta/HWAlpha//4*BW252	*pinA,*
Glenn, BW406	ND2831/Steele-ND	*Lr21*,*
BW407	BW252*2/94B92-Y3A	*Lr16, 22a, Sr23,*
BW408	BW267//BW257/94B92-Y3A	*Lr16, Sr23,*
BW410	McKenzie//BW257/94B92-Y3B	*Lr16, 22a, Sr23,*
BW412	BW252*2/94B92-Y3A	*Lr16, 22a, Sr23,*
BW414	Superb/98B19*J191	*Lr16, 21, Sr23, Fhb2?,*
Vesper, BW415	A/HWA//*3ACBarrie/6/BW150*2//Tp/Tm/3/ 2*BW252/4/98A190/5/Sup	*Lr21*, pinA,*
BW421	CDC Bounty/FHB9	*Lr16, 34, Sr23, Yr18,*
BW424	McKenzie/Quantum//Superb	*Lr21*,*
BW425	AC Domain/BW257	*Lr16, Sr23,*
BW428	Superb/98B19*J191	*Lr21, 34, Yr18, Fhb2?,*

Cardale, BW429	McKenzie/Alsen	*Lr21*, Fhb1*, 5AS,*
BW430	Alsen (ND716)/BW313 (RL4979)	*Lr34p, Fhb1, 5AS?, pinA, pinB,*
BW431	00H01*F57/98B19*T99	*Lr21, Fhb5AS, Sm1, pinA,*
BW432	BWS/KDT/GLO/ Selpek/Kavkas/Granat	*Lr34, Yr18, Fhb1?,*
SY433, BW433	[BW275W/N99-2587]2-	*Lr16, 21, Sr23,*
BW449	00H01*F57/98B19*T99	*Lr16, 21, Sr23, Fhb5AS,*
BW450	00H01*D26/00H04*J3	*Lr34, Yr18,*
BW451	98B19*N22//C2723/98B19*N22Lr52	*Lr16, Sr23,*
BW452	00H01*P61/98B19*T99	*Lr21, Fhb5AS, Sm1,*
BW454	HC736/98B69-R28//2*Prodigy/3/HC374/3*98B69-L47	*Lr16, 34, Sr23, Yr18, Fhb1, 2,*
BW455	98B34-T4B/98B26-N1C01B	*Lr16, 22a, Sr23, Fhb2?,*
BW461	98B34-T4B/98B50-H4D//98B50-H4D	*Lr16, 22a, 34, Sr23, Yr18, Fhb1,*
BW483	00H01*D26/BW342	*Lr16, 21, Sr23, Sm1,*
BW486	BD97/BW361G-031	*Fhb5AS,*
Leader, BW535	Fortuna/Chris	*LrCen, 34*, Yr18*, SSt*, PI,*
Kenyon, BW571	Neepawa*5/Buck Manantial	*Lr13, 16, Sr23,*
Lancer, BW572	Fortuna/Chris	*LrCen, 14a, 27, 34*, Sr2, 9d, 17, Yr18*,*
Laura, BW593	BW15/BW517	*Lr1, 10, 34*, Yr18*, PI, Vrn-A1a*
CDC Teal, BW616	BW514/Benito//BW38	*Lr1, 13, 34*, Yr18*,*
CDC Merlin, BW636	RL4386//BW525/Columbus	*Lr16, Sr23,*
AC Eatonia, BW642	Leader/Lancer	*LrCen, 34*, Yr18*,*
AC Barrie, BW661	Neepawa/Columbus//Pacific	*Lr13, 16, Sr23,*
AC Elsa, BW685	Pacific/Laura	*Lr1, 10, 34*, Yr18*, PI,*
AC Cadillac, BW689	Pacific*3/BW553	*Lr27, 34*, Sr2*, SrCad*, Yr18*, Bt10*,*
AC Abbey, BW691	BW608/93464//BW591	*SSt*, PI,*

AC Intrepid, BW693	Laura/RL4596//CDC Teal	*PI,*
CDC Bounty, BW720	Katepwa/W82624//Kenyon	*Lr13, 34*, Yr18*,*
CDC Imagine, BW758	CDC Teal*4/FS2	*Lr34*, Yr18*, Als1*,*
Lillian, BW776	BW621*3/90B07-AU2B	*Lr34*, Yr18*, 36*, SSt*, Gpc-B1*,*
Infinity, BW799	Kulm/8405-JC3C//AC Elsa	*Lr16, Sr23,*
CDC Abound, BW824	Superb*2/BW755	*Als1*,*
Goodeve, BW841	98A-164-B/AC Intrepid	*Lr16, Sr23, Sm1*,*
BW852	W98085/AC Barrie	*Lr16, Sr23,*
BW853	BW248/AC Elsa	*Lr22a, 34, Yr18,*
Stettler, BW867	Prodigy/Superb	*Lr16, Sr23,*
Carberry, BW874	Alsen/Superb	*Lr16, 34*, Sr23, Yr18*, Fhb1*,*
Muchmore, BW875	Alsen/Superb	*Lr34*, Yr18*,*
CDC Stanley, BW880	W95132/AC Barrie	*Lr37*, Sr38*, Yr17*,*
CDC Kernen, BW881	CDC Bounty/FHB4	*Lr34, Yr18,*
BW882	BW661//BW749/W95132	*Lr16, Sr23,*
CDC Utmost, BW883	AC Elsa//CDC Teal/Seneca DH#10	*Lr34, Yr18, Sm1*,*
BW897	Prodigy/2*Alsen	*Lr16, 34, Sr23, Yr18, Fhb1*, 5AS,*
BW314	RL4763*2/Howell	*Lr34, Yr18, Sm1,*
HW341	BW275/Sunmist//Snowbird	*Lr16, Sr23,*
Lovitt, PT205	8405-JC3C*2/AC Cora	*Lr16, 21*, Sr23,*
Helios, PT211	BW674/AC Cadillac//AC Barrie	*Lr16, Sr23,*
Peace , PT416	BW165/RL4660	*Lr1, 13, 27, 34*, Sr2*, SrCad*, Yr18*, Bt10*, Vrn-A1a*
PT459	BW314a/Peace	*Lr34, Yr18, Sm1, PPO18,*

CDC Osler PT555	AC Cora/PT534	*LrCen, 21*, 34*, Yr18*,*

Lr = Leaf rust, *Sr* = Stem rust, *Yr* = Strip rust, *Bt* = Bunt resistance, *Fhb* = Fusarium head blight, *Gpc* = Grain protein content, *Cdu* = Cadmium content, *Sm1* = Midge resistance, *Vrn* = Vernalization, *Sst* = Solid stem (sawfly resistance), *Ovp*= ovipositor, *Als*= Acetolactate synthase, *Pin*= Puroindoline, *PI, =Photo Insensitive, PPO* = Polyphenol oxidase.*-presence of gene confirmed by genetic analysis.

Table 2-3. Markers employed to develop new wheat cultivars in Canada.

Trait	*Locus*	Marker Name	Size (bp)	Ch	Reference
Biotic Stress					
Leaf rust	*Lr21*	*Ksu-D14*	885	1DS	Talbert et al. (1994)
	Lr22a	*Gwm296*	110		*
	Lr32	*Wmc43*	300		*
		Barc135	262		
	Lr34	*csLVLr34*	150+		Lagudah et al. (2006)
		cssfr1	229-517	7DS	Lagudah et al. (2009)
		caIND11	394		Dakouri et al. (2010)
	Lr46	*csLV46*			*
		Wmc44			
Stem rust	*Sr2*	*Gwm533*	*120*		*
		stm598tcac	*61*		*
		stm559gag	*85*		*
	Sr2	*csSr2*	225,112-172,*112,53+*		*

	SrCad	*FSD-RSA*	*275*	6D	Hiebert et al. (2011)
		cfd 49-F	180, 212		
		cfd 49-R			
	Sr39	*Sr39-F2*	900		Gold et al. (1999)
		Sr39-R3			
		Sr39#22r-F	487		Mago et al. (2009)
		Sr39#22r-R			
	Sr30	*CFD12-F*			*
		CFD12-R			
	Sr40	*Wmc344-F*			(Wu et al. 2009)
		Wmc344-R			
		Wmc474-F			
		Wmc474-R			
	Lr24-Sr24	*12-F*	500		Mago et al. (2005)
		12-R			
Stripe rust	*(Lr37-Yr17-Sr38)*	*VENTRIUP/*	259	2AS	Helguera et al. (2003)
		LN2			
		URIC/LN2	285		
			275		
FHB	*Fhb: Qfhs.ndsu-3BS*	*Gwm493*	290	3BS	*
		Gwm533	140		
		STS 142			
		UMN10			Liu et al . (2008)
	Fhb2	*Wmc398*		6B	Cuthbert et al. (2007)

		Gwm133			
		Gwm644			
	Fhb-5AS	*Gwm293*		5AS	McCartney et al. (2004)
Common bunt	*Bt10*	*FSD-RSA*	*275*	6D	Laroche et al. (2000)
	Blizzard	*Gwm374*		1B	Wang et al. (2009)
		Gwm264			
		Barc128			
	McKenzie	*Gwm573*		7B	Knox et al. (2013)
		Wmc17			
Loose smut	*Utd1*	*Gwm234*		5BS	Randhawa et al. (2009)
		Gwm443			
Ergot	Unpublished				Unpublished
Insect Resistance				3BL	
Wheat stem sawfly	*Qss.msub-3BL* *Qsf.spa.3B*	*Gwm247*	145	3BL	Cook et al. (2004) Houshmand et al. (2007)
		Gwm114		3BL	

Orange wheat blossom midge	*Sm1*	*WM1*	232	2BS	Thomas et al. (2005)
		Barc 35	351		
Quality					
Gpc-B1* and *Yr36		*Ucw108*	217	6BS	Uauy et al. (2006)
		Uhw89	126		Distelfeld et al. (2006)
HMW glutenin	*Bx7*	*Bx7OE*	562	1B	Ragupathy et al. (2008)
Low cadmium	*Cdu1*	*Usw47*	345	5BL	Wiebe et al. (2010)
	Cdu1	*ScOPC20*	394	5BL	Knox et al. (2009)
Lipoxygenase					
	Lpx-B1	*LOXA*	900		Carrera et al. (2007)
Waxy starch	*Wx-A1*	AFC	389		Nakamura et al. (2002)
		AR2	370		
	Wx-B1	BDFL	425		
		BRD	-		
	Wx-D1	BDFL	2307		
		DRSL	1731		
Preharvest sprouting resistance		*Gwm397*		4A	Singh et al. (2012b)
		Wmc650			
		Barc170			

Earliness					
Verbalization	*VRN-A1*	Intr1/A/F2: Intr1/A/R3	1170		Fu et al. (2005)
	VRN-B1	Intr1/B/F: Intr1/B/R3:	709	5BL	
	VRN-D1	Intr1/D/F: Intr1/D/R3	1671	5DL	Fu et al. (2005)

* source: http://maswhat.ucdavis.edu Ch = chromosome

2.6 Figures

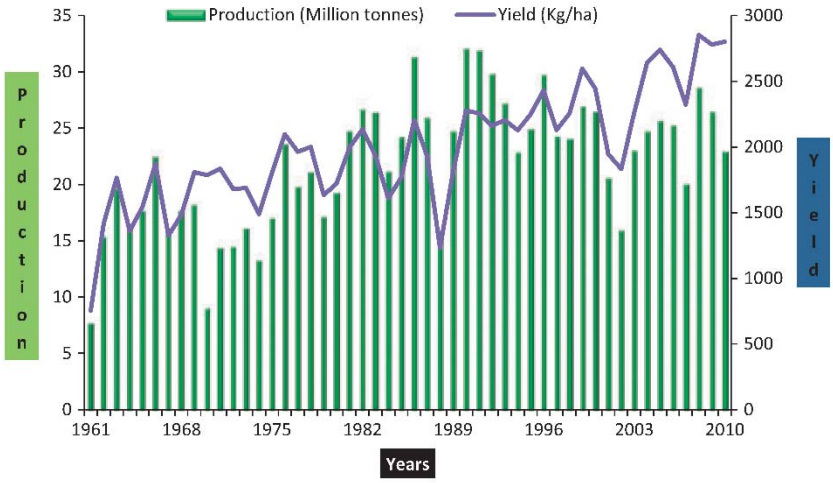

Fig. 2-1. **Trend of wheat production and yield in Canada during last 50 years.**

Source (FAOSTAT 2010)

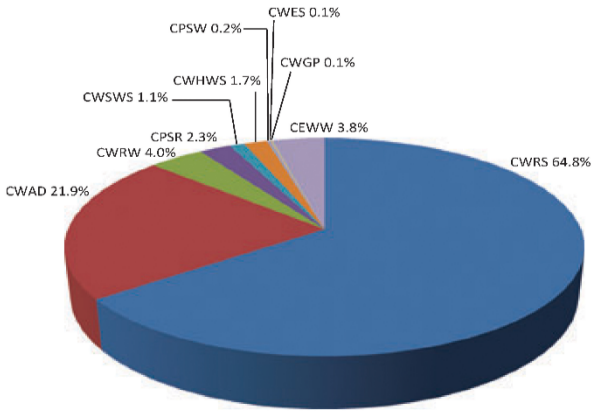

Fig. 2-2. **Percentage of total seeded area for wheat market classes in Canada from 2005-10.**

Source: (Canadian Wheat Board Variety Survey Report, 2011)

3.0 Mapping QTL, genetic differentials and the effect of *Rht-B1* under organic and conventionally managed systems in the Attila × CDC Go spring wheat mapping population

3.1 Introduction

Weeds are one of the major production constraints in organic systems and are responsible for significant grain yield losses. Wheat (*Triticum aestivum*) covers the largest area under any single crop in the world and thus contributes towards the use of huge amount of herbicides/pesticides for optimum production. In organic management systems, lack of efficient and effective weed management strategies are considered the major constraint to grain production (Gianessi and Reigner 2007; Hiltbrunner et al. 2002; Hiltbrunner et al. 2007; Wszelaki et al. 2007). Weed control in organically managed lands is challenging due to a variety of reasons. Firstly, application of synthetic herbicides is against the norm of organic agriculture (Kruidhof et al. 2008). Secondly, only a few herbicides have been approved that are costly, nonselective and potentially harmful to crops (Knezevic 2009). Thirdly, hand weeding is expensive (price varies from \$300-800 ha^{-1}), time consuming and laborious (Kruidhof et al. 2008). Lastly, herbicide application may result in herbicide tolerance in weeds (Preston et al. 1999; Coleman et al. 2001). Therefore, breeding for improved weed suppression/competitive ability is gaining enormous importance among wheat breeders to minimize yield losses in both conventional and organic systems.

The presence of genetic variability is a prerequisite for improvement of any trait. Previous studies have reported the existence of considerable genetic variation for competitive ability among wheat cultivars. Wheat exhibited poor competitive ability against annual ryegrass (*Lolium rigidum*) as compared to barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), canola (*Brassica napus* L.), rye (*Secale cereale* L.) and triticale (x *Tritticosecale*) (Lemerle et al. 1995). In a study to determine the competitive ability of 63 historical and modern wheat cultivars, it was reported that wheat cultivars with a high competitive ability suppressed weed biomass by 573% than cultivars with lowest weed suppression ability (Wissuwa et al. 2009). Mokhtari et al. (2002) studied the genetic

basis of variation for tolerance against annual ryegrass (*L. rigidum*) in two $F_{2:3}$ wheat populations derived from crosses between locally adapted Australian wheat cultivars with good and poor competitive abilities. They found significant genetic variation for competitive ability in both wheat populations with heritability estimates of 0.57 and 0.27 for percentage of yield losses (tolerance) in late and early crosses, respectively.

Studies on the relationship between agronomic/morphological traits and their effect on competitive ability with weeds have been well documented in cereals. Traits that can contribute to competitive ability include early season vigor , plant height (Huel and Hucl 1996), flowering and maturity time (Mokhtari et al. 2002), light interception (Lemerle et al. 1994; Reid et al. 2009b) and number of tillers (Lemerle et al. 1996a; Lemerle et al. 1996b). Plant height has a profound effect on wheat grain yield and is also associated with competitive ability of wheat. Taller plants exhibit better competitive ability against weeds than shorter ones mainly due to better light interception that directly affects photosynthetic activity of crop plants (Cousens et al. 2003a; Cousens et al. 2003b; Gooding et al. 1993; Mason et al. 2007b; Cudney et al. 1991). Huel and Hucl (1996) reported a significant positive correlation between plant height and competitive ability. They observed that shorter cultivars suffered greater yield losses than taller cultivars while growing with weeds in a competitive environment. The effect of height reducing *Rht* genes in wheat was investigated in an organic environment (Gooding et al. 1997). The authors found a greater weed (*Alopecurus myosuroides*) infestation in wheat genotypes with *Rht* genes and attributed it to the reduction in shading ability of short statured wheat plants that allowed an increased penetration of photosynthetically active radiation through wheat plants. In order to be competitive, short statured wheat genotypes must have erect leaves with higher leaf area to maximize light interception (Watson et al. 2006). Early season vigor is another important trait that contributes to competitive ability. Early season vigor is highly dependent on relative growth rate (*i.e.*, ability to increase biomass per unit of time) of crop plants (Grime 1979). To breed for organically managed lands, plant traits that efficiently utilize resources during early growth stages and improved competitive ability are required. These traits include greater photosynthetic active radiation (PAR), increased biomass and tillering capacity. In a population derived from early flowering parents, Mokhtari et al. (2002) reported that the effectiveness of indirect selection based on total dry weight and number of

heads per plants in monoculture was higher than direct selection. On the other hand, none of the traits had high heritability or a correlation with weed tolerance in a population derived from late flowering parents that was required to achieve greater results from direct selection. Moreover, a positive correlation between time to anthesis and percentage yield losses in both populations suggested that earliness is linked to competitiveness/tolerance (Mokhtari et al. 2002).

The environment influences the expression of various agronomic/morphological traits which confer competitive ability. Such traits are quantitative in nature which makes it difficult to breed wheat cultivars with enhanced competitive ability. Nevertheless, a number of studies have been conducted to identify quantitative trait loci (QTL) for traits conferring competitiveness so as to facilitate the development of improved cultivars. In one such study, QTL for various competitive ability traits including plant height, flag leaf size, anthesis and size of first two leaves have been reported in a population of 161 doubled haploid (DH) lines derived from a cross between Cranbrook and Halbred. The QTLs were mapped on similar positions for two years at chromosomes 2B and 2D (Coleman et al. 2001). In another study, Steege et al. (2005) reported 85 QTLs controlling early season vigor on chromosomes 1D, 2D, 4D, 5D and 7D of *Aegilops tauschii*, the D genome donor of hexaploid wheat. Spielmeyer et al. (2007) reported that early season vigor was associated with greater seedling leaf area and longer coleoptiles in wheat and found a QTL on chromosome 6A that explained 6 and 14% of the phenotypic variation for seedling leaf width and coleoptile length, respectively. The SSR marker “NW3106” was linked to the QTL, and can be used in marker assisted breeding (MAB) for coleoptile length in early generations to select lines for increased early season vigor.

Our group started field experiments exploring the competitive ability of spring wheat on 4 ha land that has been organically managed in Edmonton, AB Canada since 1999 (Asif et al. 2012; Mason et al. 2006; Mason et al. 2007a; Mason et al. 2007b; Reid et al. 2009a; Reid et al. 2011; Reid et al. 2009b). Our previous findings provided a basis for the present study, designed to i) uncover differences in heritability, selection response and genetic gains between organic and conventional management systems, ii) identify and map genomic regions (QTL) associated with various agronomic traits iii) compare and uncover QTL behaving differently in both systems, and iv) examine the

effect of *Rht-B1* on various traits in both systems in order to assist the development of competitive and high yielding wheat cultivars for organically managed lands.

3.2 Materials and Methods

3.2.1 Population Development

A population of 163 recombinant inbred lines (RILs) derived from a cross between the CIMMYT spring wheat cultivar Attila and the Canadian spring wheat cultivar CDC Go was used in the present study. The original population consisted of 171 RILs, but genotyping of 163 RILs were received from Diversity Arrays Technology (DArT) Pty. Ltd., Yarralumla, Australia. CDC Go is a hollow-stemmed, short-strawed and high yielding wheat cultivar with high grain protein content. It belongs to the Canadian Western Red Spring (CWRS) class and was grown on 5.9% of the wheat production area of the Canadian Prairies regions in 2010 (Canadian Wheat Board Survey Report, 2010). In three years of testing in the Western Bread Wheat Cooperative Test, CDC Go was reported to be lodging resistant, had medium height (85 cm), was high yielding (3.62 t ha^{-1}), early maturing (100.3 days) with high test (80.3 kg hl^{-1}) and kernel weight (42 g). Attila is an awned, semi-dwarf bread wheat cultivar widely grown in Southeast Asia (Rosewarne et al. 2008). Reid et al. (2009b) reported that Attila was high yielding (5.34 t ha^{-1}), semi-dwarf (84 cm) with average maturity for the regions tested (135 days). The population consisted of 163, F_4 derived F_6 genotypes (RILs), which were advanced to F_4 through single seed descent (SSD) to a point of near homozygosity. The RILs and two parents were planted in double head rows to multiply seed for experimental use as F_7.

3.2.2 Phenotyping

Field experiments were conducted at the Edmonton Research Station (ERS), University of Alberta, South Campus, Edmonton, Alberta, Canada (latitude: 53'34^0 N, longitude: 113' 31^0 W, elevation: 723.3 m) from 2008 to 2010 in organically and conventionally managed lands. In all three years, the trials were conducted on paired sites, one organically and one conventionally managed, located approximately 500 m apart on the same soil and with the same weather conditions. Sowing was done on May 9th, 13th and 18th at the organically managed site and on May 9th, 13th and 17th at the conventionally managed site in 2008, 2009 and 2010, respectively. Experiments were harvested on September 19th (organic) and September 29th (conventional) in 2008;

September 2-3rd (organic) and September 9th (conventional) in 2009 and on September 23rd (organic) and September 18th (conventional) in 2010. The experiments were arranged in randomized incomplete blocks within each of three (2008 and 2009) and two (2010) replications. The plots were seeded with 300 viable seeds m^{-2}. The plot (5.4 m^2) consisted of six rows 4m in length with a row spacing of 22.5 cm between rows.

The three year crop rotation on the conventionally managed site was wheat-pea-wheat, whereas on the organically managed site, wheat followed rye plow-down as green manure. On the conventionally managed site, fertilizer (11-52-0 N-P_2O_5-K_2O) was banded with seed at the time of planting at a rate of 36, 40 and 40 kg ha^{-1} in 2008, 2009 and 2010, respectively. The organically managed site did not receive any chemical fertilizer or herbicide. No compost was applied on the organically managed site. Soil nutrient levels of N, P, K and S were 34, 60, 555, and 20 mg kg^{-1}, respectively. Thus, all nutrient level were optimum except N which was reported to be marginal. Soil pH, electric conductivity and organic matter were 6.7 (neutral), 0.70 dS m^{-1} (good) and 10.8 % (high), respectively. The soil at both sites is an Orthic Black Chernozem which is typical of central Alberta (AAFRD 2004). Other agronomic practices were carried out as needed during the growing seasons to obtain a good/even crop stand. Precipitation and temperature data for each year was obtained from Edmonton Canada data archive (ftp://arcdm20.tor.ec.gc.ca/pub/dist/CDCD/).

Data were collected for days to flowering, physiological maturity, plant height, test weight, kernel weight, number of tillers, grain protein content, weed biomass, light capture and grain yield. Days to flowering were recorded as the number of days from seeding to when 75% of the spikes in a plot had visible peduncles. Physiological maturity was recorded as the number of days from seeding to a point when 75% of the peduncle in the plot had lost green color and turned to yellow/brown. Plant height was recorded on a plot basis at the completion of stem elongation from the soil surface to the tip of spike excluding awns. Test weight was calculated by weighing a 1-pint (473 ml) subsample of plot yield. Number of tillers m^{-2} was calculated by counting fertile tillers from a randomly chosen 0.5 m length of the centre of two rows. Grain protein content was determined using Near Infrared Reflectance (NIR) spectroscopy using a Monochromator NIR system model 6500 (NIRSystems, Inc., Silver Springs, MD, USA). Weed biomass was recorded to measure suppressive ability only in organically managed

plots. For this, weeds samples were taken at physiological maturity and were dried for 3 days at 50 ^{0}C to determine dry weight. The harvested grain samples were dried at 60^{o}C for ~24 hours and grain yield on a plot basis was determined by weighing the clean seeds/sample. Photosynthetically active radiation (PAR) was recorded only in conventionally managed plots using LI-COR LI-191SA Line Quantum Sensor (LI-COR Bioscience, Lincoln, NE). The Line Quantum Sensor was held in the middle of field plot at ground level and above the crop canopy with PAR recorded in µmol s^{-1} m^{-2}. The proportion of captured light was calculated using following formula:

$$Light\ Capture = 1 - \frac{PAR\ Below\ Canopy}{PAR\ Above\ Canopy}$$

3.2.3 Genotyping

Genomic DNA of 171 RILs along with parents was extracted from the leaves of 3 weeks old seedlings using DArT protocol (http://www.diversityarrays.com). DNA concentration of each sample was measured using a NanoDrop (ND-1000) spectrophotometer, adjusted to 100 ng/µl and shipped to Diversity Arrays Technology Pty. Ltd., Yarralumla, Australia for genotyping against 7000 cloned sequences. Initially 718 markers were found polymorphic, however, 136 markers were either redundant or distorted, thus discarded. Approximately 582 markers were found polymorphic within the 163 RILs. The number of polymorphic markers for each chromosome is given in Fig. 3-1. The DArT technology followed protocols similar to those illustrated by Akbari et al. (2006).

3.2.4 Statistical analysis

Phenotypic data were first tested for normality and homogeneity of the error variances using Kolmogorov–Smirnoff and Levene's tests, respectively (Steel et al. 1997). Analysis of variance (ANOVA) for all traits was performed using PROC MIXED in SAS (SAS 2003). Data were analyzed for individual years (2008, 2009, and 2010) in organically and conventionally managed sites separately and then combined over years. PROC MIXED of SAS was used for better estimation of least square means (LS Means) (Yang 2010), where lines/RILs were used as fixed effects, and years, replications, blocks within replications as random effects.

Broad-sense heritabilities were calculated on plot a basis as: $H = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE}^2 + \sigma_e^2)$, where σ_G^2, σ_{GE}^2 and σ_e^2 are among lines, line × environment and error variances, respectively . Genetic correlations among the traits were calculated using the multivariate restricted estimation of maximum likelihood (REML) method applying the MIXED procedure of SAS (Holland 2006). The estimated genetic ($\hat{r}_{g(xy)}$) and phenotypic correlations ($\hat{r}_{p(xy)}$) between traits *x* and *y* are given as follow:

$$\hat{r}_{g(xy)} = \frac{\hat{\sigma}_{G(xy)}}{\sqrt{\hat{\sigma}^2_{G(x)} \cdot \hat{\sigma}^2_{G(y)}}},$$

$$\hat{r}_{P(xy)} = \frac{\hat{\sigma}_{G(xy)}}{\sqrt{\hat{\sigma}^2_{P(x)} \cdot \hat{\sigma}^2_{P(y)}}} = \frac{\hat{\sigma}_{G(xy)} + \hat{\sigma}_{GE(xy)} + \hat{\sigma}_{e(xy)}}{\sqrt{\hat{\sigma}^2_{G(x)} + \hat{\sigma}^2_{GE(x)} + \hat{\sigma}^2_{e(x)}} \cdot \sqrt{\hat{\sigma}^2_{G(y)} + \hat{\sigma}^2_{GE(y)} + \hat{\sigma}^2_{e(y)}}},$$

Where $\hat{\sigma}_{G(xy)}$, $\hat{\sigma}_{P(xy)}$, $\hat{\sigma}_{GE(xy)}$ and $\hat{\sigma}_{e(xy)}$ are the estimated genetic, phenotypic, genotype x environment and error co-variances, respectively, between the two traits *x* and *y* whereas $\hat{\sigma}_G^2$, $\hat{\sigma}_P^2$, $\hat{\sigma}_{GE}^2$ and $\hat{\sigma}_e^2$ are estimated genetic, phenotypic, genotype x environment and error variances calculated for both traits (Holland 2006). The genotypic correlation coefficients were then transformed as suggested by Fisher (1925) : $Z_{xy} = [\ln(1 + r_{xy}) - \ln(1 - r_{xy})]/2$ to test whether correlation coefficients are significantly different from zero. The new variable Z_{xy} was tested under the null hypothesis of no correlation with $Z' = \frac{Z_{xy}}{1/\sqrt{n-3}}$ where n is the number of recombinant inbred lines.

3.2.5 QTL detection/analysis

Composite interval mapping (CIM) was carried out using QTL Cartographer (WinQTL) version v2.5.010 (Wang et al. 2010) to detect/identify QTL influencing each trait using LS Means of each trait individually for each site (organic and conventional) for three years (2008, 2009, and 2010) and then combined over years. The overall

significance level for declaring a definitive QTL was obtained using the permutation test (Churchill and Doerge 1994) wherein the analysis was replicated 1,000 times on data sets generated by random reshuffling of the trait values in the original data. The chromosome scan or walk for detecting QTL was carried out at an interval of 1 cM at a time for all traits under study. The position at which the peak of LOD scores reaches the maximum was used to declare a QTL location. The percentage of phenotypic variation explained by a QTL was calculated using the coefficient of determination (R^2) based on the marker that is closest and thus linked to the QTL. The QTL identified in the study were named according to the guidelines illustrated in the catalogue of gene symbols for wheat (McIntosh et al. 2003).

3.3 Results

3.3.1 Phenotypic evaluation

Weather data of experimental location at Edmonton is presented in Fig. 3-2 and 3-3. Temperature was normal during the wheat growing season over three years of experimentation (Fig. 3-2), whereas total precipitation varied significantly (Fig. 3-3). The total precipitation during 2008, 2009 and 2010 was 182, 133, 411 mm, respectively. From 50 years average precipitation data, the highest rainfall in Edmonton occurs during the month of July and that was true in 2009 and 2010 where 66 and 147 mm of rain fall received, respectively (Fig. 3-3).

On average, the parents (Attila and CDC Go) yielded more grain, were taller with higher test and kernel weights under conventional than organic systems (Table 3-1). The parents also took more days to mature under conventional systems. Grain protein content of Attila was higher in organically managed systems. Differences between the two parents were observed for plant height (11 cm), test weight (2 kg hl^{-1}), kernel weight (5 g), early season vigor (1), days to flowering (3), weed's biomass (20 g m^{-2}) under organic, and for test weight (2 kg hl^{-1}), grain protein content (2.1 %), early season vigor (1) and days to flowering (3) in conventional management systems. The mapping population (n=163) differed for all traits except days to flowering between the two management systems (Table 3-1). Transgressive segregation was observed in the population for all traits under conventional as well as organic management systems. The frequency distribution of grain yield and plant height for RILs showing transgressive

segregation is presented in Fig. 3-4. Heritability estimates were found to be different for grain yield (37% and 18%), tillers (32% and 16%), plant height (58% and 43%), kernel weight (27% and 44%) and grain protein content (64% and 27%) between conventional and organic management systems. (Table 3-1).

Spearman's rank correlations between the two management systems showed a high correlation for kernel weight (0.70), early season vigor (0.80) and days to maturity (0.74), a moderate correlation for tillers (0.51), test weight (0.60) and days to flowering (0.54), but a low correlation for grain yield (0.20), plant height (0.10) and grain protein content (0.24) (Table 3-2). Direct selection in conventional and organic management systems (up to 15% selection intensity) resulted in 50% or less lines/RILs selected in common (except for flowering time) for nine studied traits. For instance, if the top yielding 9 (at 5% selection intensity), 17 (at 10% selection intensity) and 26 (at 15% selection intensity) RILs of the population were selected from each management system, only half or even less lines (3, 5 and 13, respectively) were in common when selection was based on both systems (Table 3-2; Fig. 3-5). This suggests that differences exist between conventional and organic management systems for the RILs/lines studied. More precisely, the lines showed a crossover interaction between the two management systems.

Phenotypic and genotypic correlation coefficients among different traits were estimated for each management system separately (Table 3-3 and 3-4). Grain yield was moderately correlated with plant height (r_g = 0.44) and days to maturity (r_g = 0.49) in conventionally managed systems. Negative genotypic and phenotypic correlations were found between grain yield and grain protein content in both management systems. Similarly, genotypic correlations of early season vigor with both days to flowering and maturity were negative in both management systems. Negative genotypic correlation was found between plant height and number of tillers in conventional (r = -0.74) as well in organic (r = -0.39) management systems. Significant negative genetic correlation of weed biomass with plant height (r = -0.98), grain yield (r = -0.64) and test weight (r = -0.57) was observed in organically managed land. Significant positive genotypic correlation between days to flowering and maturity was found in both conventional (r = 0.91) and organic (r = 0.92) management systems. Light capture (measured only in the conventional system) showed positive genotypic correlation with plant height (r = 0.57),

kernel weight (r = 0.58). Low to moderate genotypic correlations between organic and conventional management systems for grain yield were observed in 2008, 2009 and 2010; however, a high genotypic correlation was found between same systems over three years (Table 3-5).

3.3.2 Marker analysis, map construction and QTL detection

In total, 582 polymorphic DArT markers (of 7000 clones) were used in the Attila x CDC Go population. The linkage map was constructed by DArT (http://www.diversityarrays.com). Initially, 718 markers were found polymorphic; however, 136 DArt markers were either distorted or redundant and were thus discarded. The map spanned a total distance of 1831.16 cM (A genome = 651.49 cM, B genome = 782.74 cM and D genome = 396.93 cM) and covered 19 wheat chromosomes with an average distance of 3.14 cM between the markers. Average map length was 96.37 cM/chromosome. A total of 46 QTL were found (5 for grain yield, 1 for number of tillers, 8 for plant height, 6 for test weight, 9 for kernel weight, 6 for grain protein content, 1 for early season vigor, 4 for days to flowering, 5 for days to maturity and 1 for light capture) in various management x year environments (Table 3-6). Out of these 46 QTL, 6 consistent QTL were mapped on chromosomes 6A, 1B, 3A and 5B for grain yield, test weight, kernel weight and days to flowering, respectively (Table 3-7).

3.3.3 Effect of *Rht-B1* in conventional and organic management systems

After detecting a consistent QTL (QHt.dms-4B) for plant height over various management systems and years, the parents along with the population were screened for *Rht-B1* locus on chromosome 4B using the PCR-based markers identified by Ellis et al. (2002) and the population was found segregating for this locus. In conventional management system, lines carrying wild type (tall) and mutant (shorter) alleles behaved similarly for all traits except plant height, whereas lines possessing a wild type allele produced more grain yield (3.73 t ha^{-1}), had higher grain protein content (14 %), suppressed weed biomass (5.2 g m^{-2}) and were taller (70 cm) than mutant types (Table 3-8) in the organic management system. The wild type and mutant alleles also behaved differently in both systems for various traits except for days to flowering and early season vigor. Less grain yield reduction was observed in wild types (1.11 t ha^{-1}) as compared to mutant types (1.71 t ha^{-1}). A similar trend occurred for number of tillers,

plant height and test weight. Lines with the wild type allele had 1.1% higher grain protein content and matured 5 days earlier in organic management systems, whereas grain protein content of mutant types between the two systems remained the same but matured 6 days earlier (Table 3-8).

3.4 Discussion

The Attila × CDC Go mapping population was studied in organic and conventional management systems to investigate genetic differences among lines, map genomic regions controlling agronomic and quality traits and investigate the effect of the *Rht-B1* locus on other traits. Here, we report four main findings: i) heritability estimates differed between the two systems for 5 of the 11 measured traits, ii) direct selection in each management system resulted in fewer lines selected in common for 9 traits, iii) 46 QTL were found (5 for grain yield, 1 for number of tillers, 8 for plant height, 6 for test weight, 9 for kernel weight, 6 for grain protein content, 1 for early season vigor, 4 for days to flowering, 5 for days to maturity and 1 for light capture) in various environments (management systems and years) and out of these 46 QTL, 6 consistent QTL were mapped on chromosomes 6A, 1B, 3A and 5B for grain yield, test weight, kernel weight and days to flowering, respectively, and iv) the effect of *Rht-B1* was more pronounced with respect to grain yield, tillers, test weight and kernel weight in organic than conventional management systems.

The genotypic correlations between traits are due to pleiotropy and/or genetic linkage. It represents the direction and magnitude of correlated response to selection as well as the relative efficiency of indirect selection (Holland 2006). In the case of highly correlated traits, plant breeders have the opportunity to make a direct selection for traits with high heritability to maximize genetic gain in a segregating population. In our study, heritability and correlation estimates differed between management systems. Overall, grain yield, number of tillers, plant height and grain protein content exhibited higher heritability estimates in conventional than organic management systems. The opposite trend was noted for kernel weight. Heritability estimation offers an opportunity to estimate gain from selection for a particular trait. High heritability estimates were recorded only for days to flowering in both systems, indicating higher genetic gain is possible for flowering time when selection is done on either system. It

has also been previously reported that indirect selection i.e., selection in an environment different from the target environment, can lead to higher genetic gains for highly heritable traits than direct selection (Hill et al. 1998). Thus, direct selection is highly desirable for low heritability traits. Moreover, direct selection in each management system resulted in few RILs in common at 5% selection intensity. For example, grain yield, number of tillers, plant height and early season vigor are considered important traits conferring competitive ability and had only 3, 2, 4 and 1 RIL in common at the 5% selection intensity. Spearman's rank correlation values for all traits except early season vigor were below 0.80 and we also found low to moderate genetic correlations for complex traits like grain yield between organic and conventional management system over a period of three years. These results suggest that the two systems were quite different for these traits. Thus, indirect selection in conventionally managed systems would be less efficient than direct selection in organic systems to identify superior genotypes. In a study to evaluate 800 barley breeding lines in low and highly yielding environments, Ceccarelli and Grando (1991) reported that the best lines selected in low yielding environments outperformed superior lines selected in high yielding environments. In a similar study on a large number of German variety trials, substantial differences were found in the varietal rankings that were grown under high input, low input and organic conditions (Baresel and Reents 2006). In another study, Mason et al. (2007b) tested 27 spring wheat cultivars in organic and conventional management systems in Canadian environment and reported 63% more grain yield in conventional than organic system. The study further reported that a competitive spring wheat ideotype for organically managed lands should be taller in height, early maturing, have fast early season growth and elevated fertile tiller number. Breeding on organic lands exclusively use organic conditions and major differences from conventional lands include weed competition and limited availability of nitrogen. Only a few cereal breeders make direct selection on organic lands. For example, the Association of Biodynamic Plant Breeders (ABDP) have made direct selection on organic lands and released 12 cultivars exclusively for organic farming. These cultivars were mostly taller, had low harvest indices and higher grain protein content than cultivars released for conventional farming (Wolfe et al. 2008). Similar findings of direct selection under low input farming have been reported in other cereals like *Zea mays* (Burger et al. 2008),

Hordeum vulgare (Ryan et al. 2008), *Avena sativa* (Atlin and Frey 1990) and *Oryza sativa* (Mandal et al. 2010). On the basis of our findings, we conclude that indirect selection of spring wheat lines on conventionally managed systems may not be useful for organically managed systems. Therefore, wheat breeding programs for organic systems need to select promising lines directly on organic lands.

We also subjected the Attila × CDC Go population to genome wide QTL analysis to uncover putative QTL for traits conferring competitive ability. The grain yield QTL identified in this study are positioned between 82.3 and 93.9 cM on chromosome 6A and most probably are the same QTL previously reported. Heidari et al. (2011) mapped three grain yield QTL on chromosome 6A that explained up to 20.9% phenotypic variation for grain yield in wheat. In a similar study, Baenziger et al. (2011) developed a full set of chromosome substitution lines using two historically important wheat cultivars, Wichita and Cheyenne. They reported a grain yield advantage of 19% and 14% when Wichita chromosomes 3A and 6A were substituted for Cheyenne chromosomes, respectively. Reciprocal substitution lines showed the opposite trend (e.g., grain yield reduction of 17% and 23% for 3A and 6A chromosomes, respectively) leading to the conclusion that these chromosomes contain important genomic regions influencing grain yield in wheat.

One of the major plant height QTL, QHt.dms-4B, in our study was mapped at 68.8 cM and it explained 11% to 22.5% of the variation for plant height, respectively. This QTL was consistent across management systems and years. It was associated with *Rht-B1* locus with Attila carrying the *Rht-B1b* (semi-dwarf) allele. Considering the large influence of the *Rht-B1* locus on plant height, no major influence of *Rht-B1b* (dwarf) was noticed on traits other than plant height in conventional management systems; however, a significant influence was observed under stressed very-weedy organic management systems where it reduced plant height along with grain yield and resulted in an increased weed biomass. Therefore, *Rht-B1* interacted with the management (cropping) systems and exhibited pronounced negative effects on the wheat grain yield and competitive ability in organic systems. Several studies reported that wheat cultivars carrying dwarfing alleles on the *Rht-B1* and *Rht-D1* loci exhibit shorter coleoptiles, lower rate of dry matter accumulation and have reduced leaf elongation rate when grown in hot and dry (stressed) environments of North America and Australia (Richards 1992; Bai

et al. 2004; Botwright et al. 2001; Ellis et al. 2004). The effects of dwarfing alleles, *Rht-B1b* and *Rht-D1b* were compared and it was reported that the effects of *Rht-B1b* were not as high as those linked with *Rht-D1b* and not significantly different from *rht* (tall) (Addisu et al. 2010). We found that both wild type and mutant alleles behaved in similar manner in conventional (free from competition/stress) management systems except for plant height but the effects of mutant allele was more pronounced in stressed, very-weedy organic management systems.

The number of tillers, early season vigor and light capture are considered important traits in conferring competitive ability. We found one QTL for each of these traits on chromosomes 4A, 3B and 6B, respectively. None of these QTL was found to be consistent over management systems and years probably due to QTL x environment interaction. The early season vigor QTL, Qesv.dms.3B identified in organic management systems in our study might be the previously reported early vigor QTL that was mapped on 3BL in the RAC875 × Kukri doubled haploid mapping population tested under drought and heat stress conditions (Bennett et al. 2012; Bonneau et al. 2013). To the best of our knowledge, QTL identified for number of tillers and light capture in this study have not been previously reported. In our study, there were differences in test weight and kernel weight between the two parents and the population in conventional and organic management systems. Variation in test weight is of special interest to wheat millers due to its positive correlation with flour yield, whereas kernel weight is associated with grain yield. Test weight QTL have been previously reported on chromosomes 1A, 1B, 1D, 2D, 3B, 3D, 4A, 4D, 5A, 5D, 6B, and 7A (Elouafi and Nachit 2004; Huang et al. 2006; McCartney et al. 2005a; Narasimhamoorthy et al. 2006; Zhang et al. 2008).

In our study, consistent QTL for test weight were mapped on chromosome 1B at the same position (79.4 cM) in both systems. This QTL was linked to the DArT marker wpt-5279. It accounted for 7.4% variation in the organic system and 10.2 % of the phenotypic variation in the conventional management system for test weight. The additive effect of this QTL was more pronounced in the organic (0.53) than in the conventional (0.41) system. The QTL (QTwt.dms-1A) linked to marker wpt-7339 was also detected under the conventional management system. Although, QTwt.dms-5B was only found in the conventional management system in 2008, it explained 29.9% of the variation for test weight and has not been previously reported. Similarly, a kernel weight

QTL mapped on chromosomes 6A and 1B was detected only in the organic system, whereas a QTL on 7A was found only in the conventional management system. A consistent QTL, QGwt.dms-3A, for kernel weight linked to wPt-8593 was mapped on chromosomes 3A across management systems in 2008. It behaved in a similar manner in both systems, where the additive effect (-1.16 and -1.43) and phenotypic variation (10.3 and 9.6) explained by this QTL do not differ to a greater extent in both systems.

Grain protein is one of the most important quantitatively inherited trait and is an important consideration in the development of wheat cultivars in the Canada Western Red Spring (CWRS) class of wheat. Grain protein content is negatively correlated with grain yield and, therefore, poses a serious challenge to wheat breeders (Steiger et al. 1996). Grain protein content is greatly influenced by the rate and time of nitrogen application and moisture availability in the soil. In this study, we mapped four of the six QTL for grain protein content during 2009, the year with the least rainfall. Three QTL identified in 2009 had positive additive effect on grain protein content. The QTL, *QGpc.dms-6A.1* and *QGpc.dms-6A.2* mapped on chromosome 6A in 2009 in the organic management system and collectively explained 26% of the phenotypic variation for grain protein content. Several attempts were made previously to identify minor and major QTL for grain protein content that resulted in the mapping of 10 QTL on chromosomes 1A, 2AS, 3AL, 3BS, 4AS, 4DL, 5BL, 6AL, 7AS, 7DL, 13 QTL on chromosomes 2AS, 2BL, 2DL, 3DS, 4AL, 6BS, 7AS and 7DS, 2 QTL on chromosomes 1BL and 6AS, 7 QTL on chromosomes 3AL, 4AL, 4BL, 5DL, 7BS and 7DS, 3 QTL on chromosomes 2AS, 6AS and 7BL and 2 QTL on chromosomes 4DS and 7BL QTL that explained up to 10.4, 35.8, 17.1, 22.4, 21.7 and 32.7% phenotypic variation, respectively (Blanco et al. 2006; Groos et al. 2003; Huang et al. 2006; Kunert et al. 2007; Prasad et al. 2003; Sourdille et al. 2003). Most of these QTL were not consistent across environments due to QTL X QTL epistatic and/or QTL X environment interactions. In our study, grain protein content QTL on chromosomes 3B, 5B, 6A and 6B were also mapped only in one year and/or management system, most probably due to a QTL by environment/management interaction for grain protein content.

Earliness is also one of the most desirable traits in western Canadian wheat breeding programs due to very short growing season (Randhawa et al. 2013). Nine QTL, four for days to flowering and five for days to maturity were mapped in this study. Most

of the QTL were mapped on Chromosome 5B at 49 to 54.7 cM positions. The days to flowering QTL mapped during 2008 in organic and conventional management system explained 32.8% phenotypic variation in days to flowering. The days to maturity QTL (Qmat.dms-5B) was mapped on a similar region (53.7 cM) in 2008 in organic management system that reduced maturity by 6.6 days, leading to the conclusion that chromosome 5B contains an important genomic region (49-54.7 cM) influencing earliness in wheat. All flowering and maturity time QTL identified in this study except *QMat.dms-2B* (conventional 2009) on chromosome 2B induced early flowering and maturity. Among these flowering time QTL, two QTL (Qflt.dms-5B linked to wPt-666939 and Qflt.dms-5B linked to wPt-3569) were found consistent in both systems. The flowering time QTL on chromosome 5B linked to wPt-666939 behaved in a similar manner and reduced flowering time in both systems (-2.67 and -3.18 days in organic and conventional systems, respectively), whereas other flowering time QTL on 5B chromosome linked to wPt-3569 reduced and delayed flowering by -2.12 and 2.2 days in organic and conventional management systems, respectively. The phenotypic variation explained by this QTL also varied between the two systems leading to the conclusion that same flowering time locus is involved in inducing early flowering in organic (weedy/stress conditions) and while delaying it in conventional management system.

Various studies have reported genotype by environment interactions for various agronomic and quality traits in spring wheat. This implies that some genes have different effects according to the specific/niche environment. These environmentally dependent gene/QTL effects can be of special interest to the wheat breeders. The detection and mapping of such genes/QTL might have practical implications for wheat breeding programs aiming to develop cultivars specifically for organically managed lands. In the present study, most of the QTL were either specific to conventional or organic management systems. For instance, one QTL for early season vigor, 5 out of 9 QTL for kernel weight and 4 out 6 QTL for grain protein content were specific to the organic system and the additive effects of consistent QTL for grain yield, test weight, kernel weight and days to flowering also varied between two systems suggesting that QTL express differently in different environments. Therefore, spring wheat breeders should test and select genotypes on production traits within environmental conditions which are comparable to where candidate lines are intended to perform.

3.5 Conclusion

Results of this study suggested that selection, heritability and correlation differences exist between management systems for various agronomic and quality traits which confirm previous findings of our group that breeding for spring wheat cultivars is more effective in a system which reflects its target environment. Under conventional management systems, no differences except plant height were found between wild and mutant type alleles of the locus *Rht-B1*; however, in organic systems, genotypes carrying *Rht-B1b* (mutant) exhibited reduced plant height and lower grain yield as compared to conventional management system. Overall, we found 46 QTL for various agronomic and quality traits. The consistent QTL detected across management systems for grain yield, test weight, kernel weight and days to flowering on chromosomes 6A, 1B, 3A and 5B, respectively, can be further used in marker assisted breeding through fine mapping of the specific region.

3.6 Tables

Table 3-1. Least square means, range and heritability estimates of various traits for parents and 163 RILs of Attila × CDC GO mapping population under conventional and organic management system in Edmonton, Alberta, Canada during 2008-2010.

Variables	Attila[a]		CDC Go[a]		Diff. Between Parents[b]		Population[a]		Con[c]		Org[c]		Heritability (%)[a]			
	Con	Org	Con	Org	Con	Org	Con	Org	Min	Max	Min	Max	Con	SE	Org	SE
Grain yield (t ha^{-1})	4.51**	2.74**	4.93*	3.81*	-0.42	-1.07**	4.63**	3.24**	0.43	8.32	0.12	6.94	37**	3	18**	3
Tillers (m^{-2})	481	408	549	404	-68	4	513**	454**	217	853	217	403	32**	4	16**	3
Plant height (cm)	71*	61*	73	72	-2	-11*	77**	72**	30	114	28	116	58*	3	43*	4
Test weight (kg hl^{-1})	77*	75*	79*	77*	-2**	-2**	78**	76**	71	88	41	82	18	3	24	3
Kernel weight (g)	39*	37*	42**	41**	-3	-4*	41**	39**	27	62	22	50	27**	4	44**	4
Grain protein (%)	12.3**	14.4**	14.4	13.4	-2.1**	1.0	13.2**	14.4**	8.4	17.7	7.2	17.9	64**	3	27**	3
Weed biomass (g m^{-2})		70		50	-	20**		40			10	360			83	5
Early season vigor	3	4	4	5	-1*	-1*	3.5**	4**	1	5	2	5	8	2	7	3
Days to flowering	60	61	57	58	3**	3**	59	59	42	81	42	80	76	2	70	3
Days to maturity	106**	99**	102**	95**	4	4	104**	97**	59	127	72	122	38	4	47	4
Light capture	0.62		0.66		-.04		0.57		-.01	0.98			55	3		

[a] Statistical differences tested between management systems
[b] Statistical differences tested between Attila and CDC Go
[c] Min and max represents LS means recorded for a particular trait in any of the years
Con Conventionally managed systems
Org Organically managed systems
*, ** Significant at P=0.05 and P=0.01, respectively

Table 3-2. Spearman's rank correlations (rs) between two management systems along with the number of RILs common at three selection intensities (5, 10 and 15%) for 9 traits in Attila × CDC Go population.

Variables	Rank (r_s)	Line Selected in Common		
		5%[a] (9)[b]	10% (17)	15% (26)
Grain yield (t ha^{-1})	0.20	3	5	13
Tillers (m^{-2})	0.51	2	3	7
Plant height (cm)	0.10	4	9	13
Test weight (kg hl^{-1})	0.60	3	6	11
Kernel weight (g)	0.70	1	5	10
Grain protein (%)	0.24	4	8	12
Early season vigor	0.80	1	4	6
Days to flowering	0.54	5	13	18
Days to maturity	0.74	2	5	12

[a] Selection intensity applied within each management system

[b] Maximum number of lines selected from the experimental population of 163 RILs at the given selection intensity

Table 3-3. Genotypic and Phenotypic correlations estimated under conventional management systems in Attila × CDC Go population in Edmonton, Alberta, Canada during 2008-2010.

Variables	Grain yield	Tillers	Plant height	Test weight	Kernel weight	Grain protein	Early season vigor	Days to flowering	Days to maturity	Light
Grain yield (t ha^{-1})		0.31	0.44	0.52	0.39	-0.63	-[a]	0.31	0.49	-
Tillers (m$^{-2)}$	0.28		-0.74	-	-	-	-	0.35	-	-
Plant height (cm)		-		-	-	-	-	0.41	-	0.57
Test weight (kg hl^{-1})	-	-	-		-	-	0.52		-	-
Kernel weight	0.29	-	-	-		0.34	0.54	-0.28	-	0.58
Grain protein (%)	-0.47	-0.37	-	-	-		0.29	-0.47	-0.55	-
Early season vigor	0.35	-	-	-	0.29			-0.53	-0.64	-
Days to flowering	-	-	0.32	-	-	-0.35	-		0.91	-
Days to maturity	0.28	-	-	-	-	-0.31		0.6		

Values above and below the diagonal represents genotypic and phenotypic correlation coefficients, respectively

[a] Correlation coefficient not different from zero ($P \leq 0.05$)

Table 3-4. Genotypic and Phenotypic correlations calculated under organic management systems in Attila × CDC Go population in Edmonton, Alberta, Canada during 2008-2010.

Variables	Grain yield	Tillers	Plant Height	Test weight	Kernel weight	Protein percentage	Early season vigor	Days to flowering	Days to maturity	Weed biomass
Grain yield (t ha^{-1})			0.46	0.35		-0.75	0.36			-0.64
Tillers (m^{-2})			-0.39							
Plant height (cm)	0.38	-0.31		0.42				0.36	0.33	-0.98
Test weight (kg hl^{-1})							0.29			-0.57
Kernel weight				0.28			-0.37			
Grain protein (%)	-0.39									
Early season vigor								-0.63	-0.53	
Days to flowering			0.29						0.92	
Days to maturity								0.66		
Weed biomass										

Values above and below the diagonal represents genotypic and phenotypic correlation coefficients, respectively

[a] Correlation coefficient not different from zero ($P \leq 0.05$)

Table 3-5. Genotypic correlation coefficients for grain yield among management systems in Attila × CDC Go population in Edmonton, Alberta, Canada during 2008-2010.

		Conventional		Organic		
		2009	**2010**	**2008**	**2009**	**2010**
	2008	0.91[a]	0.85	0.50	0.55	0.43
Conventional	2009		0.91	0.40	0.42	0.44
	2010			0.46	0.46	0.32
Organic	2008				0.90	0.85
	2009					0.90

[a] Correlation coefficients were different from zero ($P \leq 0.05$)

Table 3-6. Summary of quantitative trait loci (QTL) identified for 10 different traits in Attila × CDC Go mapping population in Edmonton, Alberta, Canada during 2008-2010.

S.No.	Trait	QTL	Systems	Years	Chromosome	Flanking Markers	Map Position	LOD	R^2 (%)	Additive effect
1	Grain yield	**Qyld.dms-6A**	Organic	2010	6A	wPt-4836-wPt-1375	93.6	3.2	7.0	-0.31
		Qyld.dms-6A	Organic	2009	6A	wPt-666773-wPt-741026	91.3	3.7	9.0	-0.22
		Qyld.dms-6A	Conventional	Combined	6A	wPt-666773-wPt-741026	83.4	4.0	17.0	-0.32
		Qyld.dms-6A	Conventional	2010	6A	wPt-1375-wPt-7204	93.9	3.6	7.9	-0.24
		Qyld.dms-6A	Conventional	2008	6A	wPt-666773-wPt-741026	82.3	4.6	22.2	-0.50
2	Tillers	QTil.dms-4A	Conventional	2009	4A	wPt-2903-wPt-671707	84.4	3.2	7.0	2.91
3	Plant Height	**QHt.dms-4B**	Organic	Combined	4B	wPt-733038-wPt-744595	68.8	8.5	18.1	-3.56
		QHt.dms-4B	Organic	2010	4B	wPt-733038-wPt-744595	68.8	6.0	13.6	-4.34
		QHt.dms-4B	Organic	2009	4B	wPt-733038-wPt-744595	68.8	5.5	12.8	-2.22
		QHt.dms-4B	Organic	2008	4B	wPt-733038-wPt-744595	68.8	7.3	14.3	-4.99
		QHt.dms-4B	Conventional	Combined	4B	wPt-733038-wPt-744595	68.8	8.6	22.5	-4.23
		QHt.dms-4B	Conventional	2010	4B	wPt-733038-wPt-744595	68.8	10.8	22.1	-5.00
		QHt.dms-4B	Conventional	2009	4B	wPt-733038-wPt-744595	68.8	4.7	11.0	-2.53
		QHt.dms-4B	Conventional	2008	4B	wPt-733038-wPt-744595	68.8	7.4	22.4	-4.45
4	Test weight	**QTwt.dms-1B**	Organic	Combined	1B	wPt-5279-wPt-1346	79.4	3.7	8.3	0.43
		QTwt.dms-1B	Organic	2008	1B	wPt-5279-wPt-1346	79.3	3.4	7.4	0.53
		QTwt.dms-1A	Conventional	Combined	1A	wPt-7339-wPt-733904	133.7	4.6	10.9	0.41
		QTwt.dms-1A	Conventional	2010	1A	wPt-7339-wPt-733904	133.7	3.8	8.7	0.47
		QTwt.dms-1B	Conventional	2009	1B	wPt-5279-wPt-1346	79.4	4.2	10.2	0.41
		QTwt.dms-5B	Conventional	2008	5B	wPt-666268-wPt-3569	52.0	4.1	29.9	-0.25
5	Kernel weight	**QGwt.dms-4A**	Organic	Combined	4A	wPt-3398-wPt-9305	86.1	4.3	11.2	1.11
		QGwt.dms-6A.1	Organic	Combined	6A	wPt-733151-wPt-667618	38.1	3.2	7.5	0.92

		QGwt.dms-1B	Organic	2010	1B	wPt-0359-wPt9903	36.3	6.7	13.9	1.94
		QGwt.dms-6A	Organic	2010	6A	wPt-733151-wPt-667618	31.1	3.8	8.0	1.37
		QGwt.dms-3A	Organic	2008	3A	wPt-8593-wPt-3978	87.5	4.9	10.3	-1.16
		QGwt.dms-3A	Conventional	2009	3A	wPt-8593-wPt-3978	87.5	3.9	7.7	-0.77
		QGwt.dms-4A	Conventional	2009	4A	wPt-3398-wPt-9305	86.1	4.9	12.4	0.98
		QGwt.dms-7A	Conventional	2009	7A	wPt-2100-wPt-0514	78.3	4.5	12.5	-1.00
		QGwt.dms-3A	Conventional	2008	3A	wPt-8593-wPt-3978	87.4	4.1	9.6	-1.43
6	Grain protein	QGpc.dms-6A	Organic	Combined	6A	wPt-7127-wPt-730368	29.6	4.0	10.9	-0.49
		QGpc.dms-6A.1	Organic	2009	6A	wPt-729904-wPt-9679	11.2	5.0	15.8	0.67
		QGpc.dms-6A.2	Organic	2009	6A	wPt-7127-wPt-730368	29.6	4.0	10.2	-0.63
		QGpc.dms-6B	Organic	2009	6B	wPt-3130-wPt-9971	86.3	4.3	9.2	0.51
		QGpc.dms-5B	Conventional	2009	5B	wPt-4091-wPt-2373	150.6	3.7	8.0	0.29
		QGpc.dms-3B	Conventional	2008	3B	wPt-0751-wPt-0912	111.9	4.2	9.3	0.28
7	Early season vigor	Qesv.dms.3B	Organic	2008	3B	wPt-741750-wPt-798970	25.1	3.5	9.7	-0.17
8	Days to flowering	**Qflt.dms-5B**	Organic	2010	5B	wPt-666268-wPt-3569	52.0	4.2	14.0	-2.12
		Qflt.dms-5B	Organic	2008	5B	wPt-666939-wPt-1457	54.7	4.8	32.8	-2.67
		Qflt.dms-5B	Conventional	2010	5B	wPt-666268-wPt-3569	49.0	3.5	21.2	2.20
		Qflt.dms-5B	Conventional	2008	5B	wPt-666939-wPt-1457	54.7	3.6	32.8	-3.18
9	Days to maturity	Qmat.dms-5B	Organic	2009	5B	wPt-666268-wPt-3569	50.0	3.5	4.9	-2.78
		Qmat.dms-5B	Organic	2008	5B	wPt-666939-wPt-1457	53.7	5.9	15.8	-6.66
		Qmat.dms-4B.1	Conventional	2010	4B	wPt-667593-wPt-6209	42.4	4.2	19.8	-3.43
		Qmat.dms-4B.2	Conventional	2010	4B	wPt-6209-wPt-9067	48.2	3.6	8.3	-1.90
		Qmat.dms-2B	Conventional	2009	2B	wPt-1919-wPt-4199	77.4	4.4	9.1	0.68
10	Light capture	Qlig.dms-6B	Conventional	2009	6B	wPt-0357-wPt-744302	44.4	4.1	8.9	-0.03

Stable QTL over organic and conventional management systems are bolded

Table 3-7. Summary of quantitative trait loci (QTL) identified for 10 different traits in Attila × CDC Go mapping population in Edmonton, Alberta, Canada during 2008-2010.

Trait	Year	QTL	Linked Dart Marker	R2		Additive effect	
				Organic	Conventional	Organic	Conventional
Grain yield	2010	Qyld.dms-6A	wPt-1375	7.0	7.9	-0.31	-0.24
	2008 and 2009	Qyld.dms-6A	wPt-741026	17.9	22.2	-0.44	-0.50
Test weight	2008 and 2009	QTwt.dms-1B	wPt-5279	7.4	10.2	0.53	0.41
Kernel weight	2008	QGwt.dms-3A	wPt-8593	10.3	9.6	-1.16	-1.43
Days to flowering	2008	Qflt.dms-5B	wPt-666939	32.8	32.8	-2.67	-3.18
	2010	Qflt.dms-5B	wPt-3569	14.0	21.2	-2.12	2.20

Table 3-8. Effect of *Rht-B1* (wild type and mutant) on various traits in Attila × CDC Go population grown under conventional and organic management systems in Edmonton, Alberta, Canada during 2008-2010.

	Conventional		Organic		Difference	
Trait	Wild Type	Mutant	Wild Type	Mutant	Wild Type	Mutant
Grain yield (t ha^{-1})	4.84	4.61	3.73*	2.90*	1.11**	1.71**
Tillers (m^{-2})	111	112	99	97	12**	15**
Plant height (cm)	76**	66**	70**	65**	6**	1*
Test weight (kg hl^{-1})	77	78	76	76	1**	2**
Kernel weight (g)	41	41	39	39	2**	2**
Grain protein (%)	12.9	12.9	14*	12.8*	-1.1**	0.1
Weed biomass (g m^{-2})			5.2**	8.2**		
Early season vigor	3.7	3.5	4.01	4.36	-0.31	-0.86
Days to flowering	53	52	53	52	0	0
Days to maturity	96	96	91	90	5**	6**
Light capture	0.5	0.47				

*Significant at P≤0.05

** Significant at P≤0.01

3.7 Figures

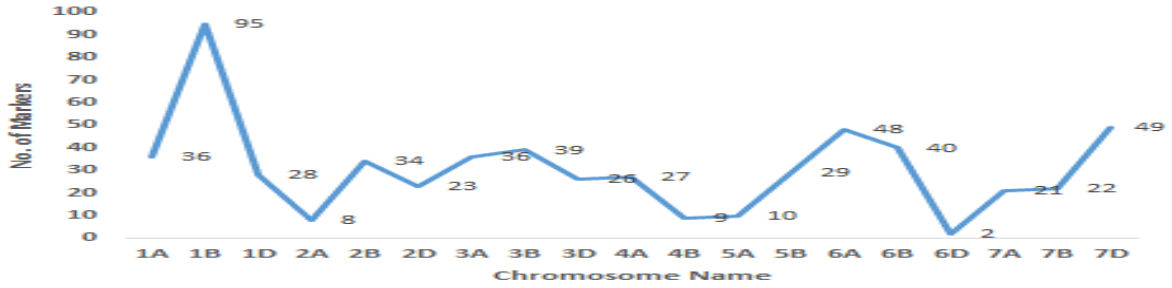

Fig. 3-1. **Number of Polymorphic DArT Markers per chromosome in Attila × CDC Go RIL population.**

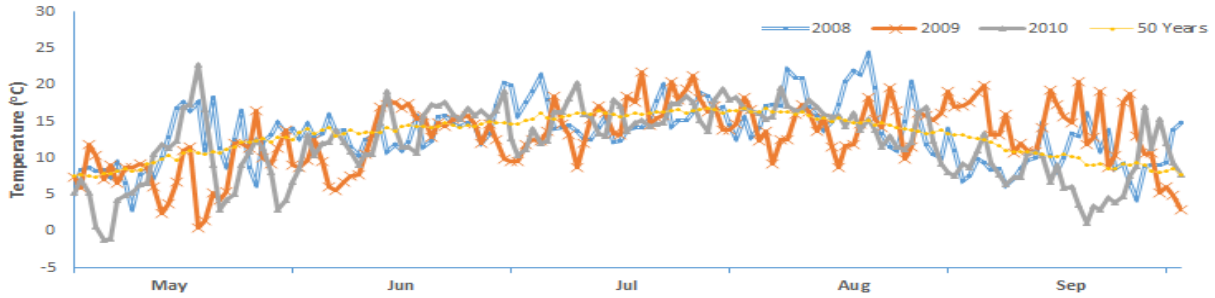

Fig. 3-2. **Mean daily temperature for the months of May to September during 2008-2010.**

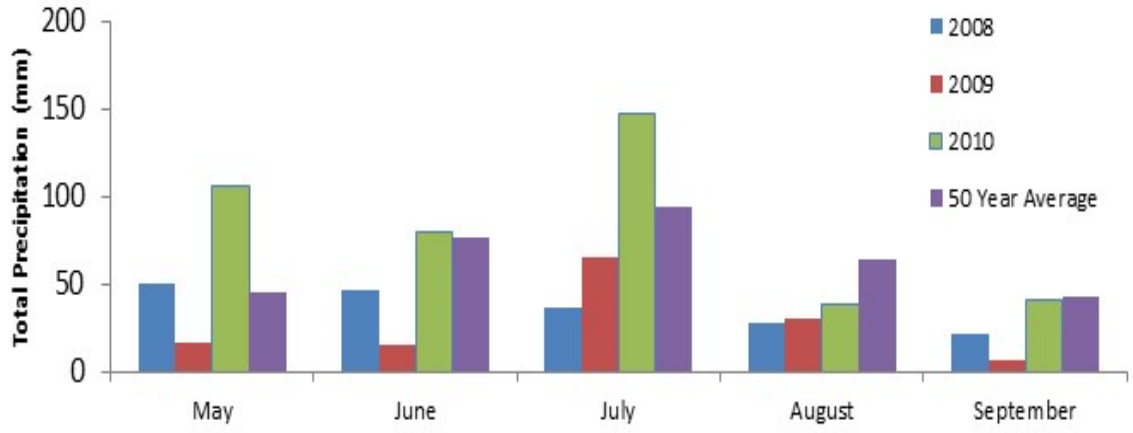

Fig. 3-3. Total precipitation for the months of May to September during 2008-2010.

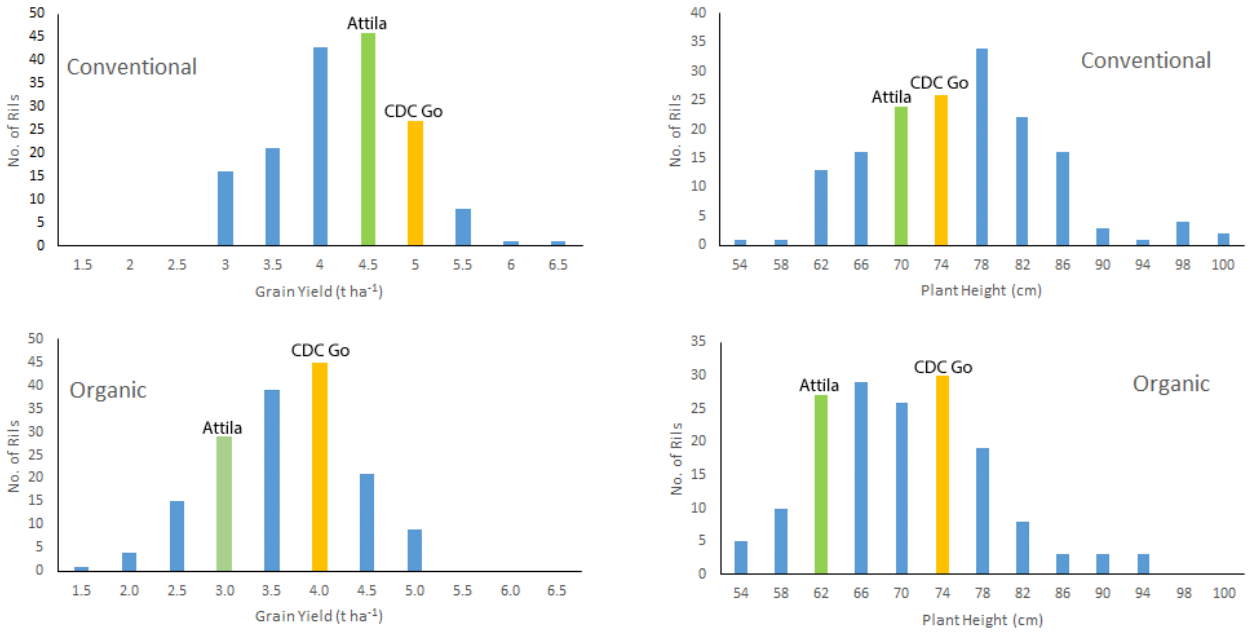

Fig. 3-4. **Frequency distribution showing transgressive segregation for grain yield and plant height using data combined over three years for 163 RILs derived from a cross between Attila and CDC Go in Edmonton, Alberta, Canada from 2008-2010.**

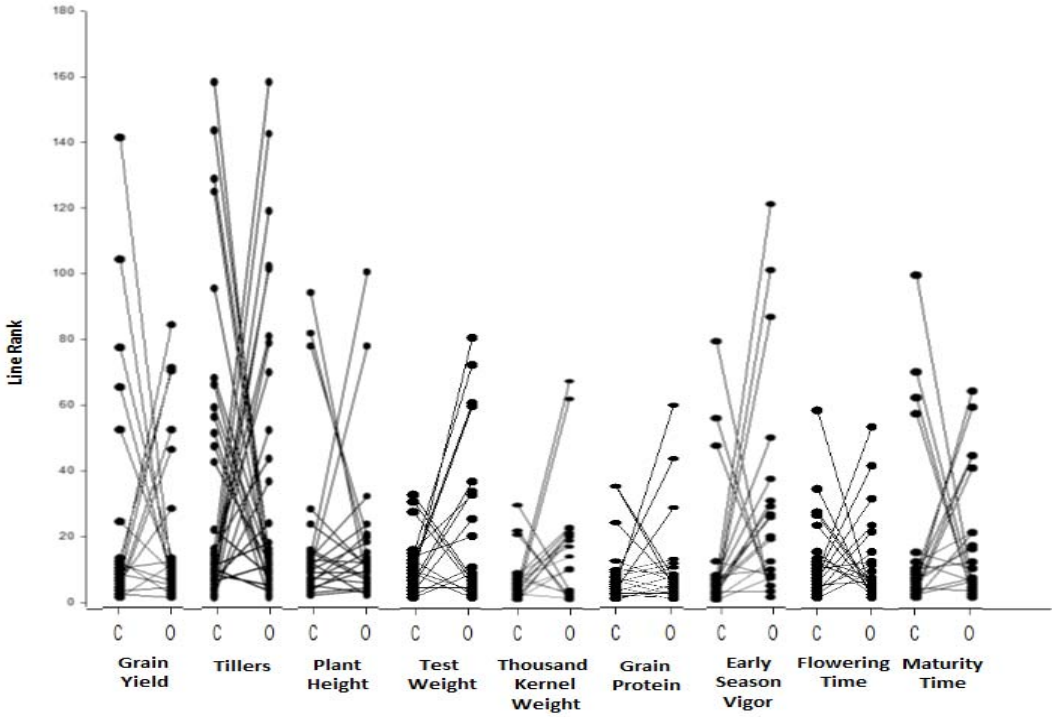

Fig. 3-5. **Observed rank changes in the top 10% RILs ranked under conventional (C) and organic (O) management systems for nine different traits measured in both systems. RILs were ranked according to the desired direction of selection (e.g. rank one for grain yield was the highest yielding RIL).**

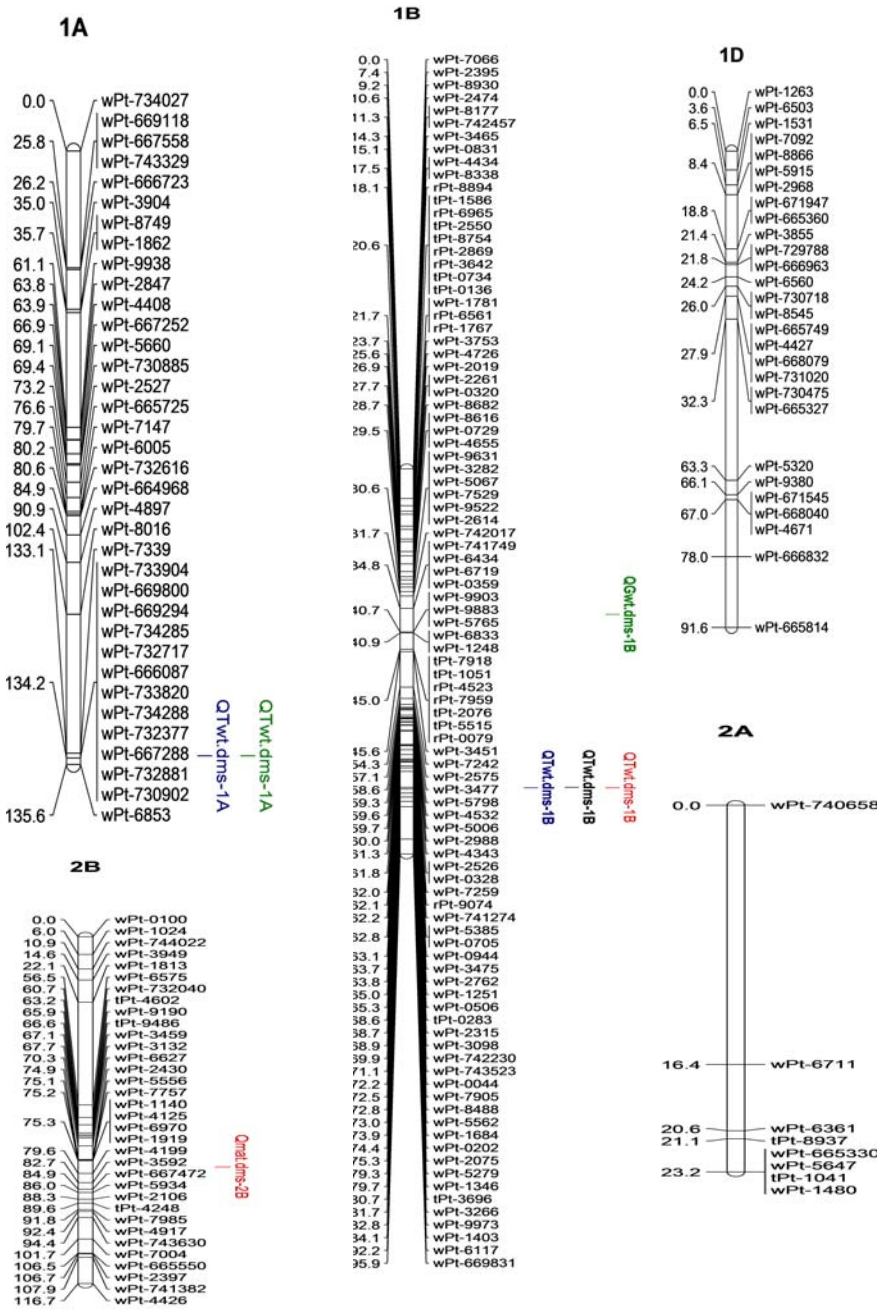

Fig. 3-6. Linkage map for Attila x CDC Go population

(Bold QTL were mapped in organic management systems whereas blue, green, red and black QTL were mapped in combined, 2010, 2009 and 2008, respectively)

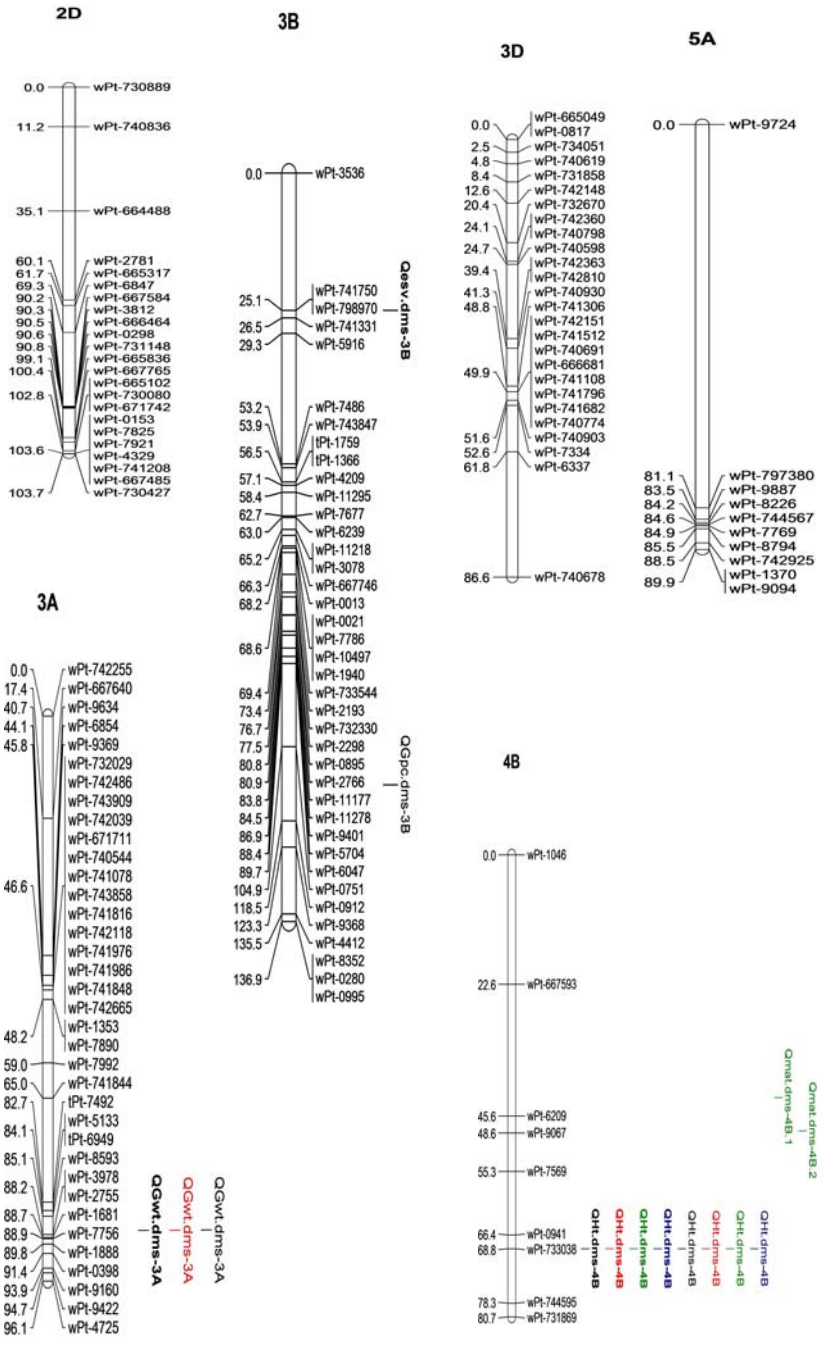

Fig. 3-6 (Continued) Linkage map for Attila x CDC Go population

(Bold QTL were mapped in organic management systems whereas blue, green, red and black QTL were mapped in combined, 2010, 2009 and 2008, respectively)

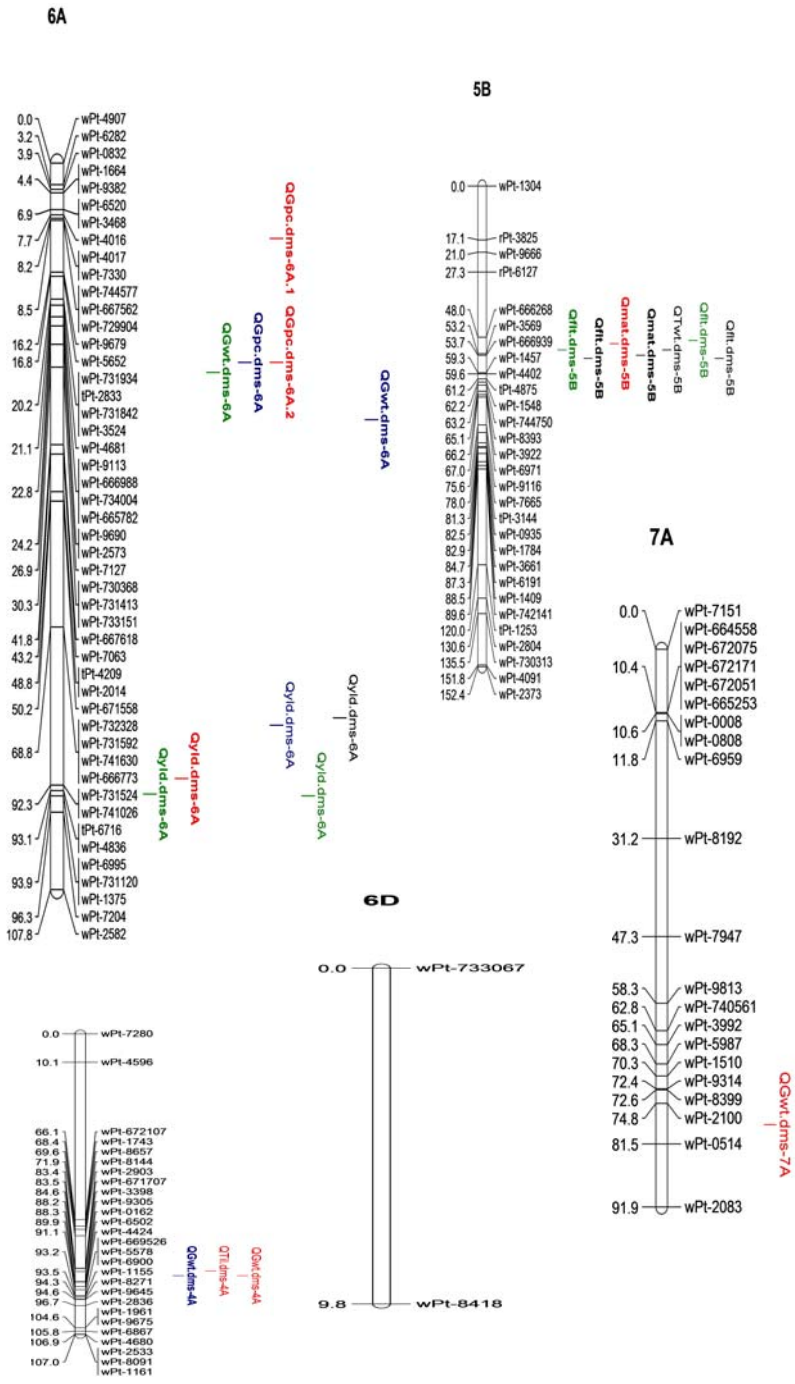

Fig. 3-6 (Continued) Linkage map for Attila x CDC Go population

(Bold QTL were mapped in organic management systems whereas blue, green, red and black QTL were mapped in combined, 2010, 2009 and 2008, respectively)

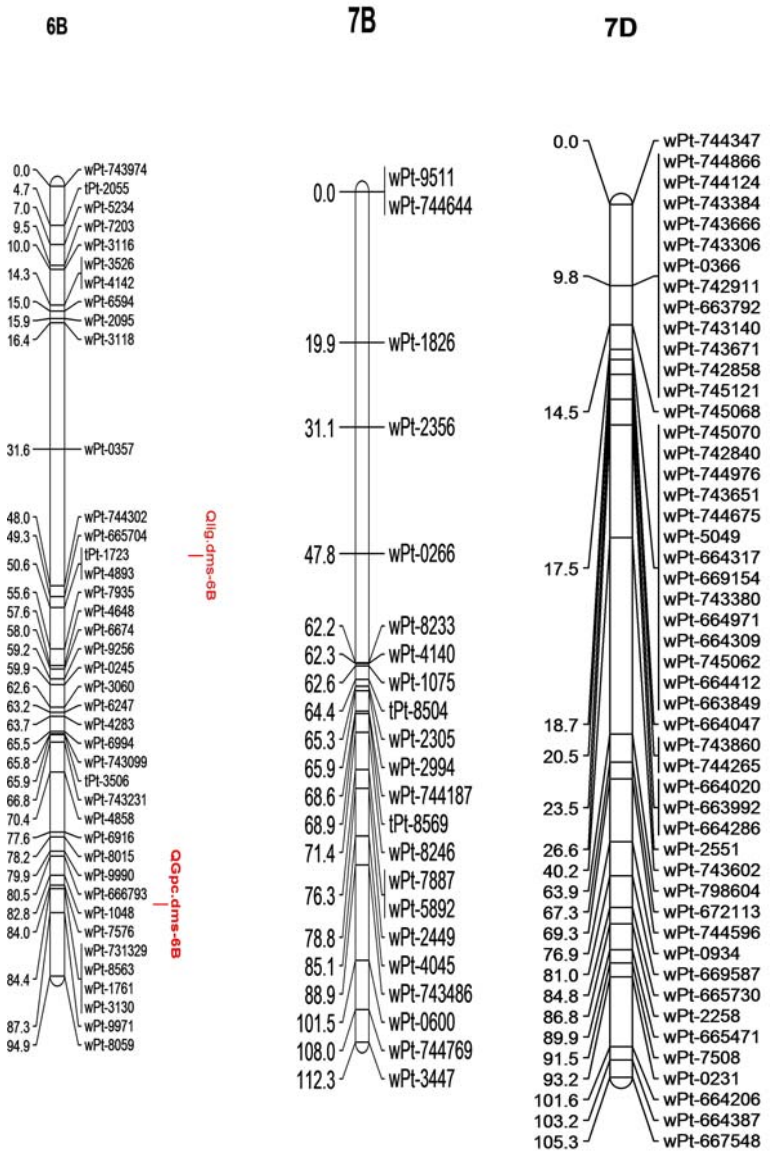

Fig. 3-6 (Continued) Linkage map for Attila x CDC Go population

(Bold QTL were mapped in organic management systems whereas blue, green, red and black QTL were mapped in combined, 2010, 2009 and 2008, respectively)

4.0 Importance of doubled haploid production in wheat breeding

The importance of doubled haploid (DH) production is well known to scientific community engaged in the field of agriculture and related disciplines. It helps to reduce time scale for development of new cultivars and also provide opportunities to develop mapping populations for genetic studies. Few research studies have been conducted employing DH populations with the objectives to map quantitative trait loci (QTL) for traits related to competitive ability for organically managed lands. In one of study, Coleman et al. (2001) reported QTL for size of first two leaves, flag leave size, plant height and anthesis (traits related to competitive ability) using a DH population in wheat derived from a cross between Cranbrook and Balbred that consisted of 161 DH lines. All QTL were reported on chromosomes 2B and 2D for two years at the same positions. Similar study was conducted where authors also employed a DH population to identify and map QTL for nitrogen uptake efficiency under low and high nitrogen regimes in the field conditions and reported nine and eight QTL for nitrogen uptake in the low and high nitrogen conditions that can be used in marker assisted breeding (MAB) to develop wheat cultivars with enhanced early season vigor (An et al. 2006).

In Canada, DH technology integrated into wheat breeding programs about 22 years ago and McKenzie was the first Canada Western Red Spring (CWRS) wheat cultivar that was developed in 1997 using anther culture (DePauw et al. 2011a). Since then, 25 wheat cultivars have been developed and released for commercial cultivation in seven market classes (DePauw et al. 2011a). Among these 25 cultivars, only two were developed through anther culture and 23 were developed using wide crossing method with corn as a pollen donor. Therefore, corn based pollination method is predominant in Canadian wheat breeding programs to develop wheat cultivars. However, this method is time consuming and laborious because it needs a lot of emasculation, pollination and embryo rescue. Moreover, it also requires an extra growth chamber to grown corn. On the other hand,

isolated microspore culture (IMC) is much efficient (in terms of space, time, labor and cost) as compared to other DH production methods but the problem of contamination, occurrence of albinism, genotypic dependency, low frequency of embryos and green plant production in cereals, especially wheat restrict wheat breeders to use this fascinating phenomenon in their breeding programs to develop new cultivars. Therefore, we were interested to improve IMC so that it can be integrated into wheat breeding programs to develop new wheat cultivars for both conventional and organically managed lands. The results of four different studies aimed to improve IMC in wheat have been presented in the next four Chapters.

5.0 Organelle antioxidants improve microspore embryogenesis[2]

5.1 Introduction

Microspore embryogenesis involves the development of embryos or embryo-like structures from isolated microspores leading to the production of haploid and doubled haploid (DH) plants. The gametophytic development of microspores is arrested and switched towards embryogenesis by means of various pretreatments. Microspore embryogenesis can be achieved either through isolated microspore culture (IMC) or anther culture. Microspore culture is of special interest to plant breeders, molecular biologists and geneticists because it is a rapid way to fix homozygosity and may also provide a platform for cost competitive production of DH lines and for genetic engineering of the haploid genome. Such a platform would hasten the development of mapping populations and facilitate genetic studies. Nitsch (1974) isolated and cultured microspores of *Nicotiana* sp. for the first time by natural shedding, free from anthers. During the last two decades, the research community has witnessed a tremendous amount of work to improve the overall process of IMC in various crops; including wheat, (Cistue et al. 2009), barley (Davies 2003), oat (Sidhu and Davies 2009), triticale (Eudes and Amundsen 2005), brassica (Agarwal et al. 2006) and pepper (Lantos et al. 2009). This research resulted in many recalcitrant crops becoming responsive to IMC. Nevertheless, IMC is not efficient enough to be broadly adopted in wheat breeding programs. Rates of embryogenesis and albinism still pose major bottlenecks in doubled haploid production and deter wheat breeders from adopting IMC.

Many factors affect microspore embryogenesis, including growth and developmental conditions of donor plants, microspore stage at the time of floral organ collection, pretreatments in the form of sugar or nitrogen starvation, heat and cold shocks, microspore isolation procedures, microspore purification and media composition (Ferrie and Caswell 2011). A stress applied before or after microspore

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isolation play an important role in microspore development; in particular reprogramming the microspore towards embryogenesis. Despite the presence of large numbers of cells or microspores in culture [e.g. 3 X 10^4 - 2 x 10^5/ml in triticale (Eudes and Chugh 2009), 4 x 10^4/ml in *Brassica napus (Huang et al. 1990)*, 5 x 10^4/ml *B. oleracea* (Ferrie et al. 1999) and 8-10 x 10^4/ml in *Capsicum annuum (Kim et al. 2008)*], cell death occurs at the very early phase of induction and continues during the entire process. This may limit the development of embryos or embryo-like structures. Microspores exposed to a stress pretreatment and isolation could suffer in oxidative damage in the form of lipid peroxidation, protein oxidation, nucleic acid damage, invagination, appearance of apoptotic-like bodies, mitochondrial alteration, which may eventually lead to cell death (Varnier et al. 2009; Wang et al. 1999). Plastids and mitochondria are the target/production sites of reactive oxygen species (ROS) during abiotic stresses (Foyer et al. 1994; Tuteja et al. 2012). In mitochondria, abiotic stresses cause severe impairment that lead to increased electron transport, H_2O_2 production, ATP depletion, and ultimately cell death (Bartoli et al. 2004; Tiwari et al. 2002). The increased level of ROS in plastids can be correlated to albinism as albino plants are devoid of chlorophyll and ROS are also linked to the overall programmed cell death in microspores (Caredda et al. 2000; Varnier et al. 2009). Therefore, it can be assumed that organelle antioxidants can lead to improved microspore viability, resulting in a greater number of embryo and embryo-like structures, less albinism and ultimately an increased frequency of green DH plants.

IMC follows a series of steps in which microspores undergo various physiological, morphological and cytological changes. The whole process can be divided into two distinct phases i) early embryogenesis involving the acquisition of embryogenic ability followed by their multiple symmetric or asymmetric cell division within the exine wall and ii) late embryogenesis consisting of cell elongation followed by a disruption/break in the exine wall. Cellular redox status during early and late embryogenesis has been studied in detail and the antioxidant response of Glutathione (GSH) and Ascorbate (ASC) redox systems, associated with the plastid metabolism, have been shown to positively affect embryo development in *Pinus palustris* and *Triticum turgidum* (Tommasi et al. 2001; De Gara et al. 2003). Alterations in endogenous GSH and ASC redox status [defined as the ratios of the reduced forms of GSH and ASC to the

oxidized forms of glutathione disulfide (GSSG) and dehydroascorbate (DHA), respectively] have been associated with improved embryogenesis response (Belmonte et al. 2003; Stasolla et al. 2004). The early phase of embryogenesis usually occurs in a reduced environment that enhances cell proliferation and division. Redox status promotes continual embryo development and deposition of storage products (Belmonte et al. 2005; De Gara et al. 2003). The contribution of Salicylic acid (SA) or 2-hydroxybenzoic acid has also been documented in many plant biological processes, including positive influence in androgenesis and organogenesis (Reis et al. 2008), and reduction of ethylene production (Hosseini 2009). The addition of SA in the induction medium has improved somatic embryogenesis of *Medicago sativa* (Meijer and Brown 1988), *Pelargonium* x *hortorum* Bailey (Hutchinson and Saxena 1996) and *Daucus carota* (Nissen 1994).

Various antioxidants, active in the mitochondria, have been studied in animal cells to improve growth. Methylene blue (MB) extends the life span of human IMR90 fibroblasts cells in tissue culture by enhancing mitochondrial functions; MB increases the mitochondrial complex IV by 30%, enhances cellular oxygen consumption by 37-70%, increases heme synthesis, and reverses premature senescence caused by HO or cadmium (Atamna et al. 2008). Similarly, N-t-butyl hydroxylamine (NtBHA) improved mitochondrial function in vivo, delaying senescence-dependent changes in human lung fibroblasts (IMR90). NtBHA is an antioxidant that is recycled by the mitochondrial electron transport chain and prevents radical-induced toxicity to the mitochondria (Atamna et al. 2001). The role of amino acids has been well documented in the literature; especially with respect to increasing the levels of reduced nitrogen in the medium that stimulates the process of embryogenesis (George 1993). Proline is considered as one of the most important amino acids in initiating plant embryogenesis, and may have a positive effect on osmotic tolerance (Hita et al. 2003). Media supplemented with proline resulted in a decrease in the water potential, allowing more essential nutrients to accumulate in the cells and promoting embryogenesis (Santos et al. 1997). A similar role for proline in embryo formation has been reported in *Zea mays* (Suprasanna et al. 1994), *Panicum miliaceum* (Vikrant 2002) and *Rosa hybrida* L. (Marchant et al. 1996).

In light of the importance of antioxidants in the process of embryogenesis or embryo development, we have conducted a series of experiments in triticale and wheat to investigate the effects of exogenous supplies of antioxidants at various concentrations. We have divided the present study into two experiments focusing on the contribution of mitochondrial and plastid antioxidants in microspore embryogenesis. The present study was conducted to establish a base line for the role of antioxidants in microspore embryogenesis. This research also included uncovering singular mitochondrial or plastid antioxidants supplemented to the induction media that could increase the number of embryo-like structures and green plants in wheat and triticale species.

5.2 Materials and methods

The experimental material consisted of one triticale cultivar (AC Ultima) and four soft white spring wheat genotypes (Fielder, Sadash, and the breeding lines SWS 411 and SWS 366). The triticale cultivar AC Ultima gives better response to isolated microspore embryogenesis (Eudes and Amundsen 2005), therefore, we tested two series of treatments first in triticale and then validated them in wheat, including recalcitrant genotypes i.e. SWS411, SWS366 and Sadash. Donor plants were grown in a growth cabinet with a photoperiod of 19h day (intensity 300 µE m^{-2} s^{-1}), and temperature 15-12^{o}C (day-night) with a relative humidity at about 70%. Plants were fertilized every 2 weeks (20-20-20, NPK 200 ppm). The plants were treated with 2.5 ml l^{-1} Tilt™ (propiconazole, Syngenta) during the tillering stage (Zadok's growth stage 2) (Zadoks et al. 1974) and Intercept™ (0.004 g per liter of soil, Imidacloprid, Bayer) once sufficient root development was established. The first 10 tillers from each pot were harvested when the spike was 1 to 3 cm emerged from sheath on the stalk in triticale but in wheat when the awns began to emerge. The remaining spikes were allowed to grow half their length out of the boot and then harvested for ovaries. Tillers for microspore isolation were kept in the refrigerator (4°C) for 21 days with their bases in distilled water and their heads wrapped in aluminum foil. Tillers for ovaries were used fresh or stored in the same manner as those for microspore isolation. After 21±3 days, the spikes were excised from their tillers and after an evaluation of their general

appearance, only the most homogenous spikes were used. The mid to late uninucleate microspore stage was verified from a median floret using acetocarmine staining.

5.2.1 Microspore isolation

Awns were removed with scissors in a laminar flow hood. Eight spikes for microspore isolation and four spikes for ovaries were sterilized with 10% bleach (5.25% sodium hypochlorite) for 3 min and rinsed four times for 1 min with sterile double distilled water with constant agitation. Anthers from eight spikes were aseptically dissected and transferred to a sterile and refrigerated 110 ml Warring blender cup (VWR international, #58983-093) containing 50 ml filter sterilized NPB99 (Konzak et al. 1999) liquid medium at 4°C. Anthers were then blended twice for 7 s at low speed (18000 rpm). The suspension was poured through 100 µm sterile mesh (VWR International, #CA21008-950) into two 50 ml centrifuge tubes (25 ml each). The blender cup was rinsed with 50 ml NPB99 at 4°C and poured through the 100 µm mesh and added to the first aliquot in the 50 ml tubes. The cells were then pelleted by centrifugation (100 x g for 5 min at 4^{o}C) using a swinging bucket rotor. The supernatant was poured off and the microspore pellets pooled into one 15 ml tube filled with NPB99 for a wash and centrifugation under the same conditions as before. The supernatant was poured from the tube and the pellet mixed in about 2 to 3 ml of NPB99 medium. These cells were then layered over 2.5 ml 30% percoll solution containing 0.4 M mannitol and 10 mM MES pH=7. The cells were spun again as described above and the band formed at the interface between the lower and upper phases was collected and transferred to a new 15 ml centrifuge tube, which was then topped with NPB-99 and centrifuged again at 100 x g for 5 min at 4^{o}C. The supernatant was again poured off and the remaining cells suspended in a total volume of 1.4 ml (NPB-99). The cells were counted using a haemocytometer and adjusted to 1x10^{5} cells per ml. Cells were then dispensed in 50 mm Petri dishes with 3.5 ml NPB-99 supplemented with 10% ficoll 400 (NPB99-10F), at a final microspore concentration of 28,000 cells per ml (100,000 cells per dish). On average, each microspore extraction generated enough cells to inoculate 14 and 7 Petri dishes of triticale and wheat, respectively.

5.2.2 Treatments

The whole study was divided in two experiments using different mitochondrial and plastid antioxidants. In both experiments, the effect of antioxidants supplemented in the induction medium (NPB99-10F) was first tested in triticale to see their effect on microspore embryogenesis and then extended to wheat genotypes. The mitochondrial antioxidants used in Experiment 1 were a) 10nM of methylene blue (MB), b) 10mM of proline c) 100 µM of N-t-butyl hydroxylamine (NtBHA), d) 100µM of 6-(γ,γ-Dimethylallylamino) purine, and e) 100µM of 6-(γ,γ-Dimethylallylamino) purine + 10mM of proline + 10nM MB + 100µM NtBHA. The plastid antioxidants used in Experiment 2 were ascorbate (ASC), glutathione (GSH) and salicylic acid (SA) at concentrations of 200nM (1X) and 2 µM (10X).

5.2.3 Induction and regeneration

Four ovaries from sterilized spikes taken directly from the wheat and triticale plants were added to each dish containing microspores. The dishes were sealed with parafilm and placed in a 150 mm Petri dish around an open 50 mm Petri dish containing sterile distilled water. The 150 mm dish was also sealed with parafilm and incubated in the dark at 28°C for 20 to 30 days. Embryos larger than 1 mm were removed from the petri dishes and plated onto GEM medium (20 ml in 10 cm petri dishes) (Eudes et al. 2003). The petri dishes were again sealed with parafilm and placed 30 cm beneath Sylvania Gro-lux wide spectrum bulbs (40 watts) delivering 80 µmol m^{-2} s^{-1} (16h light period) with a room temperature at 16°C. Once the embryos turned green, they were aseptically transferred onto 50 ml rooting medium (Eudes et al. 2003) in Magenta Vessels (VWR International ref), in the same growing conditions. Once the plants reached the 2-3 leaf stage and had sufficient root growth, they were transplanted into a 4x8 Spencer-Lemaire root trainer (Spencer-Lemaire Industries Ltd., Edmonton) and placed into a growth cabinet with the same growing conditions as the mother plants.

5.2.4 Data recording and statistical analysis

Development of multicellular structures and embryos were first verified at 10-14 days. The number of embryos or embryo-like structures was determined three to four weeks after isolation when the tissue was large enough to transfer to GEM

medium. The number of green and albino plants was determined following germination of the embryos (approximately 3 weeks after transfer).

The experiments were laid out in completely randomized design with different replications in triticale and wheat genotypes. The experimental unit was one Petri dish containing $1x10^5$ microspores. The number of replications for each genotype in each experiment is given in the results section. The data were analyzed separately for each species. The data were subjected to normality and homogeneity of the variance using Kolmogorov–Smirnoff and Levene's tests, respectively. Analysis of variance was conducted using the GLM procedure of SAS for all variables studied to find the effect of antioxidants, and genotypes and interaction in wheat (SAS 2003). Means were separated using Duncan Multiple Range Test ($\alpha \leq 0.05$). In wheat experiments, where interaction between genotypes and antioxidants were found significant, the interaction was partitioned by antioxidant using SLICE command in PROC GLM to quantify the effect of antioxidants individually. If antioxidant effects using slice were found significant, least square means of control was compared with other antioxidant (planned comparisons) using PDIFF statement (PDIFF=Control) in SAS.

5.3 Results

5.3.1 Experiment 1. Evaluation of mitochondrial antioxidants

Wheat genotype x antioxidant interaction was not significant for the three traits including embryo-like structures, green plants and albino plants. However, development of embryo-like structures and green plants were affected by mitochondrial antioxidants (proline, MB, DMAP, NtBHA and a combination of all) in triticale and wheat (Table 5-1). As a result of proline treatment in triticale, the number of ELS and green plants per Petri dish increased from 30 to 39 and 5 to 11, respectively (Table 5-2). In recalcitrant wheat genotypes SWS 411 and SWS 366, no green plants were produced from the control Petri dishes. The proline treatment produced two green plants per Petri dish for both genotypes. DMAP and NtBHA in SWS 411, and DMAP in SWS 366, also resulted in the production of two green plants per dish. Antioxidants also affected the number of ELS and albino plants in Sadash. The maximum number of embryos (13) was obtained in Sadash using NtBHA, however, this may not significantly different from the control (11). In triticale the antioxidants did not changed the production of albino plants, while in the

wheat cv. Sadash two antioxidants (DMAP and All) significantly reduced the number of albino plants and a trend was observed with proline (Table 5-2).

5.3.2 Experiment 2. Evaluation of plastid antioxidants

Experiment 2 was conducted in order to assess the effects of a second series of antioxidants on number of ELS, green and albino plants. The results presented in Table 5-1 revealed that the plastid antioxidants used in this experiment affected the number of ELS, green and albino plants in triticale. GSH10X produced significantly higher number of embryos (292.6) than the control (152). With 12 replicates, GSH1X and GSH10X also significantly increased the production of green plants as compared to the control. GSH10X also showed a reduction in albinism in triticale but the results were not significantly different from SA10X and ASC1X treatments and control (Table 5-2).

In wheat, a strong interaction of genotype x antioxidants was observed for the number of ELS and green plants indicating that wheat genotypes responded differentially to the various antioxidants for these dependent variables. This interaction was sliced by antioxidants in order to identify the specificity of wheat genotype response to a particular plastid antioxidant. The slicing (Table 5-2) revealed that genotypes responded differently to each antioxidant for number of ELS and green plants. To further elucidate the effect of antioxidants, the treatments were compared in each genotype. ASC10X, GSH1X and GSH10X produced greater numbers of ELS in SWS 411 as compared to the control (Fig. 5-1). Similarly, GSH10X also led to higher number of ELS in genotypes SWS 366 and Fielder (Fig. 5-1). It also dramatically increased the number of green plants per Petri dish in these two genotypes (Fig. 5-2). No interaction was observed for the number of albino plants in wheat (Table 5-1).

5.4 Discussion

Various types of pretreatments are commonly employed in IMC to induce embryogenesis. Among these pretreatments, sugar starvation, cold and heat shocks are widely applied in small grain cereals. In most of the studies, the authors treat spikes with a cold pretreatment at 4^{o}C for 1 to 4 weeks or with a heat shock for 2-3 days at 33^{o}C (Gustafson et al. 1995; Touraev et al. 1996; Zheng 2003). Sugar starvation for 4-5 days using mannitol rather than maltose or sucrose has also been used in cereal crops (Cistue et al. 2009; Dunwell 2010). Other pretreatments such as osmotic shock or use of

microtubule disruption agents during IMC are also common (Jahne and Lorz 1995). It has been reported that oxidative stress induced by pretreatments could affect the rate of microspore embryogenesis to a considerable degree by influencing cell viability and metabolism (Zur et al. 2009). The physical removal of floral organs from the donor plants, the microspore isolation and purification procedures generate stresses that are the main cause of microspore death during their early phase of culture. It is also known that the composition of the induction medium markedly affects the efficiency of microspore embryogenesis and helps to improve the frequency of embryos and green plants to a larger extent. Therefore, we supplemented the induction medium with four mitochondria and three plastid antioxidants to reduce oxidative stresses and to evaluate their contribution to triticale and wheat microspore culture and doubled haploid production. All candidate antioxidants evaluated in this cell culture system have been chosen for their abilities to control these oxidative stresses, as reported in earlier studies (Belmonte et al. 2003; Belmonte et al. 2005; Stasolla et al. 2001; Chen and Dickman 2005). Our results indicated that glutathione, at 2μM, had the most significant effect in increasing the production of embryos and green plants. Proline, DMAP and NtBHA contributed in a genotype dependent manner to more modest increases.

Among the group of mitochondrial antioxidant candidates, proline increased the number of ELS in triticale and increased green plant production in triticale and most wheat cultivars (Table 5-2). Proline is different from other amino acids because it is an α-amino acid and it is an osmoprotectant and maintains a balance during stress (Yoshiba et al. 1997). Reactive oxygen species (ROS) are constantly produced in plants due to cellular metabolism, and plants are equipped with scavenging enzymes to maintain their level in a limit for normal plant growth and developmental processes. Stresses in the form of heat and cold elevate the level of ROS that can damage cells (Yoshiba et al. 1997). In such cases, proline acts as a potent antioxidant and inhibits stress related apoptotic responses (Chen and Dickman 2005). Interestingly, the other candidate mitochondrial antioxidants appeared to negatively influence the success of microspore culture in triticale, while DMAP and NtBHA enhanced green plant production in the most recalcitrant wheat cultivars used in this study. This would suggest that the differences in embryo development leading to the germination of green plant could be associated with mitochondrial metabolism.

Some plastid antioxidants employed in this study increased ELS production in triticale and wheat. Glutathione in triticale and the wheat cultivars SWS 366, SWS 411 and Fielder; and ascorbic acid (10X) and Glutathione (1X and 10X) in SWS 411, significantly increased the number of developing ELS as compared to control (Fig. 5-1). The antioxidant defense system in plants includes various enzymatic and non-enzymatic metabolites (GSH and ASC) to cope with ROS signaling in plastids (Vranova et al. 2002) and there is a direct correlation between the resistance to oxidative damage and the resistance against abiotic stresses by a plant's defense system (Kranner et al. 2002). Meyer (2008) suggested that GSH is an important antioxidant to maintain the normal reduced form of the cells in order to counter act the inhibitory and harmful effects of ROS-induced oxidative stress. Glutathione has also been termed as one of the most effective plastid scavengers of ROS such as H_2O_2, O_2^- (Noctor and Foyer 1998) and OH^- (Larson 1988). Increased levels of ROS during the early phases of somatic embryogenesis have been associated with increased microspore embryogenesis (Pasternak et al. 2002; Ganesan and Jayabalan 2004). Once embryogenesis is initiated, increased ROS level can limit further development of these embryos. Exogenous application of GSH in the induction medium changed the endogenous glutathione redox state and maintained the desired GSH to GSH+GSSG (oxidized form) ratio that was necessary to increase the number of embryos from microspores (Belmonte et al. 2005), and also helped to prevent deterioration of embryos development in the later stages of the culture. Glutathione also regulates cell cycle in plants (Vernoux et al. 2000); these cells arrest at G1 phase once GSH is depleted from the growth medium. The exogenous supply of GSH in microspore culture kept the GSH levels high enough to promote continuous cell division, which resulted in a high number of embryos and ultimately green plants. Furthermore, it has been pointed out that organelles, especially plastids, are a specific risk of O_2^- (Mehler 1951) and the risk of this toxicity increases up to 25% under stress conditions (Robinson 1988; Biehler and Fock 1996). Thus, the exogenous supply of GSH helped to reduce the oxidative stress in plastids that led to the production of more green plants in recalcitrant wheat genotypes (Fig. 5-2). On average, one triticale microspore extraction from eight spikes resulted in 14 induction Petri dishes, which produced a total of 42 green plants per extraction, using 2 µM glutathione supplemented-induction medium, compared to 28 green plants from the control.

Similarly, one extraction from eight spikes of wheat resulted in seven Petri dishes, which produced 35 green plants of Fielder, SWS366 and Sadash per extraction using glutathione supplemented induction medium. Glutathione had a dramatic effect on these recalcitrant wheat genotypes and takes IMC to a level of productivity per spike similar to the well-established corn pollination method, addressing to some degree concerns with IMC genotypic effect for doubled haploid wheat production.

5.5 Conclusion

A series of antioxidants associated with either the oxidative stresses of the mitochondria or plastid have been evaluated in wheat and triticale microspore culture. Glutathione had the most dramatic effect on green plant production across triticale and wheat cultivars, while a few mitochondrial antioxidants, including proline, DMAP, and NtBHA, also increased the embryo and green plant formation in a genotype dependent manner. However, this study did not explore the dose effect and the timing of these treatments. Given this limitation, we presently recommend supplementing the induction medium for these species with glutathione or proline. Glutathione supplemented induction medium led to green plant production efficiencies that would compare well with doubled haploid production via the corn pollination method.

5.6 Tables

Table 5-1. Analysis of variance of experiment no. 1 and 2 for embryo like structures (ELS), green and albino plants.

Experiment No. 1: Evaluation of mitochondrial antioxidants					
	Source	*df*		Mean Squares	
Experiment No. 1a			**ELS**	**GP**	**AP**
Triticale	Antioxidant (Ant)	5	293**	71**	15ns
	Error	42	2395	494	464
Experiment No. 1b					
Wheat	Genotype (Gen)	3	120*	28**	37ns
	Ant	5	257*	15**	81*
	Gen x Ant	15	95ns	4ns	23ns
	Error	207	17788	725	6005
Experiment No. 2: Evaluation of plastid antioxidants					
Experiment No. 2a					
Triticale	Ant	6	74438**	8**	7*
	Error	83	1872552	197	229
Experiment No. 2b					
Wheat	Gen	3	30036**	2049**	1979**
	Ant	6	321**	30*	24*
	Gen x Ant	18	218**	23*	13ns
	Error	148	15601	1872	1442

*Significant at P≤0.05

** Significant at P≤0.01

ELS, GP and AP stands for embryo-like structures, green plants and albino plants, respectively

Table 5-2. Effect of antioxidants on embryo like structures (ELS), green and albino plants in triticale and wheat genotypes.

Experiment No. 1: Evaluation of mitochondrial antioxidants

				Wheat							
	Triticale			SWS 411		SWS 366		Sadash		Sadash	
Antioxidants	N	ELS	GP	N	GP	N	GP	N	ELS	N	AP
Control	16	30b	5bc	11	0b	10	0b	17	11ab	16	8ab
Proline	17	39a	11a	9	2a	8	2a	15	8abc	15	5bc
MB	16	26bc	7b	7	0b	7	0b	17	10abc	17	6abc
DMAP	6	23bc	3c	9	2a	7	2a	12	7c	12	3c
NtBHA	6	19c	3c	8	2a	6	0b	17	13a	17	8a
All	7	25bc	6bc	6	0b	7	0b	9	7c	9	4c

Experiment No. 2: Evaluation of plastid antioxidants

					Wheat		
		Triticale			Mean Squares sliced by Antioxidants [a]		
Antioxidants	N	ELS	GP	AP	*df*	ELS	GP
Control	15	152b	2bc	1bc	3	2030**	488**
ASC1X	13	152b	2bcd	1bc	3	3024**	154**
ASC10X	12	90bc	1cd	2ab	3	6835**	344**
GSH1X	12	163b	3a	3a	3	5469**	517**
GSH10X	12	292a	3a	1bc	3	6770**	315**
SA1X	15	163b	2bcd	2ab	3	3774**	234**
SA10X	11	31c	1d	0c	3	3565**	173**

N represents number of repetitions. ELS, GP and AP stands for embryo-like structures, green plants, and albino plants
Values followed by same letter do not differ significantly within each genotype (Duncan Multiple Range Test ($\alpha \leq 0.05$))
[a] represents LS Means obtained from 50 mm Petri dish containing 100,000 microspores
** significant at 0.01 levels of probability

5.7 Figures

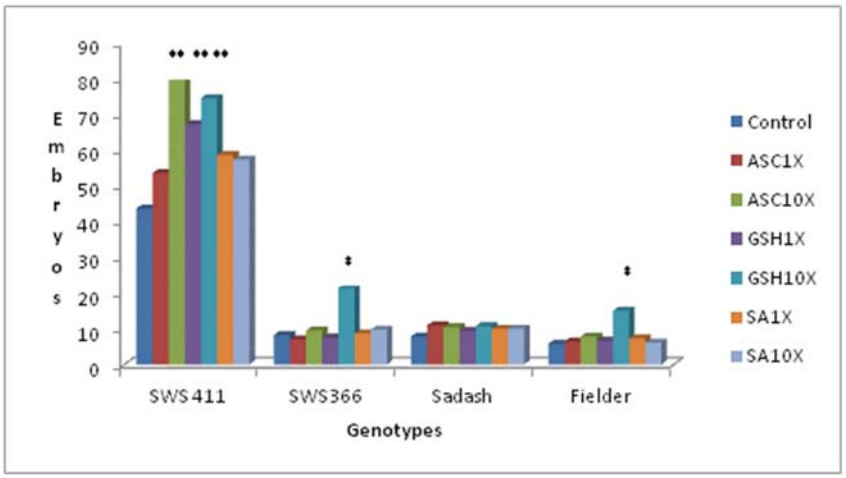

Fig. 5-1. Mean number of wheat embryos per genotype and chloroplast antioxidants.

Values significantly different from control at 0.05 (single asterisk) and 0.01 (double asterisk) levels of probability, respectively. LS mean values were obtained from 50 mm Petri dish containing 100,000 microspores. ASC1X stand for ascorbate 200 nM; ASC10X for ascorbate 2 µM; GSH1X for glutathione 200 nM; GSH10X for glutathione 2 µM; SA1X for salicylic acid 200 nM; SA10X for salicylic acid 2 µM.

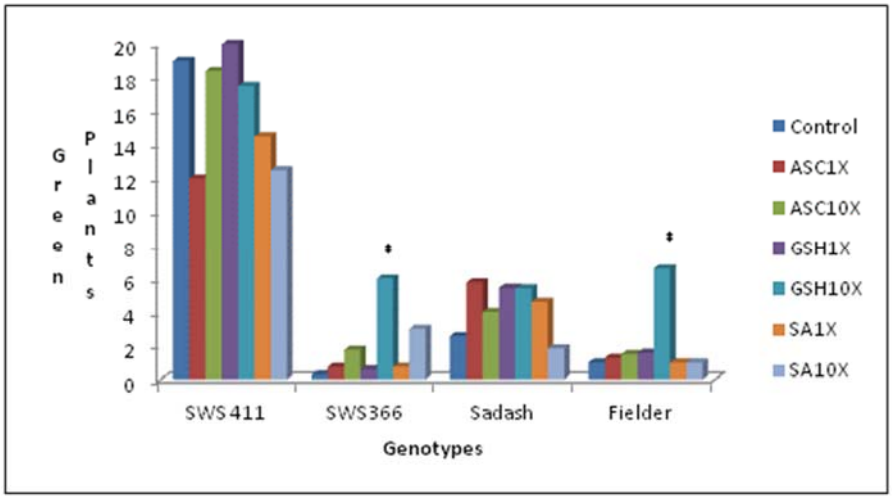

Fig. 5-2. Mean number of wheat green plantlets per genotype and chloroplast antioxidants.

Single asterisk: Significantly different from control at 0.05 level of probability. LS mean values were obtained from 50 mm Petri dish containing 100,000 microspores. ASC1X stand for ascorbate 200 nM; ASC10X for ascorbate 2 µM; GSH1X for glutathione 200 nM; GSH10X for glutathione 2 µM; SA1X for salicylic acid 200 nM; SA10X for salicylic acid 2 µM.

6.0 Induction medium osmolality improves microspore embryogenesis in wheat and triticale[3]

6.1 Introduction

Doubled haploid (DH) plants are produced in plant breeding programs throughout the world to achieve homozygosity in a single generation, reducing the breeding cycle by 3-4 years. The production of DH plants in wheat is heavily reliant on anther culture and maize pollen-based hybridization, which are labor intensive and time consuming procedures. Isolated microspore culture (IMC) is well established in various crop species like *Hordeum vulgare* (Kumlehn et al. 2006), *Zea mays (Xie et al. 1996; Zheng et al. 2003)*, *Brassica* sp. (Custers 2003; Ferrie 2003), and *Nicotiana* sp. (Garrido et al. 1995). Green plant production in *Triticum aestivum* through IMC is problematic compared to anther culture or maize pollen based hybridization. The main limiting factors are high frequency of cell death in the first 24 hours of culture, poor production of embryo or embryo like structures (ELS), genotypic effects, and high frequency of albino plantlets. Therefore, the optimization of the frequency of green plant production through the isolated microspore process is of primary concern. Research has focused on altering the growth and development conditions of donor plants, stage of microspores, flower/anther pre-treatment, microspore isolation procedures, microspore density and composition of induction and regeneration media (Cistue et al. 2009; Hu and Kasha 1999; Zheng et al. 2003) to improve the overall process of microspore embryogenesis in wheat.

Osmolality plays an important role in the inward and outward movement of water from plant cells. This depletion of water by osmotic stress is an important signal, or pretreatment, that triggers microspore embryogenesis and directs proper embryo development in various plant species (Wojnarowiez et al. 2004). Experimental manipulation of cellular water level aided embryo development in *Medicago sativa* and *Picea abies (Roberts and Dunwell 1990; Xu et al. 1990),* suggesting that the alteration of

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induction medium osmolality during microspore embryogenesis could lead to increased green plant production in wheat and triticale. Sugars play a significant role in maintaining osmotic pressure during cell and tissue culture (Zhou et al. 1991). The eliciting effect of osmolality has been recognized for a long time (Lichter 1981) and the effects of osmolality modifications have been studied in various crops, such as *Pisum sativum (Ochatt et al. 2009)*, *Cicer arietinum (Grewal et al. 2009)*, *Hordeum vulgare* (Hoekstra et al. 1993), *Asparagus officinalis* (Delaitre et al. 2001), *Brassica* spp. (Lionneton et al. 2001) and *Secale cereal* (Guo and Pulli 2000). Isolated microspores are very sensitive to osmotic pressure, and unsuitable osmolality has a severe detrimental effect, causing shrinkage or breakage, leading to death (Guo and Pulli 2000). Osmolality during the induction phase of isolated microspores also influences microspore viability and sustains cell division especially during the early stage of embryogenesis (Ayed et al. 2010; Ochatt et al. 2009). Furthermore, carbohydrates in the microspore culture medium serve a dual purpose; first as a source of energy and second as an osmoprotectant. Sugar alcohols can pass through plant cell membranes, resulting in temporary plasmolysis that is often followed by osmotic recovery (Attree and Fowke 1993). Mannitol has been reported to penetrate the cells easily and slowly due to the presence of six hydroxyl groups (Diamond and Wright 1969) and this is one of the main reasons that mannitol has been used extensively as an external osmoticum. It has been reported to improve the rate of regeneration in durum wheat (Cistue et al. 2006). A change in osmolality has also been reported using ficoll; 200 g/l of ficoll resulted in an increase of 160 mOsm kg^{-1} and more green plants during wheat anther culture (Zhou et al. 1991). Ochatt et al. (2009) analyzed different osmotic pressures using varying proportions of sucrose and mannitol in the induction medium to improve androgenesis from isolated microspores of some legume species (*Fabaceae*) and found a better response for sucrose than mannitol. The adjustment of osmotic pressure in culture medium to 350 mOsm kg^{-1} in barley increased the ratio of green/albino plants from 1:1 to 34:1 and 15% of the microspore population develop into embryo like structures (Hoekstra et al. 1993). Different optimal osmolality levels have been suggested in various species: 240 mOsm kg^{-1} in *Cajanus cajan* L. (Kaur and Bhalla 1998), 508 mOsm kg^{-1} in *Glycine max* L. (Zhao et al. 1998) and 300 mOsm kg^{-1} in *Vigna unguiculata* L. (Mix and Wang 1988).

Wheat and triticale microspore culture may benefit from an improved induction medium osmolality level, and mannitol is a natural candidate identified as an osmoprotectant for developing microspores and embryos. The present experiment was designed to identify and recommend an optimum level of induction medium osmolality to improve the overall process of microspore embryogenesis in wheat and triticale.

6.2 Materials and Methods

6.2.1 Plant materials, microspore isolation, induction and regeneration

Donor plant material consisted of the triticale cultivar AC Ultima and the spring wheat cultivar AC Andrew. The donor plants were grown as described in section 5.2 whereas microspore isolation, induction and regeneration was performed as described in sections 5.2.1 and 5.2.3.

6.2.2 Treatments

Five treatments were developed to evaluate an induction medium osmolality ranging from 300 to 500 mOsm kg^{-1}: T1, NPB99 induction medium supplemented with 10% Ficoll and 90g/l Maltose, at 300 mOsm kg^{-1}; T2 at 350 mOsm kg^{-1}, adding 9.1085 g/l Mannitol; T3 at 400 mOsm kg^{-1}, adding 18.217 g/l Mannitol; T4 at 450 mOsm kg^{-1} adding 27.3255 g/l Mannitol; and T5 at 500 mOsm kg^{-1} adding 36.434 g/l Mannitol. The pH was kept at 7.0.

6.2.3 Data recording and statistical analysis

Data were recorded for number of ELS, green and albino plants. The experiment was laid out in completely randomized design with different replications in triticale and wheat. The experimental unit was one Petri dish containing $1x10^5$ microspores. Analysis of variance (ANOVA) was conducted following Steel et al. (1997) using the GLM procedure of SAS v9.2 (SAS 2003). The data for ELS, green and albino plants were tested for normality and homogeneity of variance using the Kolmogorov–Smirnoff (Massey 1951) and Levene's tests (Levene 1960) respectively. The data were also transformed if it did not meet the assumptions of ANOVA. If the interaction between osmolality and genotype was found significant, the interaction effect was partitioned by osmolality using SLICE command in PROC GLM to estimate the effect of osmolality levels separately. In case of significant

osmolality effects, least square means (LS Means) of control was compared with other osmolality levels (planned comparisons) using the PDIFF statement in (PDIFF=Control) in SAS. In case of non-significant interaction effects, we studied the main effects and LS Means were separated using Duncan Multiple Range Test ($\alpha \leq 0.05$).

6.3 Results

The experiment was conducted in order to assess the effect of osmolality on microspore embryogenesis in terms of frequency of ELS, green and albino plants. The results presented in Table 6-1 revealed that osmolality levels used in this study affected the number of ELS, green and albino plants. There were significant differences among genotypes (AC Ultima and AC Andrew) and osmolality levels for green and albino plant production, whereas highly significant non-crossover interaction between genotype and osmolality was also observed for ELS (Table 6-1). The ELS produced were significantly more abundant at osmolality 350 mOsmKg^{-1} (450 and 331 in AC Ultima and AC Andrew, respectively) as compared to other treatments (Fig. 6-1). The standard induction medium (control) having osmolality at 300 mOsm Kg^{-1} produced only 252 and 222 ELS in AC Ultima and AC Andrew, respectively (Fig. 6-1). In both genotypes, ELS started decreasing when osmolality increased beyond 350 mOsm Kg^{-1}, and the minimum ELS were obtained at 500 mOsm Kg^{-1} (Fig. 6-1). The interaction between genotype and osmolality was found to be non-significant for green and albino plants, therefore, we investigated the main effects (*i.e*., osmolality levels and genotypes) individually (Fig. 6-2 and 6-3). Our results indicated that induction medium at 350 mOsm Kg^{-1} generated a significantly higher number of green plants (4.3) as compared to standard induction medium (control) at 300 mOsm Kg^{-1}, which gave rise to only two green plants per experimental unit (Fig. 6-2). The lowest number of albino plants was obtained from induction medium at 450 mOsm Kg^{-1} and 500 mOsm Kg^{-1} (Fig. 6-2), which was attributed to the reduced number of ELS in those treatments. Moreover, 4% of the ELS were converted into albino plants in a 500 mOsm Kg^{-1} induction medium whereas the ELS to albino ratio was only 2% at 350 mOsm Kg^{-1}. Furthermore, the number of green and albino plants also varied significantly between two species (Fig. 6- 3). Overall, one AC Ultima microspore extraction from eight spikes gave 14 induction Petri dishes, which produced a total of 60 green plants per extraction, using 350 mOsm Kg^{-1} induction

medium compared to 28 green plants from control (300 mOsm Kg^{-1}). In AC Andrew, we got about 7 Petri dishes per extraction which produced 30 green plants at 350 mOsm Kg^{-1} osmolality whereas only 14 green plants were obtained when induction medium's osmolality was at 300 mOsm Kg^{-1} (control).

6.4 Discussion

Mannitol is a sugar alcohol that is produced by a few plants as a photosynthetic product (Trip et al. 1964) and cells of various plant species take up mannitol very slowly (Cram 1984); thus it can be used as an osmolality agent to maintain osmotic pressure in *in vitro* experiments. Mannitol has also been used in the induction medium as a starvation treatment at 4°C where authors reported a significant increase in the induction frequency of triticale anther culture but in a genotype dependent manner (Immonen and Robinson 2000). Our results suggest that the application of mannitol at 9 g/l as an osmolality agent in the induction medium improves microspore embryogenesis in both wheat and triticale, yielding more ELS as compared to other osmolality levels (Fig. 6-4). It has been reported that the use of mannitol as an osmoticum to maintain osmolality at 364 mOsm Kg^{-1} yielded a maximum number of green plants in barley anther culture and plastids did not accumulate any starch that facilitated sporophytic development (Wojnarowiez et al. 2004). We also obtained the maximum number of green plants when induction medium osmolality was kept at 350 mOsm Kg^{-1} (Fig. 6-2). Genotypic differences with respect to green plants were also noticed between AC Andrew and AC Ultima (Fig. 6-3). The number of albino plants obtained from an induction medium having an osmolality of 350 mOsm Kg^{-1} were statistically similar to those obtained in a control treatment, suggesting genotypic dependency of albinism that cannot be altered with osmolality in these species. However, the conversion ratio of ELS to albino plants was reduced (1.9%) in 350 mOsm Kg^{-1} induction medium as compared to control medium (2.9%), mannitol free, suggesting a role of osmotic pressure in reducing albinism in wheat and triticale as reported earlier in barley (Wojnarowiez et al. 2004). Induction medium osmolality beyond 350 mOsm Kg^{-1} resulted in a sharp decline in the number of green plants that can be attributed to the significant reduction in ELS production.

6.5 Conclusion

The data presented in this study indicate that induction medium osmolality affected ELS and green plant production in wheat and triticale. Induction medium osmolality at 350 mOsm Kg^{-1} improved number of ELS and green plant production. These beneficial effects are possibly due to optimal environmental conditions to the microspores necessary for their survival, reprogramming and continued development into ELS during the process of embryogenesis.

6.6 Tables

Table 6-1. Analysis of variance for ELS, green and albino plants.

Source	*df*	Mean Squares		
		ELS	Green Plants	Albino Plants
Genotype	1	210358^{**}	26^{**}	163^{**}
Osmolality	4	381562^{**}	56^{**}	63^{**}
Genotype x Osmolality	4	27111^{**}	2^{ns}	14^{ns}
Error	146	5581	3	9

ns, ** not significant and significant at 0.01 levels of probability respectively

6.7 Figures

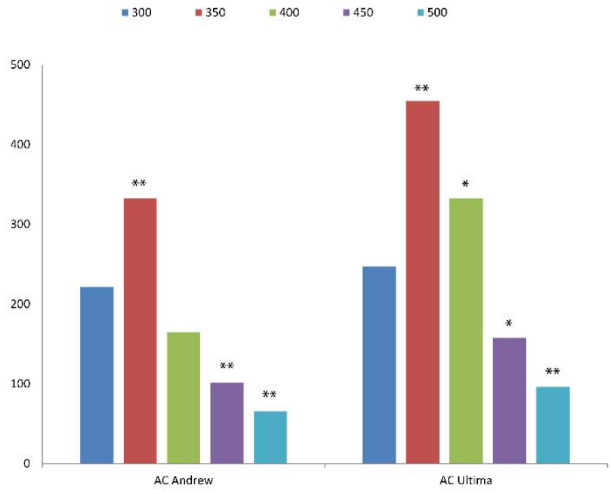

Fig. 6-1. Mean number of wheat (AC Andrew) and triticale (AC Ultima) embryos per osmolality level.

Values significantly different from control at 0.05 (single asterisk) and 0.01 (double asterisk) levels of probability, respectively. LS Means values were obtained from 50 mm Petri dish containing 100,000 microspores. 300, 350, 400, 450 and 500 represents osmolality levels in terms of mOsm Kg^{-1}

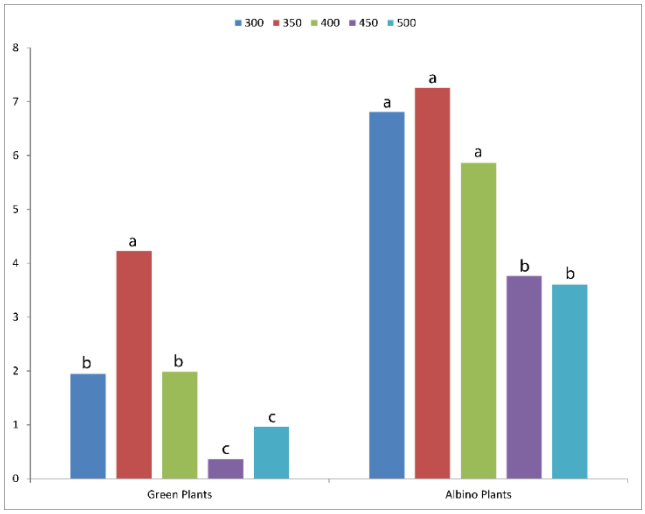

Fig. 6-2. Mean number of green and albino plants per osmolality level.

Values followed by same letter do not differ significantly from each other at 0.05 level of probability. LS Means values were obtained from 50 mm Petri dish containing 100,000 microspores. 300, 350, 400, 450 and 500 represents osmolality levels in terms of mOsm Kg^{-1}

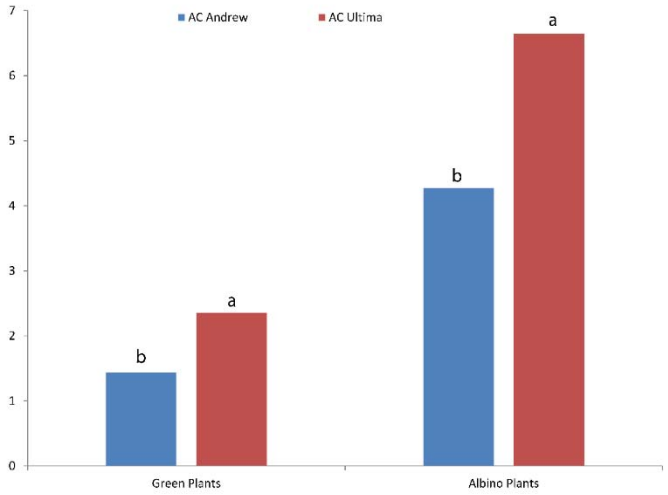

Fig. 6-3. Mean number of green and albino plants per genotype.
Values followed by same letter do not differ significantly from each other at 0.05 level of probability.

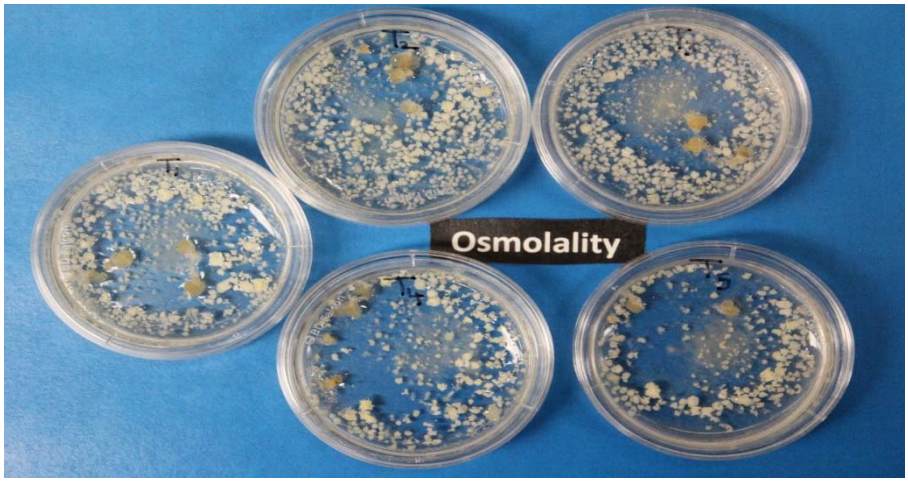

Fig. 6-4. Production of embryo-like structures at different osmolality levels in AC Ultima.

7.0 Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale[4]

7.1 Introduction

Contamination in plant tissue culture continues to obstruct successful experimentation and results in material and economic losses. Plant media are rich in sugars, amino acids, and minerals, and are thus a potential source of nutrients for microorganisms such as bacteria, yeasts and other fungi. These microorganisms compete with plant tissues for nutrients and cause tissue mortality, necrosis, reduce root and shoot proliferation, and sometimes whole tissue/plant death (Leifert et al. 1991b). The source of contaminants in *in vitro* cultures may arise from infected donor plants, manipulation in laminar flow hoods, and inadequate septic conditions (Shen et al. 2010; Thomas et al. 2011). Bacterial contaminants may be epiphytic, living on the plant surface (Hirano and Upper 1990) and chemical disinfectants can be used for their elimination. Endophytic bacteria lives in the intercellular spaces within the plants (Gunson and Spencer-Phillips 1994) and pose a great threat to tissue culture techniques (Cassells 2001; Kneifel and Leonhardt 1992) because they are very difficult to eradicate by surface sterilization (Herman 2004; Reed et al. 1995). Many environmental bacteria flourish on plant tissue culture medium, while others (like pseudomonads) are not visible but can still reduce cell growth and development (Leifert and Cassells 2001). Various measures have been used to overcome bacterial contamination in plant tissue culture by manipulating environmental (incubation temperature and pH) and nutritional factors (Leifert et al. 1991a; Long et al. 1988). The use of antibiotics to control bacterial contamination in plant tissue culture systems has been widely investigated (Falkiner 1998) but their phytotoxic effects on plant tissues often restricts their use (Silva et al. 2003; Shehata et al. 2010). In contrast, some studies have reported negligible incidence/effect of antibiotics such as cefotaxime (Danilova and Dolgikh 2004; Mittal et

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al. 2009), Vancomycin (Silva and Fukai 2001), Timentin (Supena et al. 2006), Carbenicillin (Yu and Wei 2008) and Rifampicin (Supena et al. 2006) on plant tissues and growth.

Microspore embryogenesis is a process where a haploid embryo is produced from the uninucleated cell. In this process, gametophytic pollen maturation is interrupted by pre-treatments, and cell division initiated to form embryos. The microspore divides symmetrically in majority of the genotypes to form an embryo and ultimately to form haploid or doubled haploid (DH) plants. In recent years, microspore culture has become a method of choice to produce populations of DH plants, because homozygosity is fixed in a single generation. The several advantages of isolated microspores embryogenesis over other DH production methods include i) millions of physiologically uniform microspores can be obtained in few minutes, ii) this single cell culture platform offers many opportunities for process improvement, and iii) physiologically uniform microspores can be used as targets for cell biology, embryogenesis and genetic engineering studies. In spite of a large number of published studies, the low efficiency of DH production via microspore culture in cereals (especially wheat) has limited the use of this technique in wheat breeding programs for accelerated cultivar development. Isolated microspore culture is still at the research and development stage due to problems of contamination, early cell death, low frequency of embryogenesis, rate of embryo conversion to plant, albinism and plant death during chromosome doubling. Contamination during IMC may arise due to i) microspore from contaminated donor plants ii) the occurrence of surface sterilization resistant microorganisms and, iii) the operator. While antibiotics would control growth of most of the bacterial contaminants, some antibiotics could bring additional benefits for small grain cereal microspore culture. Recently, Yu and Wei (2008) investigated the contribution of Cefotaxime and Carbenicillin to wheat calli regeneration and reported a 55% increase in regeneration of green wheat plants with the addition of 100 mg/l Cefotaxime in the regeneration medium. Similarly, Grewal et al. (2006) reported increased rates of somatic embryogenesis in indica rice, using Cefotaxime. Simmonds and Grainger (1993) studied the toxicity of seven antibiotics (Hygromycin, G418, Bekanamycin, Kanamycin, Spectinomycin, Cefotaxime and Vancomycin) in wheat protoplast culture. Vancomycin promoted cell division and enhanced protoplast plating efficiency two-fold. They further reported that Vancomycin, Cefotaxime and

Spectinomycin were least toxic while G418, Hygromycin, Kanamycin and Bekanamycin were highly phytotoxic to wheat cells (Simmonds and Grainger 1993). Supena et al. (2006) also reported a positive effect of a combination of antibiotics (timentin and rifampicin at a concentration of 200 and 10 mg/l respectively) for producing DH plants in Indonesian hot pepper (*Capsicum annuum* L.) through shed-microspore culture.

We conducted the present study to investigate the reported benefits of two antibiotics in wheat and triticale isolated microspore culture. Vancomycin and Cefotaxime were studied against recurrent sources of contamination in our lab and in the induction phase of microspore culture. The main objectives were to identify occasional contaminants from our isolated microspores cultures, evaluate two antibiotics to control bacterial growth and study the effect of these antibiotics on the formation of microspore-derived ELS, green and albino plants in triticale and wheat.

7.2 Materials and methods

7.2.1 Collection of contamination samples

We have been actively working for some years to improve IMC techniques in wheat and triticale (Eudes and Amundsen 2005; Eudes et al. 2003; Asif et al. 2013). We collected various contaminated Petri dishes of wheat and triticale microspore culture over a period of six months (example shown in Fig. 7-1a). A selection of 12 representative dishes of the visual diversity of contaminations was retained for this study. The colonies from each contaminated Petri dish was then streaked (Fig. 7-1b) onto Lysogeny broth (LB) medium and allowed to grow at 27^{o}C, as per microspore culture, for 48-72 hours.

7.2.2 Identification of bacterial isolates

The isolates were sub-cultured multiple times on tryptic soy agar (TSA; Difco) at 27^{o}C for purification. Colony and cellular morphologies were observed after 24-48 hours at both 27 and 39^{o}C. Anaerobic growth was tested on TSA at 27^{o}C in an anaerobic chamber having 5, 10 and 85% CO_2, H and N respectively. All other physiological tests were performed at 27^{o}C *for Erwinia, Panotea* and *Pseudomonas* and at 39^{o}C for *Staphylococcus*. Acid production from carbohydrates was determined with the API 50CHE gallery (bioMerieux; *Erwinia, Pantoea*) as per the manufacturer's instructions; or using purple agar base medium containing 1% carbohydrate (*Staphylococcus*) (Freney et

al. 1999). Arginine dihydrolase activity (Smibert and Krieg 1994), malonate utilization (Smibert and Krieg 1994) and urease activity (Freney et al. 1999) were tested as described elsewhere. In order to conduct chromatographic analysis of the cellular fatty acids, the isolates were grown on TSA for 24 hours and microbial identification system was used to prepare and analyze samples (Miller 1982). Bacterial DNA was isolated using a Qiagen Genomic DNA kit with the following modifications: 20 µl of lysozyme (100 mg/ml) along with 10 µl of mutanolysin (2.5 U/µl) were added to the reaction mixture during the proteinase K incubation. The 16S ribosomal RNA gene was amplified using oligonucleotide primers pairs 27f and 1492r as previously described by Gurtler and Stanisich (1996). The polymerase chain reaction (PCR) was performed by 1× Qiagen HotStarTaq plus Master Mix (Qiagen Inc.). Each PCR contained 2 µl of DNA template and 300 nM (final concentration) of each primer. The amplicons were purified and commercially sequenced using the 27f primer (Eurofins MWG Operon). Determined 16S ribosomal RNA gene sequences were at least 950 bp in a continuous stretch.

7.2.3 Bacterial isolate antibiotic susceptibility

The isolates were grown for 48-72 hours at 27^0C on LB medium in individual Petri dishes that were supplemented with different antibiotics (Table 7-1) to evaluate colony sensitivity towards antibiotics. The following antibiotic treatments were applied into the antibiotic assay on isolates and in isolated microspore cultures of triticale and wheat genotypes: T_1: Control (no antibiotic); T_2: Vancomycin at 100 mg/l; T_3: Vancomycin 500 mg/l; T_4: Cefotaxime at 50 mg/l; T_5: Cefotaxime 100 mg/l; T_6: Vancomycin 100 mg/l and Cefotaxime 50mg/l; and T_7: Vancomycin 500 mg/l and Cefotaxime 100mg/l. The isolates' growth was noted as no inhibition (+++), weak inhibition (++), strong inhibition (+) and no growth (-) relative to control, where no antibiotic was applied.

7.2.4 Plant material, microspore isolation, induction and regeneration

The experimental material consisted of one triticale (AC Ultima) and two wheat cultivars (AC Carberry and AC Andrew). The donor plants were grown as described in section 5.2 whereas microspore isolation, induction and regeneration was performed as described in sections 5.2.1 and 5.2.3.

7.2.5 Data recording and statistical analysis

Signs of contamination were checked on a daily basis and the development of multicellular structures was first verified at 10-14 days. The data were recorded for number of embryos or embryo-like structures, green and albino plants. The experiments were laid out in completely randomized designs with differing replications for each genotype (see results). The experimental unit was one Petri dish containing $1x10^5$ microspores. The data were analyzed separately for each species and were subjected to normality and homogeneity of the variance testing using Kolmogorov–Smirnoff and Levene's tests, respectively (Steel et al. 1997). Analysis of variance (ANOVA) was performed using the GLM procedure of SAS for all three variables studied in the experiments to evaluate the effect of antibiotics on number of ELS, green and albino plants (SAS 2003). Least square (LS) means of antibiotic treatments were compared with the control using a contrast statement. If the interaction effects between genotype and antibiotics (in wheat) were found to be significant, the effect was partitioned by antibiotic using the SLICE command in PROC GLM of SAS (v9.2) to measure the effect of antibiotic individually. Once the antibiotic effect using SLICE command was found to be significant, LS mean of control was compared with other antibiotics.

7.3 Results

Isolate identification and susceptibility assay

Identification of bacterial species and yeast from contaminated samples of IMC can offer important information regarding the sources of contamination along with mitigation strategies to eliminate the problem of contamination. In this study, from 12 original dishes, a total of 25 bacteria were purified and identified. Four yeast isolates were also purified (Table 7-1).

The six different treatments of antibiotics along with control (no antibiotic) were tested against the 25 bacterial isolates. Uncovered Cefotaxime (T_5: Cefotaxime 100 mg/l) was the most effective antibacterial agent in the present study against all isolates but one. No antibacterial treatment was found to be effective against the *Pseudomonas* sp. isolate C4, although the combination of Vancomycin and Cefotaxime resulted in a weak inhibition (T_6 and T_7). Bacterial isolate growth inhibition resulted from T_6 (23 isolates), T_7 (23), T_4 (21), T_3 (17) and T_2 (16), respectively. We did not observe differential growth inhibition in

the yeast isolates, as expected with eukaryotes. Since both antibiotics resulted in growth inhibition of some isolates, we were interested in further testing their incidence in embryo/embryo-like structures, green and albino plants production during IMC in triticale and wheat.

7.3.1 Evaluation of antibiotic treatments during microspore embryogenesis

We subsequently supplemented induction medium (NPB99+10F) with antibiotics treatments T_1 to T_7 and observed their effects in the control of accidental contamination and in microspore embryogenesis. Microspore culture of triticale cultivar AC Ultima is well established in our lab (Eudes and Amundsen 2005), and therefore was adopted for a rapid evaluation of the incidence of these antibiotic treatments on the cell culture, and then validated in wheat modern cultivars AC Carberry and AC Andrew. The number of accidentally contaminated Petri dishes was limited to 6 out of 249, and mainly obtained in T_1 control treatment (4 dishes), as well as T_2 (1 dish) and T_6 (1 dish). In triticale, our results indicated that antibiotics increased the frequency of ELS, green and albino plants (Table 7-2). As a result, the frequency of ELS increased from 219 (T_1: control) to 403 when induction medium was supplemented with Cefotaxime 50 mg/l (T_4). The induction medium supplemented with Cefotaxime 100 mg/l (T_5) produced 314 ELS, which was not statistically different from T_4 (Table 7-3). Similar results were obtained with respect to green plant production (7 green plants with the use of Cefotaxime 50 mg/l, 6 with Cefotaxime 100 mg/L and 4 in control). The reduced frequency of albino plants was observed for the lowest dose of Vancomycin, and could be attributed to the trend for reduced number of ELS and no different number of green plants in all Vancomycin containing treatments. In wheat, significant genotype x antibiotics interaction was noticed for ELS, green and albino plants. Therefore, we sliced the interaction effect by antibiotics, and results indicated that Cefotaxime 100 mg/l (T_5) supplemented induction medium significantly increased the number of ELS and green plants in AC Carberry and AC Andrew as compared to control (T_1). This treatment (T_5) resulted in the production of 433 ELS in AC Carberry and 411 ELS in AC Andrew whereas in the control we obtained only 275 and 218 ELS, respectively (Table 7-3). The treatment (T_5) also affected the number of green plants in these two genotypes. We report 7 and 9 green plants per Petri dish in AC Carberry and AC Andrew respectively (Table 7-3). All Vancomycin treatments resulted in a significant

reduction of ELS in AC Carberry, while in AC Andrew only T_2 and T_3 resulted in a trend of reduced number of ELS. Interestingly, the dual antibiotic treatments T_6 and T_7 resulted in an increase of AC Andrew ELS, which was significant for T_7. In AC Andrew, antibiotics did not change the production of albino plants while in cv. AC Carberry, the treatments significantly reduced the number of albino plants and minimum albino plants were obtained when induction medium was supplemented with Cefotaxime at 100 mg/l (T_5: Table 7-3). Among other treatments, T_4 (Cefotaxime: 50 mg/l) also helped to increase the frequency of ELS in AC Carberry and AC Andrew and green plant production in AC Andrew (Table 7-3). Treatments including Vancomycin resulted in inconsistent effects on green plant production, most often reducing it.

On average, one triticale microspore extraction from eight spikes resulted in 14 induction Petri dishes, which produced a total of 84 green plants using 50 mg/l Cefotaxime supplemented induction medium, compared to 56 green plants from the control. Similarly, in wheat one extraction from eight spikes resulted in seven Petri dishes which produced 49 and 63 green plants in AC Carberry and AC Andrew, respectively, using 100 mg/l Cefotaxime.

7.4 Discussion

We found five recurrent bacterial species (*Erwinia aphidicola*, *Pantoea agglomerans*, *Pseudomonas* sp., *Staphylococcus epidermis* and *Staphyoloccus warneri)* along with yeast from a selection of 29 contaminated Petri dishes. *Staphylococcus epidermis* was the most prevalent bacteria in our contaminated samples. Bacterial and yeast contaminations are particularly problematic in IMC due to rapid colony growth during the first 24-48 hours in rich liquid induction medium, and result in plant cell death and total loss of the culture. Moreover, the bacteria and yeast stains adapt very well to the cultural and environmental conditions (temperature, darkness, and pH) required for microspore development. Bacterial contamination can be introduced into the culture from various means like unhealthy donor plants, during microspore isolation and purification, and breaks in the aseptic condition (Gilbert et al. 1991). Aseptic culture practices have been developed for eliminating contamination and the complemented with antibiotics (Fossard and Fossard 1988). In microspore culture, the identification of contaminants can provide valuable information regarding their sources, in efforts to correct this problem.

Erwinia aphidicola was first isolated from the gut of pea aphid, *Acyrthosiphon pisum* (Hammes and Neuhaus 1974; Rodriguez-Serrano et al. 2012); however, it was only termed as a plant pathogen by Santos et al. (2009) who isolated it from *Phaseolus vulgaris* and *Pisum sativum* in Spain where it was held responsible for causing leaf spot diseases in these crops. *Pantoea agglomerans* is also commonly isolated from plant surfaces, seeds and fruits (Leifert and Waites 1990). *Pseudomonas* is a gram-negative bacteria with greater than 200 validly named species belonging to the genus. Their widespread occurrence is associated with numerous environments including water and plant seeds. *Pseudomonas* is also associated with wet surfaces of air conditioning systems and drain pans and these species were among the most frequently found bacteria in plant tissue culture procedures (Leifert and Waites 1992). *Staphylococcus epidermis* and *Staphylococcus warneri* are Gram positive bacteria. The genus *Staphylococcus* often resides on human skin scales (Trudeau and Fernandezcaldas 1994). Contamination due to *Staphylococcus* usually arise during medium preparation and dissection in aseptic conditions (Danby et al. 1994; Leifert et al. 1991a; Leifert and Cassells 2001; Leifert et al. 1991b). Therefore, it can be assumed that the operator was the main source of *Staphylococcus* sp. contamination.

Cefotaxime at 100 mg/l (T_5) gave the best control of these bacterial isolates, except for *Pseudomonas*, followed by T_6 (Van 100 + Cef 50), T_7 (Van 500 + Cef 100), T_4 (Cef 50), T_3 (Van 500) and T_2 (Van 100) (Table 7-1). The cephalosporin antibiotic, Cefotaxime (OmnataxTM, $C_{16}H_{16}N_5O_7S_2Na$), is a β-lactam antibiotic that inhibits cell wall synthesis in the bacterial cells and ultimately gives rise to cell lysis (Leifert et al. 1990). Third generation antibiotics like Cefotaxime have a wide spectrum against Gram-negative bacteria and are preferred agents, due to their low toxicity (Ryan et al. 2010). The inability of Cefotaxime and many other antimicrobials to overcome *Pseudomonas* spp. contamination would occur due to the presence of mutational and plasmid-mediated resistance, and also to the existence of porins that restrict their entry into periplasmic space (Ryan et al. 2010). Vancomycin is an antibiotic agent that has been classified as a glycopeptide and is effective against Gram-positive bacteria. Its mode of action involves the inhibition of cell wall synthesis in bacteria. It binds to the building blocks of peptidoglycan (monomers of N-acetylglucosamine and N-acetylmuramic acid) and prevent

the enzyme, transpeptidase forming new blocks of cell wall, causing leakage in the cellular contents of the bacteria and ultimately death (Hammes and Neuhaus 1974).

The effect of Cefotaxime and Vancomycin doses on microspore culture in triticale and wheat genotypes were also studied. These antibiotics were chosen for their abilities to control bacterial contaminations and to enhance growth in protoplast and plant somatic tissue cultures (Simmonds and Grainger 1993; Fang and Hsu 2012; Mbah and Wakil 2012; Pence 2005; Silva and Fukai 2001; Seyring 1999). Moreover, combinations of antibiotics have also been used previously in tissue culture systems (Leifert et al. 1991a; Young et al. 1984) and have been recommended (Reed et al. 1997; Wojtania et al. 2005). Our findings clearly demonstrated the greatest increase in the production of ELS and green plants using Cefotaxime at 50 mg/l in triticale and 100 mg/l in wheat. Cefotaxime also decreased the number of albino plants in wheat cv. AC Carberry (Table 7-3). Although genotypic dependency is very common in cereal tissue culture (Haliloglu 2006; Heberle-Bors 1984), the three cultivars' frequencies of ELS and green plants were all enhanced in the presence of Cefotaxime. These findings in wheat and triticale are in agreement with the previous observations where Cefotaxime has been described to improve callus growth during somatic embryogenesis and enhanced subsequent plant regeneration in wheat (Borrelli et al. 1992; Mathias and Boyd 1986), maize (Danilova and Dolgikh 2004), barley (Mathias and Mukasa 1987), millet (Pius et al. 1993), sorghum (Rao et al. 1995), cotton (Agrawal et al. 1998) and rice (Grewal et al. 2006).

Microspores are exposed to various stresses (cold, heat and/or osmotic) in order to switch their gametophytic pathway towards sporophytic development. Isolation and purification procedures during IMC result in severe oxidative damage to the microspores. These stresses could increase ethylene production (Pius et al. 1993) and the level of reactive oxygen species (ROS) and nitric oxide (NO) (Rodriguez-Serrano et al. 2012) in the culture during the induction phase of microspore embryogenesis often resulting in death. The stress treated microspores have been successfully treated with various ROS and NO scavengers during early stage of embryogenesis to reduce stress-induced programmed cell death (PCD) in barley microspores (Rodriguez-Serrano et al. 2012). The role of Cefotaxime to interfere/inhibit ethylene production in embryo cultures has also been reported by Pius et al. (1993). The exact mechanism of interference, either through the biosynthetic pathway or involvement in metabolic processes, is still unknown. A study of

three antibiotics viz. Carbenicillin, Cefotaxime and Vancomycin for controlling agrobacterium growth and determining phytotoxicity level in chrysanthemum and tobacco organogenesis revealed the phytotoxicity levels of these antibiotics as Carbenicillin>Vancomycin>Cefotaxime (Silva and Fukai 2001). In our present study, Vancomycin was phytotoxic on the microspore culture of wheat and triticale.

A Cefotaxime-related contamination-free environment allowed microspores to enter the embryogenesis pathway and reduced the occurrence of albinism. In a study conducted by Zaghmout and Torello (1992), the absence of Cefotaxime in the cultural medium yielded only albino plants. In this study, authors analyzed the effects of various plant growth regulators along with cefotaxime and reported an enhanced regeneration efficiency in ryegrass when the medium was supplemented with abscisic acid, kinetin or active charcoal but all plants produced were albino. However, pretreatment of calli with Cefotaxime (60 mg/l) prior to placing on regeneration medium for 6 weeks resulted in the production of green plants. Thus, Cefotaxime can be metabolized as a growth regulator (Mathias and Boyd 1986) and has been recommended for plant cell culture (Pollock et al. 1983) due to its role in stimulating mitotic division (Vaz et al. 1993), enhancing callus growth, embryogenesis and regeneration (Mathias and Boyd 1986; Mathias and Mukasa 1987), suppressing precocious embryoid germination (Maheshwari et al. 1995) and boosting the photosynthetic machinery (Zaghmout and Torello 1992). It has been reported that degradation of Cefotaxime by plant esterases might help to produce metabolites that can explain its growth regulator-like activity in culture (Mathias and Boyd 1986; Mittal et al. 2009). Thus, a supply of Cefotaxime in the induction medium led to increased production of ELS and green plants with less albinism.

7.5 Conclusion

We found five different and recurrent species of bacteria (*Erwinia aphidicola*, *Pantoea agglomerans*, *Pseudomonas* sp., *Staphylococcus epidermis* and *Staphyoloccus warneri)* along with yeast contaminating wheat and triticale IMC. These occasional contaminations were most likely introduced randomly by the operator. The results obtained in this study show that antibiotics especially, Cefotaxime, could help to solve the problem of microbial contamination during microspore embryogenesis in wheat and triticale. All but *Pseudomonas* sp. and yeast are successfully controlled by Cefotaxime

and/or Vancomycin. Interestingly, dramatic differences between these two antibiotics are reported in microspore culture. Only Cefotaxime resulted in consistent increases in microspore-derived multicellular structures and ratio of green plants, an effect not seen in microspore culture with Vancomycin. We concluded that induction medium supplemented with Cefotaxime is effective to eliminate most bacterial contaminations and to enhance embryo-like structure formation and green plant production during IMC in triticale and wheat. We recommend the systematic use of Cefotaxime at 100mg/l in microspore culture of these two species for production of DH plants.

7.6 Tables

Table 7-1. Antibiotic resistance profile of 25 bacteria and 4 yeast strains collected during induction phase of isolated microspore culture in triticale and wheat.

Isolate		T_1I	T_2	T_3	T_4	T_5	T_6	T_7
A1	*Staphylococcus warneri*	+++	+	+	+	+	-	+
A2	*Staphylococcus epidermis*	+++	-	-	-	-	-	-
A3	Yeast							
B1	*S. epidermis*	+++	-	-	-	-	-	-
B2	*S. epidermis*	+++	-	-	-	-	-	-
C1	*S. warneri*	+++	+	-	+	+	+	+
C2	*Pantoea agglomerans*	+++	+++	+++	+	+	+	+
C3	*P. agglomerans*	+++	+++	+++	+	+	+	+
C4	*Pseudomonas* sp	+++	+++	+++	+++	+++	++	++
D1	*S. epidermis*	+++	-	-	-	-	-	-
D2	*S. epidermis*	+++	-	-	++	-	-	-
D3	*Erwinia aphidicola*	+++	+++	+++	++	-	-	-
F1	*S. epidermis*	+++	-	-	-	-	-	+
F2	*S. epidermis*	+++	-	-	-	-	-	+
G1	*S. epidermis*	+++	-	-	-	-	-	-
G2	*S. warneri*	+++	+	-	-	-	-	-
H1	*S. warneri*	+++	+	+	-	+	+	-
H2	*S. epidermis*	+++	-	-	-	-	-	-
H3	Yeast							
I1	*S. epidermis*	+++	-	-	-	-	-	-
J1	*S. epidermis*	+++	-	-	-	-	-	-
J2	*S. warneri*	+++	+	-	-	-	-	-

J3	Yeast							
K1	*E. aphidicola*	+++	+++	+++	-	-	-	-
K2	*E. aphidicola*	+++	+++	+++	-	-	-	-
L1	*S. epidermis*	+++	-	-	-	-	-	-
L2	Yeast							
N1	*E. aphidicola*	+++	+++	+++	++	-	++	++
N2	*E. aphidicola*	+++	+++	+++	-	-	-	-

Ratings are relative to control for a given isolate. +++, ++, +, - represents no inhibition, weak inhibition, strong inhibition and no growth respectively.
Cef = Cefotaxime, Van = Vancomycin, T_1=Control, T_2=Van 100, T_3=Van 500, T_4=Cef 50, T_5=Cef 100, T_6=Van 100 + Cef 50, T_7=Van 500 + Cef 100

Table 7-2. Analysis of variance of triticale and wheat isolated microspore culture supplemented with antibiotics.

	Source	*df*	Mean Squares		
			ELS	Green Plants	Albino Plants
Triticale	Antibiotics (Ant)	6	41619**	44**	19**
	Error	27	7483	5	3
Wheat	Genotype (Gen)	1	82695**	121**	153*
	Ant	6	298375**	149**	142**
	Gen x Ant	6	90361**	19*	76*
	Error	174	7360	9	31

*, ** significant at 0.05 and 0.01 levels of probability respectively

Table 7-3. Effect of antibiotics on embryo like structures (ELS), green and albino plants in triticale and wheat genotypes.

	Triticale				**Wheat**							
	AC Ultima				**AC Carberry**				**AC Andrew**			
Antibiotics	**N**	**ELS**	**GP**	**AP**	**N**	**ELS**	**GP**	**AP**	**N**	**ELS**	**GP**	**AP**
T_1 (Control)	8	219	4	8	18	275	5	18	22	218	5	11
T_2 (Van 100)	5	135	4	4**	14	144**	2*	10**	12	149	3	6
T_3 (Van 500)	5	166	3	8	15	172**	4	10**	17	210	4	8
T_4 (Cef 50)	6	403**	7**	9	12	395**	6	12**	16	316**	8*	11
T_5 (Cef 100)	7	314*	6**	7	13	433**	7*	9**	20	411**	9**	12
T_6 (Van 100+Cef 50)	5	180	4	9	14	99**	2*	10**	12	263	6	9
T_7 (Van 500+Cef 100)	5	208	4	8	13	74**	5	12**	11	313*	7	11

N represents number of repetitions. ELS, GP and AP stands for embryo-like structures, green and albino plants, respectively.
LS Means obtained from 50 mm Petri dish containing 100,000 microspores
*, ** significant at 0.05 and 0.01 levels of probability respectively

7.7 Figures

Fig. 7-1. Microbial contamination and its prevention with antibiotics during embryogenesis in wheat and triticale isolated microspore cultures and plant regeneration.

a) Contaminated microspore culture b) Phenotypic diversity of bacterial colonies on LB medium c) Complete set of antibiotic treatments (T_1: Control (no antibiotic); T_2: Vancomycin at 100 mg/l; T_3: Vancomycin 500 mg/l; T_4: Cefotaxime at 50 mg/l; T_5: Cefotaxime 100 mg/l; T_6: Vancomycin 100 mg/l and Cefotaxime 50mg/l; and T_7: Vancomycin 500 mg/l and Cefotaxime 100mg/l) used in the experiment leading to embryo-like structures formation d) successful production of green plants using induction medium supplemented with Cefotaxime 100 mg/l.

8.0 Phytosulfokine alpha improves microspore embryogenesis in both triticale and wheat[5]

8.1 Introduction

Doubled haploid (DH) plant production is an alternative to traditional selfing for the production of homozygous lines in breeding programs. Isolated microspore culture (IMC) represents the cell culture platform of choice to produce DH plants and for studying the entire process of microspore embryogenesis; it also offers a basis for an ever increasing array of molecular and genetic studies (Ferrie and Caswell 2011). Microspore reprogramming to the embryogenesis pathway is induced by stress treatments such as cold (Jahne and Lorz 1995), heat (Touraev et al. 1996) and osmotic stresses (Cistue et al. 1994) alone or in combinations; during which microspores undergo severe oxidative stresses (Shim and Kasha 2003; Smykal 2000). As a result, small numbers of microspores may survive and ultimately develop into green plants

Development of albino plants is a major bottleneck in most of the cereals, in particular *Triticum aestivum* (Andersen et al. 1987) and *Hordeum vulgare* (Knudsen et al. 1989). Increased levels of reactive oxygen species (ROS) in plastids results in DNA damage and has been correlated with albinism (Caredda et al. 2000), and cell death (Clément et al. 2005). Early stages of the apoptotic pathways in plant cells are reversible (O'Brien et al. 1998), which might be mitigated by nursing chemicals.

The use of nurse culture systems in the form of feeder layers is well documented in the literature (Horsch and Jones 1980; Matsubayashi et al. 1999b). Feeder cells produce conditioning factors or growth enhancers in the medium that improve differentiation and proliferation of target cells. Similarly, co-culture of ovaries is required for the development of ELS and DH plants in wheat (Hu and Kasha 1997). Other explants such as anthers, florets, callus and glumes have also been used as nurse culture (Puolimatka and Pauk 1999; Torrey 1957). Phytosulfokine-α has been characterized as a universal candidate for supplementing *in vitro* plant cultures similar to the widely

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exploited growth hormones (Grzebelus et al. 2012). Phytosulfokine is a five amino acid sulfated peptide that was first isolated from asparagus (*Asparagus officinalis*) mesophyll cell cultures (Matsubayashi and Sakagami 1996) and later from *Oryza sativa, Zea mays* (Matsubayashi et al. 1997), *Daucus carota* (Matsubayashi et al. 2002) and *Arabidopsis thaliana* (Yang et al. 2001). It is a primary signal molecule responsible for cell to cell communication (Matsubayashi and Sakagami 1996) and is expressed in various tissues like leaves and roots, in addition to calli (Yang et al. 2001). It is well documented in the literature that PSK enhances colony formation of rice protoplasts (Matsubayashi et al. 1997), somatic embryogenesis (Hanai et al. 2000; Igasaki et al. 2003; Kobayashi et al. 1999), formation of adventitious roots (Yamakawa et al. 1998b), pollen germination (Chen et al. 2000), seedling development by increasing growth and chlorophyll content (Yamakawa et al. 1999), plants stress tolerance (Yamakawa et al. 1999), formation of adventitious buds in *Antirrhinum majus* (Yang et al. 1999), and tracheary element differentiation and cell proliferation (Matsubayashi et al. 1999b). Such studies appear to suggest that PSK-α plays an important role in plant growth and development, particularly due to its contribution to the formation of somatic embryos, and its role in enhanced cell division, maintenance of embryogenic cells (Igasaki et al. 2003), cellular longevity, cell division cycle that was arrested in G_0 or G_1 after isolation (Matsubayashi et al. 1999a), enhancing chlorophyll synthesis and content (Yamakawa et al. 1998a, 1999).

The present study was designed to evaluate the dose effect of an exogenous supply of PSK-α in the induction medium on the frequency of embryos/embryo-like structures, green and albino plant production from IMC in selected wheat and triticale cultivars.

8.2 Materials and Methods

8.2.1 Plant material and growth conditions

The experimental material consisted of two Canadian spring wheat (AC Carberry and Peace) and two triticale (AC Ultima and Sunray) cultivars. They were grown in controlled-environment growth cabinets with a 19h of photoperiod (intensity of 300 µmol m^{-2} s^{-1}). The day-night temperature was maintained at 15 and 12^0C, respectively. The plants were fertilized after every two weeks with a 20.20.20 NPK fertilizer blend (water soluble fertilizer). During tillering (Zadok's growth stage No. 2) (Zadoks et al.

1974), the plants were treated with 2.5 ml l^{-1} Tilt™ (propiconazole, Syngenta) to ensure a preventive control against rusts and leaf spot diseases. Once sufficient roots were established, the donor plants were also treated with 0.004 g l^{-1} (of soil) Intercept™ (Imidacloprid, Bayer) for a curative and preventive control against aphids. The first ten tillers were selected from each pot when the spike was about 1-3 cm emerged from the sheath on the stalk in triticale. Wheat tillers were selected when heads were still inside the leaf sheath with the first awn visible. The remaining heads were allowed to grow half their length out of the boot for collection of ovaries for ovary co-culture. Tillers were kept in a refrigerator at 4^0C for 21±3 days with their bases dipped in doubled distilled water. After cold pretreatment of 21±3 days, mid-to-late uninucleate stage was verified before using the eight most homogeneous spikes for microspore isolation and culture according to Asif et al (2013).

8.2.2 Dose effect of PSK-α

Microspores of AC Carberry and AC Peace, and AC Ultima and Sunray were cultured in induction medium (NPB99+10F) supplemented with PSK-α at concentrations of 10^{-7}, $5x10^{-8}$, 10^{-8}, $5x10^{-9}$ M along with the control (no PSK-α). The experiment was conducted with four co-cultured ovaries per Petri dish.

8.2.3 Dose effect of PSK-α: in absence of wheat co-cultured ovaries

Microspores of AC Carberry and Peace were cultured in induction medium supplemented with the same PSK-α concentrations along with the control, in the absence of co-cultured ovaries.

8.2.4 Dose effect of PSK-α: in absence of triticale co-cultured ovaries

Microspores of AC Ultima and Sunray were cultured in induction medium supplemented with the 10^{-7} M PSK-α along with the control, in the absence of co-cultured ovaries.

The number of embryo like structures (ELS) was recorded when transferred to germination medium, usually 3-4 weeks after microspore isolation. The number of green and albino plants was also counted following embryo germination, approximately 3 weeks after their transfer to the germination medium.

All experiments were laid out in a completely randomized design with different replications in each cultivar and PSK-α treatment. The experimental unit consisted of

one small Petri dish containing 10^5 microspores in 3.5 ml of induction medium. The data recorded for ELS, green and albino plants were subjected to normality and homogeneity of variance using Kolmogorov–Smirnoff and Levene's tests, respectively (Steel et al. 1997). Analysis of variance (ANOVA) was performed using the GLM procedure of SAS for all the variables studied individually in each experiment to determine the effect of genotypes, PSK-α, ovary co-culture and their interaction on ELS, green and albino plants (SAS 2003). If the interaction effect was found to be non-significant, it was removed from the model and rerun. Means were separated using Duncan's Multiple Range Test ($\alpha \leq 0.05$).

8.3 Results

8.3.1 Dose effect of PSK-α

The experiment was conducted with wheat cultivars (AC Carberry and Peace) and with triticale cultivars (Sunray and AC Ultima) in the presence of four fresh ovaries. In both species, a genotype x PSK-α interaction was not significant for all traits studied. Development of ELS, green and albino plants was altered by PSK-α doses (10^{-7}, $5x10^{-8}$, 10^{-8}, $5x10^{-9}$, 0) and genotypes ($P<0.01$). Induction medium supplemented with 10^{-7}M PSK-α significantly improved ELS numbers from 173 to 274 in AC Ultima, 169 to 454 in AC Carberry and 178 to 408 in Peace (Table 8-1). Induction medium supplemented with other doses of PSK-α ($5x10^8$ and $1x10^8$) also improved the number of ELS in these cultivars as compared to control, and a dose effect was observed in all cultivars. Similarly, 10^{-7} M PSK-α supplemented induction medium significantly increased green plants in Sunray, AC Ultima, AC Carberry and Peace from 8 to 11, 4 to 6, 4 to 12 and 2 to 9 plants per Petri dish, respectively, as compared to the control (Table 8-1). In wheat, $5x10^{-8}$ M PSK-α also significantly increased green plant production. In triticale, the PSK-α did not significantly change the production of albino plants whereas in the wheat cv. AC Carberry and Peace, the highest PSK-α dose significantly reduced the number of albino plants, in favor of green plants (Table 8-1).

8.3.2 Dose effect of PSK-α in the absence of wheat co-cultured ovaries

This experiment was carried out with wheat cultivars AC Carberry and Peace in the absence of co-cultured wheat ovaries. PSK-α and genotype altered the number of ELS and green plants ($P<0.05$). The treatment at 10^{-7} M PSK-α produced significantly

higher numbers of ELS in AC Carberry (115) and Peace (82) as compared with the control (2 and 0, respectively) that led to the production of 3 and 2 green plants per Petri dish (Table 8-2). We did not observe green plant production when the induction medium was not supplemented with PSK-α and ovaries.

8.3.3 Dose effect of PSK-α in the absence of triticale co-cultured ovaries

This experiment was conducted using triticale cultivars Sunray and AC Ultima to test the highest dose of PSK-α (10^{-7}M) in the absence and presence of fresh ovaries. A PSK-α effect was found to be significant in the case of ELS and green plants ($P<0.05$). PSK-α supplemented induction medium with ovary co-culture gave the highest number of ELS and green plants. With 21 replicates, 10^{-7}M PSK-α helped increase the number of ELS from 364 (control) to 429 in Sunray and 239 (control) to 326 in AC Ultima (Table 8-3). Similar findings are also reported for green plant production in Sunray and AC Ultima using co-cultured ovaries. Interestingly, in the absence of co-cultured ovaries, PSK-α supported the formation of numerous ELS in Sunray and Ac Ultima, as well as green and albino plants (Table 8-3).

8.4 Discussion

Phytosulfokine alpha (PSK-α) was chosen for this study due to its stimulatory effect on cell proliferation, cell differentiation, somatic embryogenesis, and enhanced chlorophyll formation as identified in the previous studies (Chen et al. 2000; Yamakawa et al. 1999, 1998a; Yamakawa et al. 1998b; Yang et al. 1999). Therefore, we supplemented induction medium with various doses of PSK-α, and observed increased microspore viability and growth, and an effect on the number of ELS, green and albino plants in triticale and wheat cultivars. Our results further demonstrated that the maximum number of ELS and green plants were obtained when the induction medium was supplemented with the highest dose tested of PSK-α (10^{-7}M) along with co-cultured ovaries. A downward trend in the production of ELS and green plants was observed with a decrease in PSK-α dose, and the lowest values were obtained when the induction medium was not supplemented with PSK-α (control). It has been previously reported that induction of cell division can be achieved by the addition of a nurse culture (Blakely 1964; Muir et al. 1954) and conditioned medium (Stuart and Street 1969). Moreover, most of the uni-nucleate microspores, at the time of floral organ collection from donor

plants, are at the G_1 stage of the cell cycle and many of them remain at this stage as a result of cold treatment (Shim and Kasha 2003). Therefore, PSK-α supplemented induction medium could resume the progression of the cell division cycle that was arrested in the G_1 stage, as observed in carrot (Eun et al. 2003). The induction of rice anther culture was also enhanced by supplementing NBA induction medium with an exogenous supply of PSK-α in a concentration of 100 pMol/L along with cold pretreatment (Chen et al. 2010a). In a study conducted by Hanai et al. (2000) to unravel the stimulatory role of PSK-α in somatic embryogenesis of carrot, the authors reported an accelerated somatic embryo formation and dramatic increase in cell number following application of PSK-α to an embryo inducing medium. They further found that extremely rapid cell division can only occur during the initial phase of embryogenesis, and that exogenous PSK-α stimulated such division, which resulted in accelerated somatic embryo formation. A similar role of PSK-α has also been reported in *Cryptomeria japonica* (Igasaki et al. 2003) and *Daucus carota*(Kobayashi et al. 1999) when PSK-α was used at concentrations of 32 nM and 100 nM, respectively. Therefore, we propose that an exogenous supply of PSK-α in the induction medium promoted not only cell division cycles and cell growth but also helped quiescent microspores to re-enter the cell cycle; thus decreasing their mortality rate and ultimately increasing the number of ELS in the tested cultivars in all experiments described herein. Higher green plant production can be directly correlated to a high frequency of ELS.

Removal of fresh ovaries from the wheat and triticale florets is tedious and time consuming. We tested the contribution of PSK-α to microspore embryogenesis in the absence of fresh ovaries. Our results demonstrated that production of ELS and green plants is possible; however, frequency was drastically decreased and is not as successful as medium conditioned with ovary co-culture and PSK-α. In the absence of PSK-α and ovary, no wheat embryos were able to form, even after 35 days of induction. These results suggest that PSK-α exhibits a strong stimulatory effect, similar to ovaries, and the effect is additive in the presence of ovaries.

In our study, an exogenous supply of PSK-α in the induction medium also helped to reduce the frequency of albino plants (Table 8-1), but in a genotype dependent manner. Albino plants are devoid of chlorophyll and are unable to carry on the process of photosynthesis. Supplying PSK-α in the culture medium elevated the chlorophyll

content in etiolated cotyledons of cucumber (Yamakawa et al. 1998a). Another study reported a similar finding with PSK-α in *Arabidopsis* seedlings, where it maintained chlorophyll formation even during high night-time temperature treatment (Yamakawa et al. 1999). These two reports support our observations of reduced albino plant production in the presence of PSK-α, in AC Carberry and Peace (Table 8-1).

One triticale Sunray microspore extraction from eight spikes resulted in 14 induction Petri dishes, which produced 154 green plants with ovary co-culture and PSK-α, and 14 green plants without ovary co-culture. However, the highest gains were observed in wheat. One microspore extraction with eight wheat spikes yielded seven induction Petri dishes, resulting in 84 and 63 green plants with ovary co-culture supplemented with PSK-α and 28 and 14 green plants without PSK-α in cultivars AC Carberry and Peace, respectively.

8.5 Tables

Table 8-1. Dose effect of PSK-α on mean number of embryo like structures (ELS), green and albino plants per Petri dish in triticale and wheat genotypes.

	Triticale								Wheat							
	Sunray				AC Ultima				AC Carberry				Peace			
PSK	N	ELS*	GP	AP	N	ELS	GP*	AP*	N	ELS	GP	AP	N	ELS	GP	AP
$1x10^{-7}$	9	468	11a	5ab	8	274a	**6**	4	11	454a	12a	5b	7	408a	9a	3b
$5x10^{-8}$	10	423	9ab	4bc	7	285a	4	4	11	387a	8b	8b	7	352a	5b	2b
$1x10^{-8}$	9	386	8bc	3c	7	224ab	4	3	10	312b	7bc	11ab	7	282b	3bc	3b
$5x10^{-9}$	10	386	7c	4bc	8	144b	4	2	11	290b	6cd	8b	6	216bc	2c	1b
Control	8	372	8bc	4bc	6	173b	4	4	11	169c	4d	16a	7	178c	2c	8a

* represents non-significant variable. N represents number of repetitions. ELS, GP and AP stands for embryo-like structures, green plants and albino plants.
Mean values within a column followed by the same letter are not significantly different (Duncan Multiple Range Test ($\alpha \leq 0.05$))
LS Means obtained from 50 mm petri dish containing 100,000 microspores

Table 8-2. Dose effect of PSK-α in absence of wheat co-cultured ovaries on mean number of embryo like structures (ELS), green and albino plants per Petri dish in triticale and wheat genotypes.

	AC Carberry				Peace			
PSK	N	ELS	GP	AP*	N	ELS	GP	AP*
$1x10^{-7}$	11	115a	3a	2	7	82a	2a	2
$5x10^{-8}$	11	93a	1a	2	6	63a	0c	0
$1x10^{-8}$	11	78a	1a	4	5	33b	1ab	1
$5x10^{-9}$	11	70a	1a	3	7	26b	0c	2
Control	11	2b	0b	0	7	0	0c	0

* represents non-significant variable. N represents number of repetitions. ELS, GP and AP stands for embryo-like structures, green plants and albino plants.
Mean values within a column followed by the same letter are not significantly different (Duncan Multiple Range Test ($\alpha \leq 0.05$))
LS Means obtained from 50 mm petri dish containing 100,000 microspores

Table 8-3. Dose effect of PSK-α in absence of triticale co-cultured ovaries on mean number of embryo like structures (ELS), green and albino plants per Petri dish in triticale and wheat genotypes.

	Without ovary co-culture								With ovary co-culture							
	Sunray				AC Ultima				Sunray				AC Ultima			
PSK	N	ELS*	GP	AP*	N	ELS	GP*	AP*	N	ELS	GP	AP*	N	ELS	GP	AP*
$1x10^{-7}$	21	93	1a	2	7	131a	0.3	1	29	429a	4a	5	8	326a	4a	5
Control	21	70	0b	1	7	28b	0	0	29	364b	3b	5	8	239b	2b	5

* represents non-significant variable. N represents number of repetitions. ELS, GP and AP stands for embryo-like structures, green plants and albino plants.
Mean values within a column followed by the same letter are not significantly different (Duncan Multiple Range Test ($\alpha \leq 0.05$))
LS Means obtained from 50 mm petri dish containing 100,000 microspores

9.0 Future research directions and original contributions to knowledge

9.1 Future research directions

Wheat breeders seeking superior genotypes suitable for organic agriculture mostly rely on selections from conventional lands where herbicide use is routine in breeding trials, precluding selection for traits conferring competitive ability. In the present research work, my aim was to find out differences with respect to slection response, heritabilities, correlations, and the effect of *Rht-B1* and quantitative trait loci (QTL) mapping of various agronomic and quality traits between organically and conventinally managed systems. The results indicated that differences exist between the two management systems for selection response, heritabilities and correlations. The effect of *Rht-B1* was more pronouned in organic than conventional management systems. Most of the identified and mapped QTL were either specific to organic or conventinal management systems. Moreover, consistent QTL identified in both systems differed with respect to their phenotypic variation and additive effect leading to the conclusion that wheat breeding for organically managed lands should be done in organic systems by testing and selecting genotypes based on production and quality traits within environmental conditions which are comparable to where candidate lines are intended to perform.

Fine mapping of the genomic regions identified in this study for grain yield, test weight, kernel weight and days to flowering on chromosomes 6A, 1B, 3A and 5B, respectively, will help wheat breeders to use marker assisted breeding. Future students of our group will have the opportunity to further study the Attila x CDC Go population by targeting specific QTL regions using sequence capture arrays to enrich marker depth with single nucleotide polymorphism (SNP) markers as this population is a part of the Canadian Triticum Advancement Through Genomics (CTAG) project. The SNPs will help to identify any candidate genes and facilitate the development of diagnostic or perfect markers.

Doubled haploid production is being done in many crops to reduce cultivar development time. The acceleration has been observed in research studies on DH

production over the last five years. Major research has been focused to change the status of many recalcitrant crops to responsive to DH production and to improve the overall methodology of DH production. Several model genotypes have been identified in various crops that have led to an overall improvement in the technology. In this regard, IMC is of special interest to the plant breeders, geneticists and molecular biologists due to the availability of embryonic units in a larger number. It is quick and efficient as compared to other available methods. Moreover, the single cell-culture system of IMC provides many opportunities for process improvement, and genetically identical and physiologically uniform single cells are also being used as targets for cell biology, embryology, and genetic engineering studies.

The low frequency of green plant production limits the use of IMC in wheat breeding programs. Results from the thesis research indicate that Glutathione, Phytosulfokine alpha, Cefotaxime and optimum osmolality level of induction medium helped to improve green plant production in wheat. Further research work on early embryogenesis and especially on cell/microspore death during first 24-48 hours of induction phase of IMC using video tracking systems and flow cytometry will help to address future challenges and improve our understanding on how the complex mechanism of microspore conversion from the gametophytic to sporophytic pathway takes place and ultimately converts these microspores to haploid plants.

9.2 Original Contributions to knowledge

Marker assisted breeding (MAB) has gained importance among wheat breeders in Canada for the last 15 years. It provides opportunities to the breeders to i) select superior genotypes in early segregating populations ii) select superior genotypes for traits that are difficult to select based on phenotype in the field conditions and iii) pyramid desirable combinations of genes into a single genetic background. Keeping in view the importance of MAB for wheat breeders in Canada, Chapter 2 of this thesis was written to review the history and applications of MAB in Canada. This chapter provides information regarding molecular markers that have been used to select/breed various genotypes/cultivars in Canada for various traits related to biotic stresses (rust, Fusarium head blight, tan spot, bunt, loose smt, ergot, insect resistance), phenology (earliness) and grain quality (protein content, gluten strenght, cadmium content, preharvest

sprouting, pasta color and lipoxygenase activity). The putative gene composition and pedigree of 120 experimental lines/cultivars (belong to CWRS) have been presented which will help wheat breeders to select a particular genotype to be used as parents in their wheat breeding programs.

The study summarized in Chapter 3 was developed in response to the need to identify differences with respect to various agronomc and quality traits between organic and conventional management systems in order to facilitate organic breeding. The results suggest that differences exist between the two management systems for heritability, correlations, selection response, effect of *Rht-B1* and genomic regions controlling various agronomic and quality traits. Direct selection in each management system resulted in 50% or less lines/RILs selected in common for eight of the nine (except for flowering time) studied traits. Overall, we mapped 46 QTL for various agronomic and quality traits across organic and conventional management systems for three years. Most of these QTL were specific to a given management system; however, consistent QTL for grain yield, test weight, kernel weight and days to flowering were mapped in both systems on chromosomes 6A, 1B, 3A and 5B, respectively. The effect of *Rht-B1* was more pronounced in organic systems where lines carrying the wild type allele were taller, produced more grain yield with higher grain protein content and suppressed weed biomass to a greater extent than mutant types.

Doubled haploid (DH) production technology has been used to develop mapping populations to study the inheritance and development of molecular markers linked to traits of intrest. The technology has hastened the process of cultivar development by at least 4 years through fixing homozygosity of a compeletely heterogenous (F_1) progeny in one generation. In Canada, DH technology has been integrated into wheat breeding programs for about 22 years and 25 DH cultivars have been released for commercial cultivarion since 1997 (Depauw 2011a). Keeping in veiw the growing interest in the use of DH production for cultivar development, my intent was to review the literature to identify problems associated with this fascinating phenomenon. Thus, Chapter 4 was written to discuss the importance of androgenesis and bottlenecks that restrict breeders from using this technology in their breeding programs. This chapter highlights various androgenic methods available to plant breeders for the production of DH plants. It also provides information to the reader regarding the various steps involved in each method

to facilitate DH production. I wrote this chapter as a part of my book titled "*Progress and Opportunities of Doubled Haploid Production*" recently published by Springer, USA and this will hopefully serve as a "one-stop" reference for scientists and students engaged in the field of DH production for information on the progess of doubled haploid production over a period of 90 years.

In Canada, uniparental chromosome elimination or pollination of wheat ovules with maize pollen followed by chromosome doubling is the predominant method to produce DH plants (Knox et al. 2000). Isolated microspore culture is prefered over maize pollen based DH production because i) it is not time consuming and laborious; ii) it does not require any tedious jobs like emasculation and pollination; iii) more embyonic units are available in IMC; iv) it does not require space needed to grow mazie, and; v) it provides a platform for cell tracking and targeted mutagenesis studies. However, the low frequency of green plant produciton, microbial contamination and albinism associated with IMC limit/restrict the use of this technology in wheat breeding progarms. These bottlenecks provided me with opportunities to design experimental studies to address these problems.

The results presented in Chapter 5 indiate that induction medium supplemented with proline (10mM) yielded greater numbers of embryos/embryo-like structures and green plants in triticale and wheat. Among plastid antioxidants, glutathione (2µM) proved to be the best antioxidant to increase embryo and green plant production. Salicylic acid also helped to reduce the number of albino plants in triticale and the wheat genotype SWS366. Overall, induction medium supplemented with proline or glutathione enhanced microspore embryogenesis in triticale and wheat, and increased the number of green plants in recalcitrant genotypes

The experimental study conducted to optimize induction medium osmolality (Chapter 6) demonstrated its positive effect on the production of embryo/embryo like structures (ELS) and green plants. Here, I report that the process of microspore embryogenesis can be improved by optimizing osmolality of the induction medium to 350 mOsm kg^{-1} in wheat and triticale.

The experiment in Chapter 7 examined occasional contaminants of isolated microspore cultures in our laboratory. The results indicate that five recurring species of bacteria (*Erwinia aphidicola, Pantoea agglomerans, Pseudomonas sp., Staphylococcus*

epidermis and Staphyloccus warneri) are present predominantly in contaminated cultures. On supplementing induction medium with various doses of two different antibiotics, it was found that Cefotaxime at 50 and 100 mg L^{-1} controls most recurrent bacterial contamination and also helps to increase the fomation of ELS and green plants in triticale and wheat genotypes.

The results presented in Chapter 8 provide evidence that phytosulfokine alpha (PSK-α) supplemented induction medium supports the formation of ELS and green plant production, and also minimizes the occurance of albinism in wheat genotypes studied.

Overall, the research work presented in the thesis constitutes an advancement of knowledge in the domains of QTL mapping, organic breeding, marker assisted selection and doubled haploid production.

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Appendix

Doubled haploid production via isolated microspore culture (IMC): Review of literature[6]

Androgenesis is one of the most important methods that have been extensively used in plant breeding programs to produce doubled haploids. It involves the induction of microspore embryogenesis that leads to the development of a haploid embryo instead of mature pollen grain. The microspore embryogenesis is usually brought about by modifying the environmental conditions of anthers/microspores by reprogramming their gametophytic pathway towards sporophytic growth and development. Under natural conditions, the microspore develops into a mature pollen grain that is comprised of generative and vegetative nuclei. The generative nucleus develops into two sperm nuclei. Thus, the sporophytic development should be started before the onset of cell division when the gamete cells in the microspores are still totipotent. However, the embryogenic stage of microspores varies greatly among species (Touraev et al. 2001). The microspores are amenable to androgenesis and consist of a haploid (n) number of chromosomes and therefore give rise to haploid plants. Androgenesis can be divided into three distinct steps: i) embryogenesis induction, ii) regeneration of haploids followed by iii) artificial chromosome doubling. The production of haploids or doubled haploids (DH) via androgenesis can be achieved either through isolated microspore culture or anther culture.

Microspore culture

Microspore culture (pollen culture) offers an opportunity to plant breeders to develop DH plants on a larger scale which enables them to speed up the breeding process by fixing homozygosity in one generation after a cross has been made. Thus, cultivar development period can be dramatically reduced with the help of isolated microspore culture (IMC) in crop species responsive to this method. The IMC involves

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isolation of immature pollen grains or microspores from the anthers followed by culturing them on growth medium under optimum environmental conditions necessary for their growth and development and to reprogramme their gametophytic pathway towards sporophytic by using various kinds of stress treatments. This method is preferred over anther culture (Kieffer et al. 1993; Arnison and Keller 1990) due to the following reasons:

1. Anther culture can give rise to diploid plantlets (non-haploids) from anther tissues (wall) along with haploids, whereas in microspore culture plantlets always originate from microspores that have a haploid number of chromosomes (*n*).
2. Anther culture is a lengthy, time consuming and laborious method.
3. In case of anther culture, anther tissues other than the microspores could have a negative influence on the growth and development of a developing microspore and to some extent, it deters their developmental process.
4. The developing microspores have uniform nutrient accessibility during IMC.
5. Isolated microspore culture provides an opportunity to better track the pathway of microspore embryogenesis by monitoring each embryogenesis stage separately and to better understand the most important factors (like morphological and cytological changes) contributing towards microspore embryogenesis.
6. Isolated microspore culture offers a platform for targeted mutagenesis and an effective gene transfer technique that can direct the breeder/molecular biologists to pyramid genes of their interests in a shorter period of time. Moreover, the transgenic plants can be identified at a very early stage of their life cycle.
7. Cell changes studies during a shift from the gametophytic to sporophytic pathway and the initiation of microspore embryogenesis can be easily performed/tracked during IMC.
8. The embryogenic units are ten times greater in IMC as compared to anther culture.

Microspore embryogenesis gained enormous importance and attention from the breeders after 1960. This technique developed very rapidly when Guha and Maheshwari (1964) discovered that by providing specific environmental conditions, haploid plants could be easily produced from the anthers of immature pollen grains of *Datura innoxia.* Nitsch (1974) used natural shedding phenomenon and successfully isolated microspores of *Nicotiana* sp. from its anthers that followed mechanical microspores isolation in

Brassica sp. by Lichter (1982) before culturing them on medium to produce haploid plants. This discovery opened a major breakthrough in the area of DH production. Since 1960, extensive research studies have been conducted to improve the efficiency of IMC. However, a large number of crop species such as legumes are still recalcitrant to microspore culture. Each step of IMC has been investigated in detail to improve the efficiency of this technology. A wide range of protocols have been summarized in various crops. Each crop/genotype has its own specific protocol due to distinct androgenic responses to microspore embryogenesis, but the main processes involved in this technique are the same (Fig. 1) and include i) donor plant's growth and developmental conditions, ii) removal/collection of floral organs from donor plants, iii) pretreatments, iv) isolation of microspores, v) composition of medium, vi) regeneration of haploids and vii) artificial chromosome doubling.

Donor plant's growth and developmental conditions

The donor plant's growth and developmental conditions occupy the most important position in the whole process of IMC as efficiency is directly linked to it. If the donor plant is free of insects, pests, diseases, absence of nutrient and water deficiencies and environmental stresses like temperature, humidity and photoperiod, the effectiveness of this method can be enhanced to a greater extent.

The donor plants are normally grown in optimum conditions to get a healthy crop stand, *e.g*., in cereals, strong and vigorous tillers are desirable. Other agronomic practices like watering and fertilization are done routinely. An improved embryogenic response has been observed if donor plants are planted under controlled conditions (greenhouse, glasshouse or control chambers) than plants grown under field conditions. Optimum growth and developmental conditions can be provided to the donor plants through supplying optimum light, humidity, temperature, and photoperiod under controlled conditions that also ensure minimum disease occurrence and infestation due to insects and pests. The growth chamber grown donor plants are often preferred over greenhouse plants because a higher number of green plants can be obtained from them (Dahleen 1999). Field grown plants have also been used to isolate microspores but less embryogenic response has been observed. Moreover, there are greater chances of contamination if donor plants are grown under field conditions. The growing conditions

of donor plants have a direct effect not only on the embryogenesis but also on the regeneration and the number of green plants. Among growth conditions, temperature is of major concern that has been probed in several studies. Luk et al. (1983) reported that an increase in day and night temperature from 18 to 28°C and 14 to 25°C, respectively, in triticale will diminish the regeneration process to a greater extent. The plants sown in cold temperature consequently give rise to a higher number of embryos and green plants (Bernard 1977). In this study, cold temperature (12-15°C) gave a better response in inducing embryogenesis in triticale. By contrast, no such requirement of cold temperature treatment for donor plants of pepper and asparagus has been reported (Lantos et al. 2009; Wolyn and Nichols 2003). If plants are not grown under cold temperature, the stress to isolated anthers in the form of cold temperature has been proven to have a strong positive effect on embryogenesis (Osolnik et al. 1993). Thus, on the basis of these studies it can be deduced that cold treatment is not only essential to arrest the gametophytic stage but it also helps to improve the entire androgenesis and regeneration developmental processes.

Light intensity and its interaction with temperature also affect the physiological status of donor plants. A five time decrease in embryogenic response in *Nicotiana tabacum* was noticed with an increase in photoperiod from 8-16 hours (Duncan and Heberle 1976). The status of nitrogen in the soil also has a direct relationship with embryogenic response in tobacco and it has been observed that donor plants grown under starved nitrogen conditions have given an enhanced response to IMC as compared to donor plants that were fertilized routinely (Tsay 1982). However, specific recommendations with regards to optimal conditions for the growth and development of donor plants are not possible because donor plant requirements vary significantly among various crop species.

The genotype of the donor plants also plays a crucial role with respect to the response of microspores to embryogenesis and it not only differs from species to species but also varies considerably within species and this is especially true for cereals like triticale, barley, wheat and oat. This intra and inter-specific variation in embryogenic response during IMC differs extensively with some varieties/cultivars/lines of a particular genotype/species exhibiting a greater response while others show no

response at all, and sometimes differences in microspore embryogenesis within a plant are also very high (Phippen and Ockendon 1990). The winter and spring genotypes have given varying degrees of response to embryogenesis. In *B. napus,* greater embryogenic response was noted in winter cultivars than in spring cultivars (Keller et al. 1987b; Keller et al. 1987a). Contradictory results were obtained by Ohkawa et al. (1987) in 96 genotypes. Similarly, japonica genotypes in rice are more responsive to androgenesis than indica cultivars (Miah et al. 1985) and a same trend between *B. napus and B. juncea* has been reported by Chanana et al. (2005) where the latter carry a poor response. In wheat, 32-85.6% of genotypic variation for embryogenic response was observed (Zhou 1996) and these differences were 73% in barley (Torp et al. 2001). The embryogenic response of genotypes is considered a heritable character and it can be improved by crossing a non or poor receptive cultivar with a responsive one (Petolino et al. 1988). A number of experiments have been conducted to investigate the androgenic response of cultivars/lines/varieties in different species in order to identify model species with improved DH production. The major varieties that have been recognized for their better androgenic response are Chris, Pavon 79 and Bob White in wheat (Kasha et al. 2003b), Igri in winter barley (Davies 2003), Topas in *B. napus* (Ferrie et al. 1995a), Narayen, Rupali, Kimberley in chickpea (Croser et al. 2011), CV-2 in *B. rapa* (Ferrie et al. 1995a), Green, Shogun, SDB9 in *B. oleracea* (Dias 2001) and CAV-2648 in a wild species of red oat (Kiviharju et al. 2004).

Collection of floral organs

The efficiency of microspore culture is also dependent on plant age and pollen stage at which the floral organs are collected from donor plants for microspore isolation. A greater androgenic response has been noticed if microspore isolation is done with the floral organs that emerge first (e.g. primary tillers in wheat) than those appearing in the later (secondary or tertiary tillers) stages of donor plants. A similar trend has been seen in cereal species where primary tillers have given a much better response to anther as well as microspore culture than secondary and tertiary tillers. However, in *B. rapa* and *B. napus,* pollen collected or microspores isolated from older plants perform well to the androgenesis than the young donor plants (Takahata et al. 1991). In a sowing date study on *B. juncea*, it was observed that the frequency of

embryos was increased when floral organs were collected from late sown plants than plants planted at the normal sowing date (Agarwal and Bhojwani 1993). In tobacco plants, a four time variation in the number of green plantlets was reported with a variation of 2 mm in corolla length (Dunwell 1976). The optimum microspore stage that can reprogramme the microspores from gametophytic to sporophytic pathway appears to differ among species. In the case of *N. tabacum*, first pollen grain mitosis or unicellular to bicellular (G_1) stage of microspores is considered to be the most responsive (Touraev et al. 2001), but on the other hand microspores between the mid-late uni-nucleate or early bicellular are most responsive in cereals. The microspores isolated at later stages of pollen grain are normally not responsive because they contain starch grains (Sangwan and Sangwannorreel 1987a, b), whereas in brassica pollen grains used for microspore isolation already contain starch gains and embryogenesis is successfully induced in them by a well-timed heat pretreatment (Binarova et al. 1997). The DAPI and acetocarmine stains have been extensively employed in tissue culture studies to identify the accurate microspore stage prior to their collection or before using them for isolation to induce embryogenesis (Fan et al. 1988).

Pretreatments

Pretreatments of various types are given to the floral organs to induce stresses that can ultimately help to switch gametophytic pathway of microspore to sporophytic development (Fig. 2). The commonly used pretreatments are cold and heat shocks, starvations in the form of nitrogen and carbohydrates, irradiation or chemical treatments that are often given to the floral organs like spikes or floret buds, excised anthers or even to the microspores after their isolation. Pechan and Keller (1989) pointed out that pollen irradiation is not a widely adapted stress treatment as compared to other pretreatments. There are few species that do not require any pretreatment (or stress) for embryogenic induction because their microspores exhibit a certain kind of natural capability for microspore embryogenesis (Zhou et al. 1991). However, the frequency of such tendency towards embryogenesis is exceptionally low. It is also assumed that the removal of anthers or floral organs from the donor plant is itself a stress that can guide the fate of the microspore towards sporophytic development. The pretreatments alone or in combination with each other act as triggering factors or as an

external stimulus to achieve an optimum conversion of microspores from their gametophytic growth to sporophytic pathway. It was also observed that animal cells in addition to plant cells also require some sort of pretreatment in the form of stress to induce embryogenesis because the origin of "Dolly", in the case of animal cloning, entailed stress pretreatment as one of the major component for development of an embryogenic cell in sheep (Zheng 2003). Puddephat et al. (1999) found that the donor plant developmental conditions in onion had a strong positive influence in inducing microspore embryogenesis. The pretreatments in the form of cold temperature have shown a promising androgenic response in barley (Li and Devaux 2003), wheat (Indrianto et al. 1999), durum wheat (Sibi et al. 2001) and rice (Bishnoi et al. 2000), but on the other hand heat treatment enhanced embryogenic response in brassica (Binarova et al. 1997), tobacco (Touraev et al. 1996b), cucumbers (Gemes-Juhasz et al. 2002), pepper (Barany et al. 2001), wheat (Touraev et al. 1996b) and starvations in the form of nitrogen and carbohydrates conferred an improved effect in tobacco (Touraev et al. 1996a), barley (Hoekstra et al. 1992) and rice (Raina and Irfan 1998). The use of colchicine and auxin as a pre-treatment has also induced microspore embryogenesis in few species (Obert and Barnabas 2004). The use of growth regulators such as abscisic acid, cytokinin and auxin have been employed to switch somatic cell towards embryogenic cell (Filonova et al. 2000) but their transitional response in regards to DH production via IMC is not adequate/sufficient.

Cold pretreatment

Cold pretreatment has been used in many crops to induce microspore embryogenesis. The cold pretreatment of anthers has a nursing effect on microspores that not only arrests their normal gametophytic development (Zheng 2003) but also assists in the synchronization of the whole developmental progression of microspores (Hu and Kasha 1999). The cold treatment of spikes for more than seven days in cereals stimulates microspore embryogenesis and helps to increase the frequency of embryos or multicellular structures and green plants. Substantial progress in embryogenic efficiency of microspores in cereal crops such as maize (Gaillard et al. 1991), triticale (Marciniak et al. 2003), wheat (Indrianto et al. 1999), barley (Sunderland and Xu 1982), rye (Immonen and Anttila 2000) and other crops like citrus (Germana and Chiancone

2003) and tobacco (Sunderland and Roberts 1979) has been done by applying cold pretreatment as stress. The chilling temperature helps to decrease the degradation of microspores/cells, thereby inhibiting their exposures to the decaying material and other toxic substances (Duncan and Heberle 1976). In *B. rapa*, cold temperature treatment helps to arrest the bicellular microspore stage, thereby increasing the frequency of embryogenic microspores exhibiting two equal nuclei. Sato et al. (2002) reported that a bicellular microspore stage with two equal nuclei is one of the most crucial phase to induce embryogenesis in *B. rapa*. A cold treatment of 2-4 days has improved the efficiency of microspore culture to several folds in *B. napus* whereas it is less helpful in *B. rapa* and has no effect in *B. oleracea* (Gu et al. 2003b; Gu et al. 2003a; Xu et al. 2007). The cold pretreatment of spikes in barley helped microspore separation from anthers and their free occurrence in the locule (Sunderland and Xu 1982) whereas it does not have any effect on microspore detachment in tobacco because they are previously separated and found free in the locule and they do not need any pretreatment (Zoriniants et al. 2005).

Heat pretreatment

The heat pretreatment has been found as an efficient embryogenesis inducer. It has been used alone or in combination with sugar starvation to achieve maximum output from microspore embryogenesis and has been extensively applied in brassica species not only to induce embryogenesis but also to increase the frequency of embryo or embryo like structure (ELS) and green plants during IMC. When the floret buds of *B. napus* and *B. carinata* were treated with heat shocks for 1-4 days at 32^0C, that activated the process of embryogenesis in all buds used in the experiment (Pechan and Smykal 2001). However, the heat pretreatment longer than 4 days did not yield any improvement in the cell division and lowered the frequency/number of embryos to a greater extent (Barro and Martin 1999). The heat pretreatment (33^0C for eight hours) and treatment of floret buds exhibiting microspores at the bicellular phase/stage at 42^0C in rapeseed gave better results for inducing embryogenesis. A gentle heat treatment (33^0C) of rice anthers for four days also resulted in optimum numbers of embryos and green plants (Raina and Irfan 1998). In tobacco, mild heat shocks as a separate pretreatment or with sugar starvation helped to seize gametophytic development and

improved microspore's reprogramming towards sporophytic pathway (Touraev et al. 1996c). It has been observed that heat shocks cause numerous cell modifications/alterations and of these changes, the synthesis/production of highly conserved group of heat shock proteins (HSP) carries an imperative position with respect to androgenesis. The production of HSPs was not only linked with heat treatments, but it also originated as a result of various stimuli such as osmotic stress, cold treatment and oxidative stress (Almoguera and Jordano 1992; Sabehat et al. 1998). The HSPs synthesis can take place at different phases of plant growth like embryogenesis, fruit maturation (Low et al. 2000), pollen grain growth and development (Parcellier et al. 2003), and germination (Wehmeyer et al. 1996). Major components of these HSPs, mainly HSP90 and HSP70, are known to produce elevated level of expression right from the initiation of microspore embryogenesis or soon after embryogenic induction in *N. tabacum* (Zarsky et al. 1995), *B. napus* (Segui-Simarro et al. 2003), corn (Gagliardi et al. 1995), *Capsicum annuum* (Barany et al. 2001) and this HSP synthesis continues till the first pollen mitosis. These HSPs also obstruct the synthesis of those proteins that are needed for pollen grain growth and development. Thus, HSPs play a very crucial role to reprogramme the microspores from their gametophytic development to sporophytic pathway (Telmer et al. 1993). These HSPs are also known to play a major role in coping with programmed cell death (PCD) of microspores or microspore's apoptosis throughout their induction or culture period when they are subjected to heat shock (Zoriniants et al. 2005).

Starvation

Sugar and nitrogen are major components with respect to stresses induced by starvation. The starvation stress is always applied to the uniform population of immature pollens or microspores mostly during the induction phase when the microspores are between mid to late uni-nucleate phase to induce sporophytic development. In tobacco, the heat shocks along with sugar and nitrogen starvation was given to a homogenous population of microspores between mid to late uninucleate and early bicellular stage that resulted in the induction of microspore embryogenesis in greater than 70% of the microspores. The remaining 30% of microspores either died or did not possess/exhibit embryogenic characteristics that can only be attributed to the

stress application and/or complications in the isolation process (Touraev et al. 1996c). Caredda et al. (2000) conducted a similar experiment by applying 3-4 day cold shocks instead of heat treatment in conjunction with starvation that resulted in an enhanced survival rate of microspores to a considerable degree and increased the percentage of green versus albino plantlets compared to a treatment where the floral organs of donor plants were pretreated only with cold for 3-4 weeks. The significant improvements in barley and wheat microspore cultures were reported by replacing sucrose by maltose in the induction medium that elevated the rate of metabolism leading to hypoxia and also resulted in increasing ethanol accumulation and lowering energy levels (Indrianto et al. 1999; Scott et al. 1995). Thus, the substitution slowed down the consumption of maltose that gave rise to a starvation stress in the microspores, directing them to initiate sporophytic development rather than gametophytic development. The utilization of carbohydrates during microspore culture is mainly dependent on the pH and osmotic pressure of the medium. The induction of microspores in a media exhibiting low pH levels, *i.e.*, between 5.0-6.0 can result in an effective utilization of sucrose and facilitate the conversion process of sucrose into starch, thereby leading microspores towards pollen grain development following the gametophytic pathway. On the other hand, if microspores are inducted in a media having pH levels 8.0 or higher, it will considerably reduce the sucrose utilization (creating a carbohydrate starvation) and direct the microspores towards sporophytic pathway (Zoriniants et al. 2005). Furthermore, a very slight variation in the sugar content of induction medium was reported by Zhou et al. (1991), suggesting its important role as an osmoticum in the media. These findings were followed by various research studies that reported an enhanced effect of medium osmotic pressure on embryogenic development of microspores (Croser et al. 2006; Ramirez et al. 2001). The sugar starvation initiated microspore's dedifferentiation, leading them towards sporophytic pathway but on the other hand when all necessary/required ingredients/nutrients were present in the induction medium, it resulted in the redifferentiation of isolated microspores, directing them towards normal gametophytic development (Harada et al. 1988). The nitrogen starvation induced by glutamine has shown to have a major role in suppressing or inhibiting the process of microspores maturation and enhanced their successful transfer to sporophytic development (Kyo and Harada 1986). The starvation induced by

carbohydrates is known to cause various structural and physiological cell modifications that are comprised of i) inhibition of cell growth, ii) instant/speedy carbohydrate intake, iii) reduction in the rate of cell respiration, iv) degradation of cell proteins and lipids, v) rapid accumulation of free amino acids and P*i*, phosphorylholine and vi) reduction in the activities of glycolytic enzymes (Yu 1999). Principally, these cellular modifications take place during the entire process of cellular adaptation to carbohydrate starvation. The cellular changes brought about by sugar starvation in tobacco microspores include dedifferentiation of plastids, degradation of starch and lamellar structure, emergence of large vacuole, dilution of generative cell wall, rapid decline in the size of nucleolus, loss of nuclear pore in the vegetative nuclei and various chromatin changes, that have been experienced when tobacco microspores from early and mid to bicellular phase are cultured to induce embryogenesis (Garrido et al. 1995; Kyo and Harada 1990). The other cellular changes associated with sugar starvation consisted of deregulation of protein kinase activities, decrease in energy levels especially in the form of ATPs, decrease in RNA synthesis along with status/levels of cell/microspore`s respiration and especially these physiological and structural changes that occurred when sucrose was substituted with maltose in the induction media (Scott et al. 1995; Zarsky et al. 1990). Moreover, it is also believed that cell cycle arrest along with the activation of HSP genes during tobacco embryogenesis as a consequence of stress treatment in the form of sugar starvation not only help to preclude PCD of microspores but also increase cell division as compared to cell enlargement (Zarsky et al. 1995; Zarsky et al. 1992).

Based on the above discussion, it can be recapitulated that many crop plants require stress pretreatment either in the form of cold, heat, or carbohydrates to switch their gametophytic development of microspores to sporophytic pathway whereas on the other hand the crops that do not require such pretreatment, the physical removal of floral buds/spikes/organs from the donor plants produce/generate sufficient stress that can initiate androgenesis in microspores. However, the length of stress plays a significant role in inducing microspore embryogenesis and to attain a high percentage of embryos or ELS along with green plants, but the mechanism by which these stresses influence the degree or pace of the androgenesis and regeneration processes is still unclear. However, it can be contemplated that these pretreatments facilitate the overall

process of microspore embryogenesis or trigger embryogenesis by creating various stresses that bring structural and physiological cellular changes leading them towards sporophytic event rather than normal gametophytic pathway of pollen grain development.

Microspore's isolation and purification

The floral organs such as buds, florets and spikes are treated with chemicals prior to microspore isolation in order to eliminate/remove any insect, pest, fungal or bacterial contaminants. The main chemicals used for the removal of contaminants are bleach, ethanol, sodium hypochlorite and mercuric chloride. However, care must be taken with respect to the duration of surface sterilization to avoid any lethal effect of these chemicals on the microspores. The surface sterilization procedure starts by immersing floral organs in 75% ethanol or 10% bleach for 3-5 minutes. The floral organs can also be surface sterilized with 6% sodium hypochlorite for 15-20 minutes. These surface sterilization procedures are then followed by water (doubled distilled) washings (2-3) for about 1-2 minutes. In few studies, the surface sterilization with mercuric chloride (0.1%) has been conducted but it is recommended to avoid it for surface sterilization of floral organs that are going to be used for isolation of microspores due to its lethal or toxic effect.

Four microspore isolation procedures have been reported in various studies that include shed microspore, maceration, magnetic bar stirring and blending. The shed microspore method was first reported in *N. tabacum* by Sunderland and Roberts (1977). It consists the collection of microspores shedding from anthers in liquid media that is followed by their induction in a different media separate from anthers to circumvent the toxic effect of anther (somatic) tissues. The anther tissue is critical to remove and it is recommended to keep these tissues away from microspores because they release phenolic compounds that have a lethal effect on microspores. Moreover, somatic tissues of anthers may lead to development of diploid (*2n*) rather than haploid plantlets and may give rise to some complications in the research experiments/trials. This isolation method is very simple, easy to follow, avoid any complications for isolation and always results in less injury/damage to the microspores but it is more like anther culture rather than microspore culture. Since the discovery of shed microspore in 1977, it was

quickly adopted by Datta and Wenzel (1987) for microspore isolation in wheat. The magnetic bar stirring isolation method involves a stirring force to remove microspores that are covered by anthers. This isolation procedure is more efficient and effective as compared to natural microspore shedding because it gives rise to a higher number of microspores than the shed microspore method (Cho and Zapata 1990). Lichter (1982) used a glass or Teflon rod to isolate microspores from anthers in *B. napus* by pestle maceration followed by sieving for purification. Micro blending is one of the most widely adopted methods and consists of blending the surface sterilized dissected buds or florets in mechanical blenders. It removes microspores more effectively as compared to the previously described procedures. The somatic tissues of anthers are effectively removed from microspores by sieving through the sterile mesh of various sizes (100-200 µm). The mechanical micro blending was first described by Swanson et al. (1987) in *B. napus,* followed by Olsen (1991) in barley and Mejza et al. (1993) in wheat. Currently, a couple of centrifugations are conducted for microspore purification. These centrifugations often involve density gradients such as maltose (Kasha et al. 2001) or percol (Joersbo et al. 1990) to isolate microspores that are between mid to late uninucleate or early bicellular phase/stage in case of cereals and between unicellular to mid-bicellular phase in case of tobacco. This purification results in a uniform population of isolated microspores that do not contain any anther tissue or nonviable microspores. The mechanical micro blending is an important procedure as compared to other methods as it always yields a greater number of viable microspores (75%) (Gustafson et al. 1995). Lately, one more isolation procedure has been identified in *Datura metel* by Iqbal and Wijesekara (2007) where the anthers were aseptically removed from their filaments by opening the flower buds. Then, these anthers were used to isolate microspores by applying various combinations of temperature pulses. Anthers were placed lengthwise on liquid media and squeezed out the microspores by temperature pulse followed by removal of anther tissues or debris by using stereo microscope.

Media composition

Basal media like MN6, MS, B5, A2, MMS3, P4, P2, CHB3, NLN, N6, and NPB99 has been effectively used in anther and microspores culture in many crop species. The NLN (Lichter 1982) and MS (Murashige and Skoog 1962) media with minor changes are

used for brassica and other allied species, whereas NPB99 (Konzak et al. 1999), A2 (Touraev et al. 1996b) and MMS3 (Hu and Kasha 1997) are routinely used during anther or microspore culture in cereal species like wheat, barley and triticale. In the early days of androgenesis, solid media using agar as a solidifying agent was preferred but as the time progressed, liquid media became the best choice to achieve the desired results because solid media contains agar that is proven to have a pollen inhibitory effect in a few cases and hinders pollen growth towards embryogenesis. On the other hand, liquid media offers no competition for nutrient availability among developing embryos or ELS, especially during initial induction/culture phase of anthers/microspores. The main problem associated with liquid media is microspore sinking that often results in creating an anaerobic environment leading towards slower metabolism and a decrease in energy production. However, this problem can be easily solved by adding Ficoll to the medium (Cistue et al. 2009; Kao 1981). The effectiveness of IMC is mainly dependent on the properties and characteristics of the induction medium that consists of i) nutrient constituents like mineral substances, carbohydrate, pH and osmolality ii) cultural environment like light intensity, temperature, photoperiod and duration of culture and iii) density of the medium. The role of media with respect to microspore culture is twofold: first, it supplies microspores with all necessary nutrients required for their growth and development *in vitro* and secondly, it also helps to switch the pathway from gametophytic to sporophytic. It has been recommended that microspores must be provided with all required nutrient rich media having macro and micro salts, carbohydrates, vitamins, nitrogen source and growth regulators, if required. The nutrient concentration and their presence in the media are highly variable and depend on the crop species being used.

For quite some time, hormones such as potato extract, auxins, coconut milk, cytokinins, yeast extract, and ethylene were frequently used in the media (Raghavan 1986), but recently it has been reported that these growth hormones have a major role in callus formation during the process of embryogenesis. The characteristics and functions of growth regulators/hormones in media have been investigated in detail to see their effect on increasing the efficiency of embryogenesis in many crop species. In cereals like barley, triticale and wheat, the positive role of phenyl acetic acid (PAA),

naphthalene acetic acid (NAA), indole acetic acid (IAA), abscisic acid (ABA), Benzylaminopurine (BAP), 2, 4-D and Kinetin in the media alone or in combination with each other to improve the entire process of embryogenesis has been reported in numerous research studies (Davies 2003; Hansen 2000; Kasha et al. 2001; Otani and Shimada 1994; Pauk et al. 2003). However, these hormones have not been used to a larger extent in media being used for microspore culture. Antibiotics such as cefotaxime have been successfully used in the microspore induction medium to manage/tackle the problem of contamination (Davies 2003; Lantos et al. 2006). Charcoal has also been added to media to control contamination or to remove toxin substances due to its absorption capacity but simultaneously it also absorbs other crucial nutrients from the media necessary for the development of ELS (Gland et al. 1988). The addition of antioxidants to media to promote embryogenesis has also been examined where glutathione played an important role in embryo development (Asif et al. 2013; Fletcher et al. 1998). Sucrose is a key source of carbon in media. The concentration of sucrose differs from one crop species to another. Carbohydrates are only source of energy but their role as an osmoticum to maintain a certain osmotic pressure in media cannot be overlooked and as an osmoticum, they regulate the movement of nutrients/elements from cells.

Medium alteration is one the most popular exercises that has been carried out for the last 40-50 years to seek maximum output from microspore culture for improving androgenic response especially in recalcitrant species. The substitution of sucrose by maltose between 60-90 g/l in rice, triticale, barley, rye and wheat induction media (Bishnoi et al. 2000; Chu et al. 1990; Karsai and Bedo 1997; Kasha et al. 2003a; Otani and Shimada 1994; Pauk et al. 2000) has demonstrated improved effects but on the other hand sucrose is still being used in Brassica species in a concentration of 130 g/l (Pechan and Smykal 2001). A significant improvement in the efficiency of barley embryogenesis has been illustrated by altering the sources of organic nitrogen. Olsen (1987) observed enhanced results by lowering down the concentration of ammonium nitrate (NH_4NO_3) and increasing glutamine concentration in media. This finding is still being adopted by researchers and recently glutamine in the concentration of 500 mg/l in media has revealed a positive influence on barley microspore culture (Kasha and Maluszynski 2003;

Kasha et al. 2003a; Kasha et al. 2003b). Glutamine is also a key element to develop DHs in brassica (Hansen 2003), rye (Pulli and Guo 2003) and triticale (Wedzony 2003) *via* microspore culture. In durum wheat, glutamine in combination with glutathione has given promising results with respect to the frequency of embryos and green plants (Cistue et al. 2009; Asif et al. 2013). The mineral ingredients like Fe, $ZnSO_4$ and $CuSO_4$, have also been reported to have a positive effect in inducing embryogenesis and increasing the ratio of green vs. albino plants in *Hordium vulgare* (Echavarri et al. 2008; Wojnarowiez et al. 2002). Jacquard et al. (2009) and Prem et al. (2008) reported a positive effect of induction medium supplemented with Cu and $AgNO_3$. In a similar manner, the supplementation of media with n-butanol also improved embryo yield in wheat microspore culture and boosted the frequency of green plants up to 3-5 times (Soriano et al. 2008). In tobacco, the embryogenic division of microspores is highly reliant on the presence of Fe in the induction media. Furthermore, Fe also plays a major role in the senescence of the anther wall (Vagera and Havranek 1983).

The pH and osmotic pressure of media are other important factors that play a critical role in affecting not only the process of embryogenesis but also help in improving regeneration efficiency of embryos towards green plants. The alteration of medium osmoticum is usually done using polyethylene glycol (PEG) and mannitol in different concentrations. The occurrence of albinism has been seen to be lessened by high osmolality of the medium (Jacquard et al. 2006). The media pH is normally kept around 6.0, however, slight change is needed depending on crop species being used for embryogenesis (Ferrie et al. 1995b).

The IMC in cereals is often comprised of induction medium supplemented with various types of embryogenic material such as florets, ovaries, embryogenic microspores or ovules that has shown promising results in improving an overall process of microspore embryogenesis in wheat, barley, triticale and rye (Lantos et al. 2009). Generally, it is assumed that supplementing induction medium with these tissues supplies microspores with certain phytohormones and signaling molecules to start/initiate the process of embryogenesis and thus, these tissues contribute towards embryo development, but the main mechanism by which this material/tissue converts microspores to embryos is yet not clear. However, it was pointed out that

arabinogalactans-proteins/arabinogalactans exhibit certain stimulatory functions that helped to initiate the process of embryogenesis (Letarte et al. 2006). The supplemented induction media with gum arabic and Larcoll has also shown a strong impact in improving wheat microspore culture. The addition of Larcoll with or without ovaries in the induction medium greatly reduced mortality of microspores. It also provided a genotype independent effect, reduced albinism and improved green plants regeneration (Letarte et al. 2006). In barley microspore culture, the addition of florets in the induction medium greatly improved androgenesis and has been found to be more successful than ovary co-culture (Lu et al. 2008).

The optimum density of microspores in the induction medium is another factor that ensures their further growth and development and determines the time required to produce embryos from microspores. In triticale, the microspores densities of 3×10^4 to 2×10^5 microspores/ml of the induction media is considered optimum for normal growth and development of microspores (Eudes and Chugh 2009) while densities of 8-10 $\times 10^4$ microspores/ml for *Capsicum annum* (Kim et al. 2008), 4×10^4 microspores/ml for *B. napus* (Huang et al. 1990) and 5×10^4 microspores/ml for *B. oleracea* (Ferrie et al. 1999) are considered ideal.

Regeneration

The development of embryos from microspores can be achieved via indirect/direct pathways. Regardless of these pathways, embryos are required to shift from culture to regeneration medium in order to achieve a smooth transition/switch from embryos/ELS to green plants. Regeneration is always achieved in the presence of light. This transition relies on many factors such as age or growth stage of embryos at the time of regeneration, regeneration medium, light intensity and temperature during the regeneration period/phase. The cold treatment below 10^oC during early regeneration period (1-3 weeks) and transfer of embryos at cotyledonary stage in brassica species has shown promising results (Ferrie 2003; Niu et al. 1999). However, the transition phase of microspore descended embryos (torpedo, early, mid or late cotyledonary stage) differs greatly from one species to another. The desiccation of embryos prior to their transfer to regeneration medium was recommended by Hansen (2003) who pointed out that desiccation facilitates the process of embryo germination.

The cold temperature around 10^{o}C for seven days during initial phase of regeneration has also been suggested for triticale to alleviate the process of transition, reducing albinism and enhance the frequency of green plants (Wedzony 2003). Supplementation of regeneration media with vitamins or phytohormones and drought stress to the embryoids have also been recommended to ease the overall transition from embryos to green plants (Zhang et al. 2006).

Increase in ploidy level

The number of chromosomes/ploidy level of plantlets produced by IMC can be verified/determined by several methods that include i) counting of chromosomes that is mostly done using microspore, ii) by measuring chloroplast size and number of stomata (guard cells), iii) using flow cytometer and iv) through morphological observations. A comparison of these methods were done by Sari et al. (1999) to determine ploidy level in water melon. The authors concluded that stomatal or guard cell measurement is one of the simplest and easiest methods to find/calculate number of chromosomes in plants. Spontaneous chromosome doubling is high in few crop species; thus, these species do not require any artificial chromosome doubling whereas on the other hand, most crop species need an increase in their ploidy level to covert haploid plants (obtained through IMC or anther culture) to doubled haploids that often involve the use of anti-microtubule agents like colchicine.

The rate of chromosome doubling is affected by numerous factors such type of genotype, stage of microspores at the time of floral organ collection from donor plants, the pathway of microspore during embryogenesis, pretreatment (cold, heat or starvation), exposure time of microspores/embryos to various chemical agents, concentration of chemicals during induction and regeneration phases, and methods of application. The history of induced doubling of chromosomes goes back to 1929 when Lindstorm (1929) decapitated tomato shoot and discovered that new developing shoots were tetraploid rather than diploid. This was followed by Randolph (1932) who conducted an experiment in maize and induced an artificial increase in ploidy level by giving heat treatment (using heating pot) to the developing ear shoot. This artificial chromosome doubling attracted many researchers to design studies/experiments in order to test different methodologies/protocols to induce chromosome doubling in

agricultural crops using cold treatments, heat shocks, and antimitotic agents. The discovery of colchicine from Gloriosa by Clewer et al. (1915) perfected its use in chromosome doubling and with the passage of time, it became a method of choice for artificial chromosome doubling (Blakeslee 1939) in at least 48 agricultural crops. Regardless of its extensive exploitation, effectiveness and application in agricultural crops, there are numerous disadvantages associated with its usage that include occurrence of mixed polyploids (Pei 1985), loss of sterility, decrease in fertility, abnormal growth, chromosomes rearrangements and gene mutations (Luckett 1989). However, it has been successfully used for chromosome doubling to produce/develop DH plants in wheat, sorghum, barley, maize, sugar beet and many other crops.

Principally, the doubling of chromosome is achieved by various means/pathways that include i) endomitosis that is referred as “duplication of chromosome number without nuclear division”, ii) an interference in cell cycle of plants iii) endoreduplication referred as “chromatids become double without separating from each other” iv) C-mitosis referred as “an artificially induced abortive nuclear division where separation of centromere does not take place in the metaphase stage”, v) nuclear fusion where “one nucleus forms as a result of fusion of two or more nuclei” (Jensen 1974). The entire cell cycle can been divided into 4 well-defined stages (Francis 2007); i) G_1 (Gap_1): A post mitotic stage in which cell grows and enlarges and it becomes ready for cell division ii) S (Synthesis): It is characterized by DNA replication or synthesis iii) G_2 (Gap_2): A pre-mitotic stage and vi) M (Mitosis): that consists of division of a mother cell into two daughter cells. The chemical agents that interfere with cell division at the completion of Synthesis/S stage and prior to the completion of Mitosis/M has been termed as good agents to increase ploidy level in plants (Dhooghe et al. 2011). Various pretreatments that have been applied to donor plants or microspores to initiate their sporophytic growth have revealed explicit results with respect to increases in ploidy levels, *e.g.* pretreatment of floral organs with mannitol alone in combination with cold or heat shocks have significantly increased the frequency of chromosome doubling in wheat (Li and Devaux 2003) and rye (Guo and Pulli 2000b). On the other hand, colchicine treatment alone or in combination with heat or cold shocks in the induction medium has also facilitated chromosome doubling in *Phleum pretense* (Guo and Pulli

2000a), *B. napus* (Zhao et al. 1996a, b) and Easter lily (Antoine and Beckert 1997). A few other anti-microtubule agents have also been successfully exploited for this purpose that include 2,6-Dinitroaniline in watermelon, Trifluralin in Orange Ball (*Buddleia globosa*), Surflan in *Lilium longiflorum*, amiprophos-methyl (APM) in Dianthus sp. and oryzalin in *Solanum* sp. (Greplova et al. 2009; Nimura et al. 2006; Omran et al. 2008; Takamura et al. 2002; Van 2008). These anti-microtubule agents induce chromosome doubling by creating hindrance in the separation/segregation of sister chromatids toward poles, inhibit spindle formation and nuclear fusion (Testillano et al. 2004) and offer extreme affinity to plant tubulins as compared to the most commonly used colchicine. Thus, a very little amount of these anti-microtubule agents (mostly in milimole concentration) is required to induce artificial chromosome doubling (Morejohn and Fosket 1984).

On the basis of above discussion, it can be concluded that a universal protocol cannot be identified or developed for artificial chromosome doubling mainly due to the complexity of the process and its genotypic dependency because anti-microtubule agents behave differently in different crops. Therefore, selection of a polyploidizing agent is mainly dependent upon the type of genotype being used, stage of cells/cell cycle at the time of application, application procedure/method and exposure time to these chemicals. Nevertheless, colchicine is an extensively used and widely adapted anti-mitotic agent to induce chromosome doubling in cereal, leguminous and horticultural crops but the applicator should avoid any contact during its treatment/use mainly due to its anticipated lethal and harmful effects on plants and to applicator as well.

Albinism

In plants, albinism can be defined as lack or deficiency of green pigment called "chlorophyll" or failure to carry out the process of "photosynthesis", a chemical process necessary to synthesize food (carbohydrate) from carbon dioxide (CO_2) and water (H_2O) in the presence of sunlight. Albinism eventually results in plant death. The process of photosynthesis is initiated by absorbing light energy by round, oval or disc shaped structures/organelles known as chloroplast which consists of chlorophyll. Plants store absorbed light in the form Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

(NADPH) and Adenosine Triphosphate (ATP). This captured light is then used by plants in later stages. Green pigment or chloroplast organelles are absent in albino plants and therefore, they are not able to carry out photosynthesis, a process essential for their growth and development. Thus, these plants do not reach maturity and die at a very early stage. Albinism is considered a major bottleneck in plant genetics and breeding programs that involve interspecific crosses or wide hybridization to create variation and in plant tissue culture techniques involving microspore and anther culture particularly in the case of cereals such as barley, oat, wheat, rice, rye and triticale. Varying degrees of albinism have been described in DH production via anther culture, microspore culture and wide hybridization that is characterized by a partial to a total loss of green pigments. Abadie et al. (2006) conducted an interesting experiment to compare chlorophyll contents of green and albino plants and depicted severe dissimilarities. They reported chlorophyll contents of green and albino plants as 2.97 + 0.56 and 1.9 + $5x10^{-2}$ µg/mg of fresh weight, respectively. Yao and Cohen (2000) also pointed out that albino plants have at least 1-6% less green pigments than green plants. In wheat, the importance of magnetic field was studied by Pingping et al. (2011) to improve an overall chlorophyll contents in the leaves of chlorophyll deficient plants. They reported an increase in the chlorophyll content of magnetically treated albino plants, which converted them to partially green plants and such plants attained physiological maturity as well. A similar study was carried out in date palm to investigate the influence of magnetic field on chlorophyll contents and results showed a substantial improvement in total pigment contents, carotenoid and chlorophyll a, b due to static magnetic field. However, the pigment content increase was extremely reliant on exposure duration and intensity (Dhawi and Al-Khayri 2008). A similar trend in the increase of chlorophyll contents in soybean after an exposure to a static magnetic field has also been reported by Atak et al. (2007). Mouritzen and Holm (1994) depicted that during earlier stages of plant growth, albino plants can be distinguished from green plants because of variations in their plastid DNA as a result of microspores/anthers redifferentiation and such plants have irregular chloroplast shape/structures rather that can be differentiated either by amyloplasts or proplastids (undeveloped). Therefore, these plants are not able to carry out the process of photosynthesis and cannot make carbohydrates for their growth and development. Caredda et al. (2000) described that seedlings/plantlets of albino plants

can exploit reserve food only for some time and when stored carbohydrates are exhausted, albino plants begin to die due to reason that abnormal and undeveloped plastids cannot be switched to be functional chloroplasts. The dissimilarities with respect to physiology, structure and behavior of plastids in albino and green plants have also been reported in barley (Caredda et al. 2004; Caredda et al. 2000). These findings revealed that genotypes yielding/giving green plants exhibit thylakoids (dense and undifferentiated plastids capable of multiplying quickly and accumulating starch rapidly) while albino plants were devoid of thylakoids, deficient in cytoplasm, plastids were not dividing and starch was accumulating in their stroma. Furthermore, plastids in genotypes producing green plants had much higher levels of DNA as compared to other genotypes.

The decrease in frequency of albino plants has been achieved by manipulating genetics (using different cultivars) as well as by altering growing conditions of donor plants. Various aspects like growth and developmental conditions of donor plants, genotype, stage of microspores at the time of floral organs collection, pretreatments, induction duration, microspore pathway, composition of medium, embryo age at the time of transfer to regeneration medium, temperature, oxygen and light intensity during induction and regeneration play a crucial role in tackling this challenge. The alteration in any of these components will alter the frequency of green to albino plants.

Cold shocks and starvation for three to four days have significantly decreased the frequency of albino plants as compared to a longer pretreatment of three to four weeks (Kasha et al. 2001). In an exciting study in barley, supplementation of induction media with $CuSO_4$ alone or in combination with mannitol enhanced the percentage (90%) of green plants as compared to chlorophyll deficient plants in a cultivar Igri (Cistué et al. 2003). It has been suggested that a strong relationship exist between microspore sampling stage and frequency of chlorophyll deficient plants (Caredda and Clement 1999). The frequency of albino plants increases if the donor plants of barley and oat are grown in a temperature less than 15^{o}C (Collins 1927). However, an exposure of oat albino mutants to a temperature higher than 20^{o}C results in switching to green plants (Nishiyama and Motoyosh 1966). As discussed earlier, chlorophyll deficient plants/plantlets are not capable of making their own food or enough carbohydrates to

keep and support their growth and development, therefore, it has been proposed that raising sugar (especially sucrose) levels/contents of the medium can help to solve the problem of albino plants. Saidi et al. (1997) successfully tackled this issue (albinism) in *Triticum turgidum* by manipulating the sucrose contents of the media and converted albino plants/plantlets to green. The addition of starch-melibiose and mannitol in medium in combination with cold treatment considerably enhanced green plant frequency in barley (Datta and Potrykus 1998; Hunter 1987). The green pigment content and percentage of green plants have also been significantly increased by the addition of glucose and growth hormones such as cytokinin, kinetin, IAA and benzyl adenine (BA) (Broughton 2008; Chory et al. 1991; Nishiyama and Motoyosh 1966) but kinetin and 2,4-D seemed to have no influence on the green plant percentage especially in triticale (Pauk et al. 2000). In anther culture or IMC, addition of Ficoll in the liquid induction medium has prevented microspore's sinking that in turn help to decrease DNA degradation in plastids. Therefore, the addition of Ficoll in induction medium in barley microspore culture enhanced the percentage (0 to 50%) of green plants (Kao et al. 1991). The same trends with maltose (Redha and Talaat 2008) and Ficoll (Zhou et al. 1992) have been reported in wheat. An interesting aspect in regards to albinism has been highlighted in cereals (oat, barley wheat and rice) with respect to the collection of floral organs from donor plants (Reinbothe et al. 2003a; Reinbothe et al. 2003b) where the authors stated that collection of floral organs from primary tillers give a higher number of green plants as compared to a collection from secondary or tertiary tillers that might be due to a hormonal imbalance in later (secondary or tertiary) tillers that affected the structure and behavior of plastids. Moreover, the existence of competition among secondary or tertiary tillers for hormones, and nutrients are concentrated in the center of the root zone might be a cause of albinism in later tillers (Casimiro et al. 2003) and it is also obvious because primary tillers are always more productive and healthy than later ones.

Genetics and genomics of albinism and green plant regeneration

A large number of studies have been conducted to identify quantitative trait loci (QTL) and genes in several crop plants to increase the percentage of green plants. In this regard, QTLs on chromosomes IBL/1RS, 2AL, 2BL and 5BL have been mapped to improve

the frequency of green plants in wheat. Among them, a QTL mapped on 2AL helped explained higher variation (Torp et al. 2001; Tuvesson et al. 1989). In another experiment, two genes controlling embryogenesis have been identified on chromosomes 2D and 2A using the wheat monosomic lines while 5B, 5A, 4A and 2B carry few minor genes (Zhang and Li 1984). Genes controlling frequency of albino plants and embryoids have also been mapped on 5B and 5BL, respectively (Agache et al. 1989). Recently, three QTLs were identified on barley chromosomes 6H, 5H and 2H for number and percentage of green plants (Chen et al. 2007). It was previously reported that albinism is controlled by one gene in barley (Collins 1927). Two QTLs have also been identified on rice chromosomes 1 and 9 as well (He et al. 1998) that improve green plant percentage. Ekiz and Konzak (1991) conducted studies in wheat using alloplasmic lines exhibiting different plastid but same nuclear genome and found that the plastid genome plays a critical role in microspore culture response in wheat. The phenomenon of albinism is extremely heritable (Larsen et al. 1991) in some crops while in others such as wheat (Redha and Talaat 2008), low heritability has been reported for green plant percentage. However, Chaudhary et al. (2003) argued that it is strongly influenced by non-additive and additive type of gene action. In a similar study conducted by Moieni and Sarrafi (1995), on forty nine different wheat varieties, high heritabilities ranging from 0.80-0.88 were found for characteristics such as green plant frequency, embryoid percentage, and frequency of plantlet regeneration. The specific and general combining abilities of these characteristics have also been found to be significant. The investigators demonstrated the lowest heritability for albinism proposing that proportion of albino plants can be decreased by altering environmental and cultural conditions at various stages during the process of microspore culture in wheat. A few other experiments on this aspect suggested that the frequency of albino plants is under the control of one gene in soybean, barley and maize (Barwale and Widholm 1987; Collins 1927; Neuffer et al. 1997) while two or more loci are involved in peanut (Dwivedi et al. 1984). In peanut, cytoplasmic inheritance has also been shown to control albinism (Branch and Kvien 1992).

Pathways of microspore embryogenesis

The embryogenic capability in microspores is usually attained by the application of different stresses and/or starvation that is followed by a series of steps during which the microspores are converted to embryo/embryo-like structures. During the process of embryogenesis, the microspore goes through several morphological, physiological and cytological changes that are indispensable for their further growth and development *in-vitro*. This embryogenic process of microspores can be distinguished into three distinct phases: i) attainment of embryogenic capability, ii) several asymmetric or symmetric cell divisions within exine wall that convert/lead microspores to embryo or ELC or sometime called multicellular structures (MCS) and iii) development and initiation of a certain pattern in ELS/MCS following exine wall disruption. Indrianto et al. (2001) carried out an interesting experiment to track microspores in wheat during the entire process of embryogenesis. They reported that microspores isolated from stress-induced anthers were two-fold increased in size and morphologically diverse as compared to microspores derived from freshly isolated anthers. On the basis of microspore morphology during embryogenesis, they divided microspores into three distinct kinds: Type I was characterized as vacuolated microspores because they had a huge vacuole that was present in the center while their nucleus was pushed/pressed near periphery/cell wall. Basically, these types of microspores were non-stressed that are usually present at late unicellular stage. Type II was characterized as having fragmented/broken vacuole. Basically, cytoplasmic strands were crossing/passing through vacuole from one end to another and these strands were linked to phragmosome (cytoplasmic pocket) inside the nucleus. In this type (II) of microspores, cytoplasmic pocket was adjacent to cell wall while type III microspores can be easily differentiated from others because they had a phragmosome in the center. Their study also revealed that that 62% of the type III microspores were not embryogenic as they were not capable of switching their gametophytic pathway to sporophytic and could not develop into embryos. By the contrast, this frequency was much less in microspores of type I (5%) and II (23%). A few other morphological and cytological variations in microspores during embryogenesis including cell enlargement, intine (new cell wall) development inside exine, chromatin compaction and decreases in size, amount and

magnitude of starch grains and nucleolus have also been reported (Garrido et al. 1995; Ramirez et al. 2001).

There are numerous studies that have discussed the pathway and fate of microspores regarding their switching or reprogramming from the gametophytic pathway to the sporophytic. Theories presented in these experiments are basically established on the basis of division of generative and vegetative cells. Recently, five potential pathways for conversion of embryogenic microspore to embryo or ELS have been proposed by Aionesei et al. (2005) that are basically a modification or alteration in A, B and C pathways previously defined by Sunderland (1974). A-Pathway is characterized by a symmetrical division of vegetative cells leading towards embryo development. However, sometime callus development has also been reported instead of embryo formation and this is true especially for cereals like rice (Chen 1977), barley (Sun 1978) and wheat (Wang et al. 1973). In this type of pathway, the generative cell dies at a very initial stage of embryogenesis or goes through a division that results in two sperm cells that die ultimately. The A-pathway has been noticed in the embryogenesis of *Brassica napus* (Fan et al. 1988), wheat (Reynolds 1993) and tobacco (Sunderla and Wicks 1971). B-Pathway is associated with the splitting of the cell nucleus in two identical vegetative cells, both of which contribute to switching towards embryo formation as identified by Indrianto et al. (2001) in wheat. The C-Pathway is comprised of a merging or fusion of one vegetative and one generative cell nucleus or fusion between two vegetative cell nuclei. This pathway has been reported in barley (Yao et al. 1997) and *Datura innoxia* (Sunderland 1974). The D-Pathway is an altered type of B-Pathway whereby two broken/divided nuclei divide again and again that ultimately lead to production of callus or a haploid embryo (Pan et al. 1983; Zhu et al. 1978). The E-Pathway consists of the development of an embryo from a generative nucleus. The repeated division of generative and vegetative nuclei can also give rise to an embryo but this embryo will consist of a higher number of cells from generative than vegetative nuclei (Sun 1978). The E-Pathway has been noticed in wheat, rice, barley and *Hyoscyamus niger* (Pan et al. 1983; Qu and Chen 1984; Raghavan 1978, 1976).

The preponderance of one pathway over other is governed by several elements. In this regard, pretreatments in the form of cold, heat and starvation or any kind of

stress play a critical role in deciding the fate of microspores (Kasha et al. 2001). The adaptation to a pathway varies considerably among species. Recent advancements in video cell tracking systems and the development of flow cytometry will definitely assist the molecular scientists to improve their understanding of how complex mechanism of microspore conversion from gametophytic to sporophytic pathway take place and ultimately culminate in haploid plants.

Anther Culture

The haploids plants through anther culture are usually obtained via two methods, *i.e.,* culturing anthers in liquid or semi liquid media that involves pollen separation by agitation, and placing anthers on solid media (solidification is usually obtained using agar). Basically, surface sterilized buds and florets are opened (*in vitro*) in sterile environment followed by anther removal and placement on liquid or solid medium (Sunderland et al. 1984). Once the embryo formation is completed, embryos (culture) are shifted to the regeneration medium under light conditions for organogenic differentiation (shoot and root development). This method is very similar to IMC. The only difference in microspore culture is the removal of anther tissues or anther wall (somatic tissue) to prevent any lethal effect of the maternal tissues on embryo development; and sometimes, the somatic tissue may give rise to a diploid plant rather than a haploid. Similar to IMC, anther culture also implies numerous pretreatments (vary considerably from species to species), surface sterilization, anther dissection and finally placement on induction and regeneration medium. The effectiveness of anther culture is highly reliant on growth, developmental conditions and the physiological state of donor plants, pollen or microspore stage at the time of anther dissection, genotype and medium composition. In anther culture, the pollen may give rise to callus (indirect embryogenesis) tissues or callus formation as in wheat and rice or lead to an embryo (direct embryogenesis) development as in *Brassica* spp.

Genotype, physiological state, growth and developmental stage of donor plants

Physiological state, growth and developmental conditions and genotype of donor plants are among the important factors that decide the efficiency of anther

culture because these conditions directly interfere with the overall effectiveness of embryogenic pollen grains (P-grains) by effecting the hormonal level and nutritional status of anther tissues (Sunderland and Dunwell 1977). It has been reported that donor plants grown under nitrogen starvation conditions often yield embryogenic pollen grains. These pollen grains can be easily differentiated by having a large vacuole, absence of starch grains and the presence of a thin exine wall (Heberle-Bors 1989, 1984). Contrary to IMC, a better response has been obtained from field grown donor plants as compared to greenhouse plants (Vasil 1980). The other developmental conditions such as day light, photoperiod intensity and temperature also influence anther culture to a considerable degree (Heberle-Bors 1989). A varied genetic response has also been noticed in several experiments/studies that differ not only among species but also within genus, species and cultivars thereby suggesting a major role in anther culture/embryogenesis. Germana (2007) and Bajaj (1980) conducted two different studies to identify most responsive cultivar to anther culture. They termed 2 out of 23 and 10 out of 20 varieties as responsive in citrus and wheat, respectively. In anther culture, the embryogenic time/window of pollen grain consists/starts from the first mitosis which is characterized by vacuolated microspores to bi-cellular stage. However, this embryogenic window is highly variable and depends on the genotype being used for anther culture. Moreover, pollen grains lose their embryogenic efficiency when they begin storing/preserving starch in the form of grains (Raghavan 1990; Touraev et al. 2001). The ploidy level of plants obtained through anther culture is also influenced by the developmental stage of pollen grains/microspores at the time of induction. Sopory and Munshi (1997) depicted that microspores at the uninucleate stage will give rise to haploid plants while culturing of anthers having microspores at later stages often yield higher ploidy levels.

Pretreatments and media composition

The pretreatments have been categorized as “novel”, “widely used” and “neglected”. The commonly adapted pretreatments are cold and heat shocks, starvation in the form of nitrogen and sucrose, heavy metal and chemical treatments, changes in pH, humidity, osmotic levels and water stresses (Shariatpanahi et al. 2006). Among these pretreatments, temperature shocks have been termed as widely adapted. As

discussed earlier, the anthers are usually chosen when they exhibit microspores in the embryogenic window (between first mitosis to bi-cellular phase), however, a little heat (41^{o}C) pretreatment in *B. napus* has resulted in embryogenic division in already developed vegetative cells as reported by Binarova et al. (1997) and it also appeared to be helpful in pepper (Barany et al. 2001) and *Nicotiana tabacum* (Touraev et al. 1996a; Touraev et al. 1996c) anther culture. The gamma rays have been successfully used as pretreatments for anther culture in barley (Vagera et al. 2004) and rice (Aldemita and Zapata 1991) while colchicine has also been used as a pretreatment (stress inducer) as well as to double the chromosome number in various crops such as wheat, brassica, maize, rice, sugar beet and sorghum (Germana 2011b, a).

The composition of the medium also occupies an important position in anther culture to induce embryogenesis. In this regard, B5, MS and N6 with minor changes are among the widely adapted media. The MS is mostly used in solanaceous crops while N6 has been applied in cereals (Chu 1981). Sucrose is a main source of carbohydrates and its concentration varies from 6-17%. In anther culture, medium having a high sucrose concentration has been used in species where culturing of tricellular pollen (mature) has given a high response (*e.g.* in Cruciferae) (Dunwell and Thurling 1985) but on the other hand in solanaceous species where bicellular pollen is used for anther culture, medium with a low concentration of sucrose has given optimum results (Dunwell 2010). Maltose in the medium has also indicated an explicit influence on anther culture embryogenesis in rye, rice, triticale, barley and wheat (Wedzony et al. 2009). Germana and Chiancone (2003) described explicit findings of clementine anther culture using a glactose and lactose in the medium. The same results in clementine have also been reported using sucrose in combination with glycerol (Germana et al. 2000). The effects of growth hormones have been extensively studied during the last 60-70 years and they have provided exceptional results in some recalcitrant species. However, there are few species (belonging to the Solanaceae) that do not need any growth hormones in their culture for embryogenesis. There are two main functions of growth hormones in the medium; one is to induce embryogenesis (Bajaj 1990; Bajaj et al. 1977) and other is to identify the fate of embryogenic pathway (Ball et al. 1993). Various studies have indicated that 2,4-D helps to enhance/promote callus growth while NAA and IAA

supports direct embryogenesis (Liang et al. 1987). The supplementation of medium with polyamines has also improved the frequency of embryos in clementine (Chiancone et al. 2006), *Cucumis sativus* (Kumar et al. 2004) and wheat (Rajyalakshmi et al. 1995). Similar findings in cereal anther culture have also been stated using arabinogalactans (Letarte et al. 2006) and ovary co-culturing (Broughton 2008).

An incredible advancement in anther culture methodology has been achieved in crop species such as triticale, wheat, rice, barley, rye, and several others like medicinal, vegetables, fruits, ornamental and woody plants. However, there are still many groups of species that are termed as recalcitrant to anther culture and legumes are considered as one of these (Dunwell 2010; Wedzony et al. 2009). The up to date progress and developments of anther culture have been recently reviewed in detail by Dunwell (2010); Germana (2011b, a); Touraev et al. (2009) and Wedzony et al. (2009).

Uniparental chromosome removal/elimination or wide hybridization

The uniparental chromosome elimination or wide hybridization is considered to be an important tool not only to produce DH but also to create genetic variation, introduce new species and for gene transformation studies. It consists of crossing a female parent to a distant male exhibiting haploid inducer genes. Intercrossed parents are taxonomically or ecologically similar to each other. During the process of intercrossing, chromosomes of pollen donor parent are automatically removed or eliminated. Wide hybridization becomes the best method to achieve desired results in DH production following the recovery of barley (*Hordeum vulgare*) haploid plants involving wide intercrossing using *H. bulbosum* as a male parent (Kasha and Kao 1970). During wide hybridization, endosperm is either not developed or poorly formed. Thus, the embryo must be rescued or cultured *in vitro* or otherwise it may not survive and give rise to a haploid plant. The *in-vitro* embryo culture provides a conducive environment and nurtures the immature or weak embryos allowing them to carry their growth and developmental process. Cereals such as wheat, barley, rice, maize, rye and triticale are amongst the most privileged crops in which wide hybridization has been exploited extensively along with microspore and anther culture to induce sporophytic

embryogenesis. The technique of wide hybridization has been used effectively in solanaceous crop species to recover hybrids. The key benefits of wide hybridization include absence of gametoclonal variation, genotypic independence, getting unbiased random gametes for producing mapping populations and absence of albino plants or albinism which is especially true for cereals.

Bulbosum method

The intercrossing of *H. vulgare* and *H. bulbosum* involves preferential chromosome removal of later parent following fertilization. The completely grown embryos (caryopsis) are rescued (*in-vitro*) before endosperm disintegration, usually 12-14 days after pollination, to recover hybrids or haploid plants. The chromosome elimination is genetically controlled and genes involved in the chromosome elimination of *H. bulbosum* chromosomes have been mapped on *H. vulgare* chromosomes 2 and 3 (Ho and Kasha 1975). It has been further explained that elimination or retention of chromosomes is highly genotypic dependent (Pickering 1984) and it will only take place if the parents are grown in a cold temperature below 18^{o}C together with the application/spray of growth hormones/regulators (like 2,4-D or Dicamba) 1-2 days after pollination as illustrated by Devaux and Pickering (2005). Chromosomal elimination can be tracked by distinct arrangement of species specific centromeres on multi polar spindles along with the production of nuclear extrusions in initial/early interphase (Gernand et al. 2005; Kim et al. 2002; Subrahma and Kasha 1973). Thus, consecutive cell divisions (mitosis) during the process of embryo development results in chromosomal elimination of the male parent that give rises to a haploid embryo. Barclay (1975) successfully intercrossed hexaploid wheat with *H. bulbosum* to develop haploids in hexaploid wheat. However, genotypic dependence to some extent and lack of crossability (crossability barrier) with *H. bulbosum* are among the major hurdles to use the Bulbosum method in wheat (Snape et al. 1979).

Haploids using maize as a pollen donor

The barriers with respect to crossability have not been reported between crosses of maize and wheat. Crossability genes that have been mapped on wheat chromosomes *i.e. Kr1* on 5BL, *Kr2* on 5AL, *Kr3* on 5D and *Kr4* on 1A, are not sensitive;

thereby, they do not create any barriers/hindrance. A high frequency of green haploid plants has been obtained using maize as the pollen donor (wide hybridizer) not only in wheat but also in barley (Furusho et al. 1991) and triticale (Wedzony et al. 1998). Genotypic dependence (to some extent), growth and developmental conditions (Campbell et al. 2001) and emasculation method (Knox et al. 2000) have been illustrated as major aspects affecting the frequency of green plants to a greater extent in wheat. The spray of growth regulators following pollination (2,4-D or/and Dicamba) or injection in the last internode have significantly enhanced embryo production (Wedzony et al. 1998). The frequency of haploid production in oat using maize as a wide hybridizer is low (Rines and Dahleen 1990) because maize chromosome are not completely or entirely removed/eliminated during caryopsis. Therefore, it often results in the production of more polyhaploids than haploids. These polyhaploids have been exploited in other genetic studies due to their use in the production of aneuploids (Rines 2003). A few other species have also been used as a wide hybridizer include *Zea mays* sp. Mexicana, pearl millet, Job's-tears (*Coix lachryma-jobi* L.) and sorghum (Inagaki and Mujeeb-Kazi 1997; Mochida and Tsujimoto 2001; Riera-Lizarazu et al. 1993; Ushiyama et al. 1991).

Haploids using Solanum phureja and maize inducer lines

In *Solanum tuberosum* (cultivated tetraploid potato), haploid plants are produced by crossing with a diploid species, *S. phureja*, used as a pollen donor. The cross gives rise to a functional endosperm that results from the union of both sperm nuclei with the central wall of the ovule. Maine (2003) explained that this fusion initiates growth and development of the unfertilized egg via parthenogenesis. The percentage of haploid embryos produced from a cross between *Solanum tuberosum* and *S. phureja* is extremely low, however, the haploid embryos can be simply differentiated from hybrid ones using a colored gene marker. The colored gene markers have been incorporated in the haploid embryo by male parent (pollen donor). A similar technique of color gene marker is also being used in maize to produce haploid plants that involves crossing with a haploid inducer line that transmits colored (scorable) gene markers like lec1 promoter driving CRC, anthocyanin gene, R-nj and GFP (US Patent 20060185033). The genotypes

RWS (Geiger and Gordillo 2009) and stock 6 (Eder and Chalyk 2002) have been used commercially to produce haploids on a larger scale in maize.

Based on the above discussion, it can be concluded that the importance of doubled haploidy is well known in all fields of agriculture and related disciplines. The acceleration has been observed in research studies on DH production over the last five years. Major research efforts have been focused on changing the status of many recalcitrant crops to responsive and to improve the overall methodology of DH production. Several model genotypes have been identified in various crops that have led to an overall improvement in the technology. In this regard, IMC has been of special interest to the plant breeders, geneticists and molecular biologists due to the availability of embryonic units in a larger number. It is quick and efficient. Moreover, genetically identical and physiologically uniform microspores provide a target for cell biology and genetic engineering studies. Molecular studies increase our knowledge on the pathways by which gametophytic development is converted to the sporophytic pathway during microspore embryogenesis. The genomic studies have identified various genes/QTLs associated with androgenic induction that will help further improvement in the production of doubled haploids.

Figures

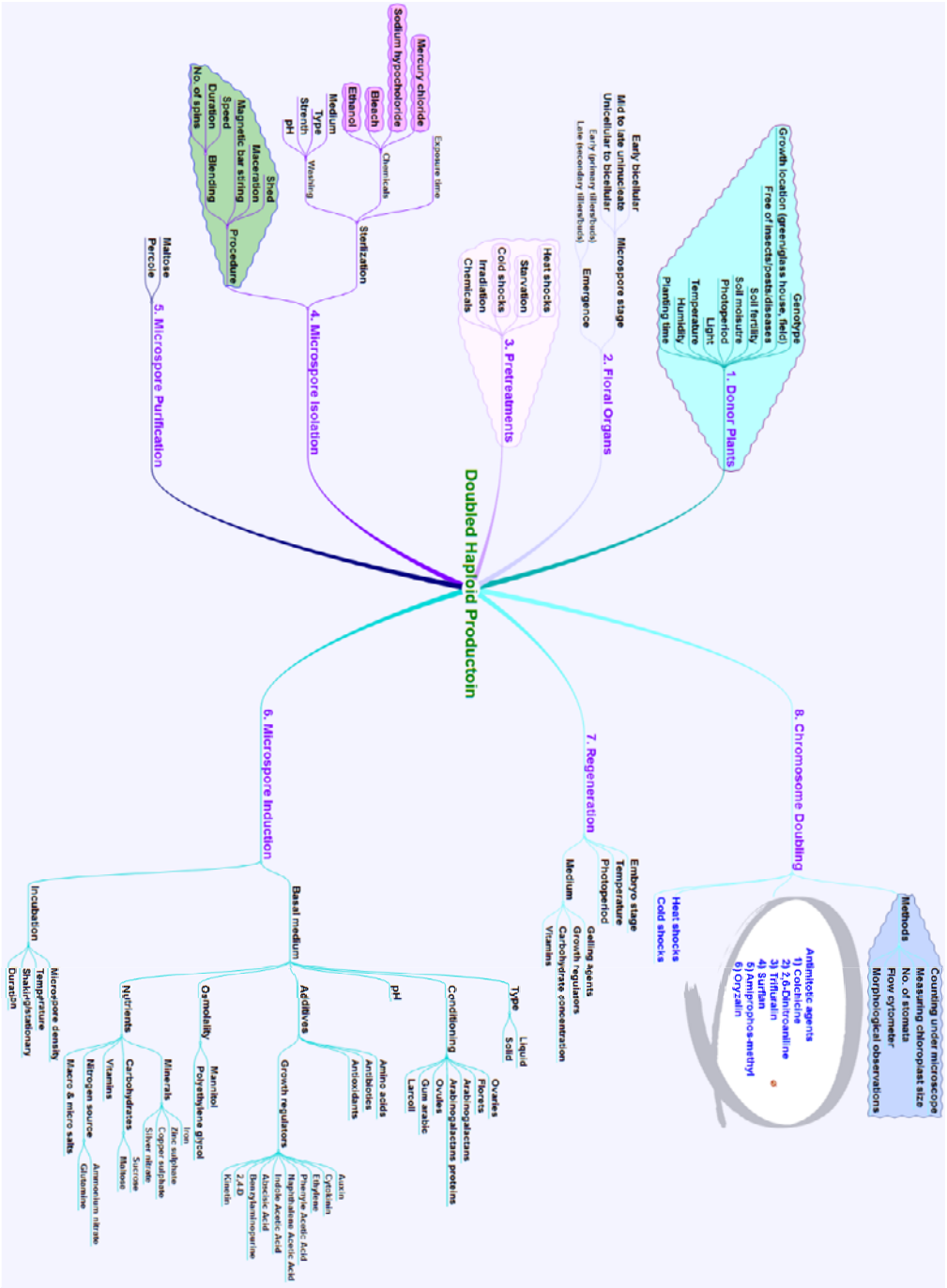

Fig.1. Steps involved in IMC to develop doubled haploid plants.

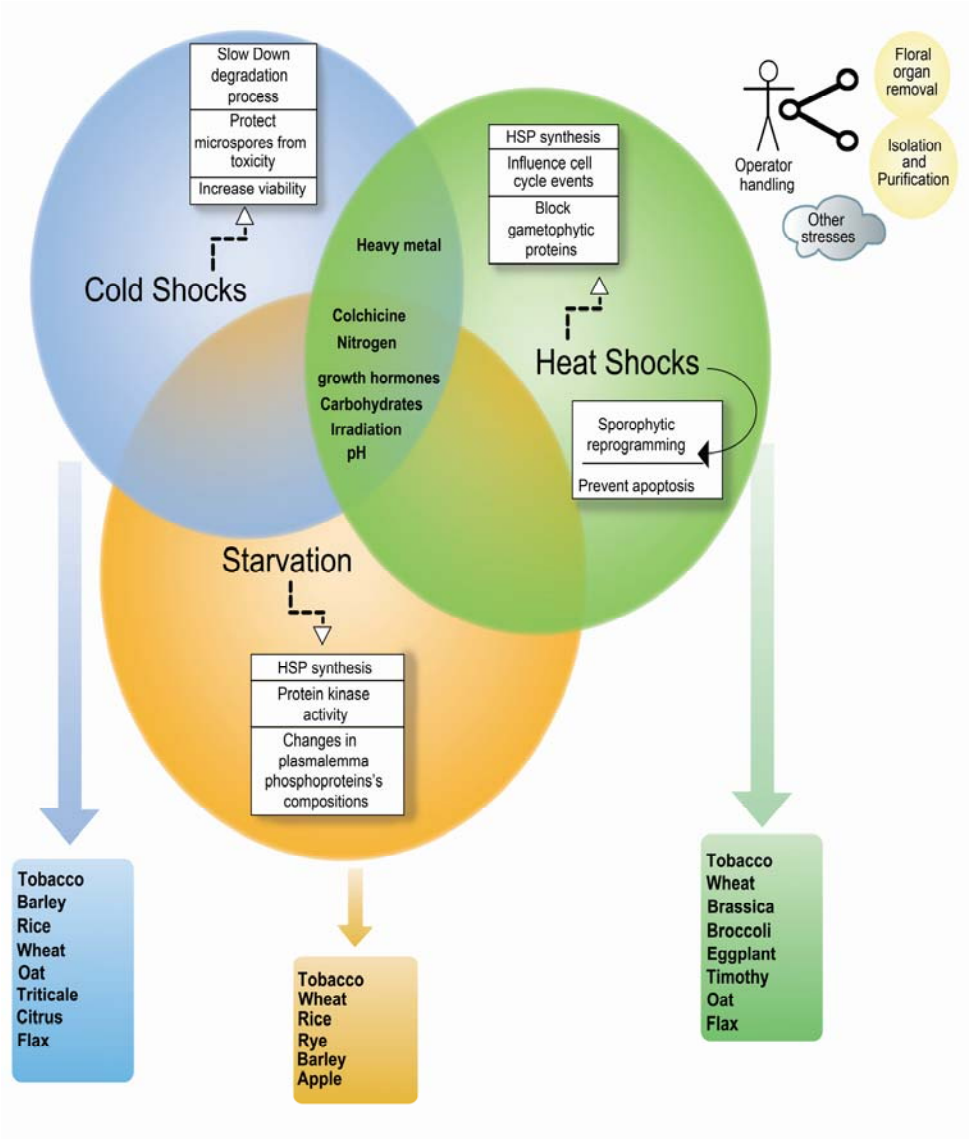

Fig.2. **Schematic representation of pretreatments/stresses employed during IMC in various crops.**

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