2008

Effects of ammonium nitrate upon direct somatic embryogenesis and biolistic transformation of wheat

Greer, Michael S.

Lethbridge, Alta. : University of Lethbridge, Faculty of Arts and Science, 2008

EFFECTS OF AMMONIUM NITRATE UPON DIRECT SOMATIC EMBRYOGENESIS AND BIOLISTIC TRANSFORMATION OF WHEAT

MICHAEL S. GREER
Bachelors of Science, University of Lethbridge, 2005

A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

MASTERS OF SCIENCE

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

Dedicated to my parents, Mike and Dianna Greer,
for their love, inspiration and support
throughout my education.

Abstract

Triticum aestivum is of major importance both nutritionally and economically globally. Traditional breeding mechanisms have been unsuccessful at keeping pace with the increasing demand for better yielding and more resilient wheat varieties. The introduction of foreign genes into systems has provided a new tool for crop improvement, but has been difficult to apply to elite wheat varieties mainly as result of their recalcitrance to prerequisite tissue culture. Investigations here demonstrate that modification of the ammonium nitrate content in direct somatic embryogenesis induction medium can increase the number of primary embryos produced by over two fold in the elite hard red wheat cultivar Superb. The number of primary embryos which were capable of transitioning into shoot development also increased by two fold. Biolistic transformation efficiency was also improved when targeted scutellar tissue was exposed to elevated ammonium nitrate levels.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic Acid
cv.	cultivar
CWB	Canadian Wheat Board
CWRS	Canadian western red spring
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double strand break
DSBR	double strand break repair
DSEM	direct somatic embryogenesis in monocots
EDTA	ethylenediamine tetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FEN1	fenpropimorph resistance
GEM	germination of embryo of monocot
HR	homologous recombination
IR	illegitimate recombination
Ku	Ku (patient name)
MAPK	mitogen activated protein kinase
NAA	napththaleneacetic acid
NHEJ	non-homologous end joining
PARP	poly(ADP-ribose)polymerase
PEG	polyethylene glycol
PGR	plant growth regulator

PIN	PIN-FORMED
PPE	plantlet producing embryo
PVP	polyvinylpyrrolidone
RAD1	Radiation 1
RAM	root apical meristem
RecA	recombinase A
SAM	shoot apical meristem
SDSA	synthesis dependant strand annealing
SE	somatic embryogenesis
SEM	secondary embryogenesis in monocots
sp.	species
SSA	single strand annealing
ssDNA	single-stranded deoxyribonucleic acid
XRCC4	X-ray repair cross complementing protein 4

Chapter 1. Literature Review

1.1 Biolistic Transformation

1.1.1 Technique

Microparticle bombardment, alternatively known as biolistic transformation, is a physical delivery system which makes use of small biologically inert microcarriers such as gold or tungsten to carry foreign nucleic acids into the cells of targeted tissues. This is done by first coating the particles with the nucleic acid in addition to PVP (Polyvinylpyrrolidone) which binds the particle to the casing of the bullet. The particles are then accelerated at high speeds by helium or air pressure discharged into a target tissue (Helenius *et al.*, 2000). This can either be done in a very controlled environment lacking air (Chawla, 2002) or in a less controlled but more practical ambient environment (Helenius *et al.*, 2000). In either event, a heterogeneous mixture of transformed and un-transformed cells is produced in the target tissue, which necessitates that the targeted tissue be cultured to produce plants from individual cells if a non-chimeric plant is desired (Sahrawat *et al*., 2003).

1.1.2 Biolistic Transformation of Wheat

Wheat, *Triticum aestivum* L., is of the utmost importance both nutritionally and economically in Canada and in the world at large. As of 2005, 17% of the world's cultivatable land was devoted to the production of wheat (Jones, 2005). In 2004 more than 500 million metric tonnes of wheat had been produced globally (Bhalla, 2006a), which traded at a value of 22.8 billion dollars Canadian (Bhalla *et al.,* 2006b). As strong

as demand currently is for wheat, this demand is expected to rise by 40-50% from current levels by 2020 (Janakiraman *et al.,* 2001; CIMMYT). In the past, however, traditional breeding methods have only increased wheat yields by 0.9% per annum (Reynolds *et al.*1999), which would only produce at 12% increase over current levels by 2020.

The introduction of novel transgenes is a powerful tool that can help produce better yielding and more resilient wheat crops capable of meeting the anticipated rise in demand for wheat. Unfortunately, many transformation techniques that have been successful in other crops, including, microinjection, transformation via protoplast (PEG & calcium phosphate), electroporation (Altpeter *et al*., 2005), vortexing with DNA and silicon carbide bristles, pollen tube mediated introduction (Bhalla *et al.*, 2006b) and even laser mediated uptake (Jones, 2005) have all been shown to be ineffective or irreproducible in wheat. As a result, microparticle bombardment and *Agrobacterium* mediated transformation have emerged as the sole techniques capable of introduction foreign DNA into wheat.

Although wheat is not a natural host of *Agrobacterium tumefaciens*, the advent of super virulent strains and the use of *vir* inducing agents, such as acetosyringone and glucose, have enabled the bacteria to infect cereal tissues (Sahrawat *et al*., 2003, Janakiraman *et al*., 2002) and introduce T-DNA into the target genome at a comparable rate to microparticle bombardment (Bhalla, 2006a). Despite these advances, however, microparticle bombardment still possesses several desirable qualities that sets it apart from *Agrobacterium* mediated DNA transfer. Firstly, bombardment requires only a minimal cassette and therefore problematic T-DNA and whole plasmid backbones can be removed prior to transformation (Fu et *al*., 2000; Jones, 2005). The sequences contained

in vector backbones can often promote recombination events prior and post integration, as well as induce meiotic instability and silencing of the transgene (Fu *et al.,* 2000). Secondly, the success achieved using *Agrobacterium* has largely been in agronomically inferior cereal lines and can require extensive backcrossing to introduce the transgene into a more desirable background (Bhalla, 2006a) whereas microparticle bombardment is fairly genotype independent (Jones, 2005; Takumi *et at*., 1997). As well, microparticle bombardment is capable of introducing numerous transgenes at once, allowing for the introduction of polygenic traits and multi-gene pathways facilely (Sahrawat *et al*., 2003; Halpin *et al.*, 2005). Finally, microparticle bombardment is also capable of targeting foreign DNA to mitochondria and chloroplasts, providing a broader range of integration sites than is available with *Agrobacterium* mediated transformation (Altpeter *et al.*, 2005).

1.1.3 Complex Transgene Integration

Although microparticle bombardment is more expensive and technically challenging than *Agrobacterium* mediated transformation (Bhalla, 2006a), the main shortcoming of microparticle bombardment is its tendency to produce complex integration patterns (Jones, 2005; Hu *et al*., 2003). These undesirable patterns are the products of either a construct, fragmented during bombardment, being integrated into the genome, high number of copies being integrated into the genome or rearrangements that can occur before and after integration. 'Simpler' or 'cleaner' integration patterns are preferable as they are easier to characterize, produce simpler Mendelian segregation patterns and are less prone to silencing (Jones, 2005). For human consumption, and

many scientific and commercial ventures, it is necessary to introduce a transgene in such a fashion that it will stably express from generation to generation without altering the genetic background it was placed within (König *et al*., 2004). As it is less capable of producing this desired stability, many favor *Agrobacterium* mediated transformation over microparticle bombardment.

1.2 Transgene Integration

1.2.1 Non-Homologous End Joining (NHEJ)

In yeast, animals and plants, it is suggested that transgenes introduced either via microparticle bombardment or by *Agrobacterium* are inserted into the genome by the host's own replication, transcription or DNA repair machinery into natural or induced breaks in the chromosomes (Somers & Makarevitch, 2004). In plants, non-homologous end joining (NHEJ), sometimes referred to as illegitimate recombination (IR), is the predominant double strand break (DSB) repair pathway (Britt, 1996; Friesner & Britt, 2003). This pathway is initiated when a heterodimer of Ku70 and Ku80 binds to the free end of a DNA molecule protecting it from degradation and from proteins involved in other double strand break repair (DSBR) pathways (Tsukamoto & Ikeda, 1998, Li *et al.*, 2007). The Ku70/80 DNA complex then recruits a host of DNA-PKcs and a XRCC4-DNA ligase IV dimmer which completes the end-joining by linking the free ends of the broken DNA. In some instances where the free DNA ends have large single stranded overhangs, additional proteins will be recruited to 'process' the ends before they are ligated together (Wyman & Kanaar, 2006).

Non-homologous end joining, as the name implies, searches for very little homology, 2-5 base pairs, between the two free ends it links together (Britt, 1996; Ray & Langer, 2002). This entails that, although there is a possible favoring of transcriptionally active regions of the genome (Kim & Gelvin, 2007), the transgene will integrate into one of the available DSBs at random, thus making the location in the genome of transgene integration random as well.

1.2.2 Homologous Recombination (HR)

DSB repaired by HR can proceed by three different pathways, all of which reconstitute DNA strands only when they share long tracks of homology. The first pathway, single strand annealing (SSA), links homologous regions together from the same chromosome at the expense of any sequence that lies between the two regions of homology (Figure 1.1) (Tsukamoto & Ikeda, 1998; Sugawara *et al.*, 2000). The second two pathways, termed double strand break repair (DSBR) and synthesis dependant strand annealing (SDSA), make use of sequences on ectopic or allelic DNA strands to repair the double strand break (DSB) (Figure 1.2) (Lida & Terada, 2004). The DSBR and SDSA models then differ in that mobile Holliday junctions are formed in DSBR which can travel up and down the DNA strands. As a result, crossing over events are possible with DSBR, which cannot occur via SDSA where the invading DNA strand returns to anneal with its original complimentary strand (Wyman & Kanaar, 2006).

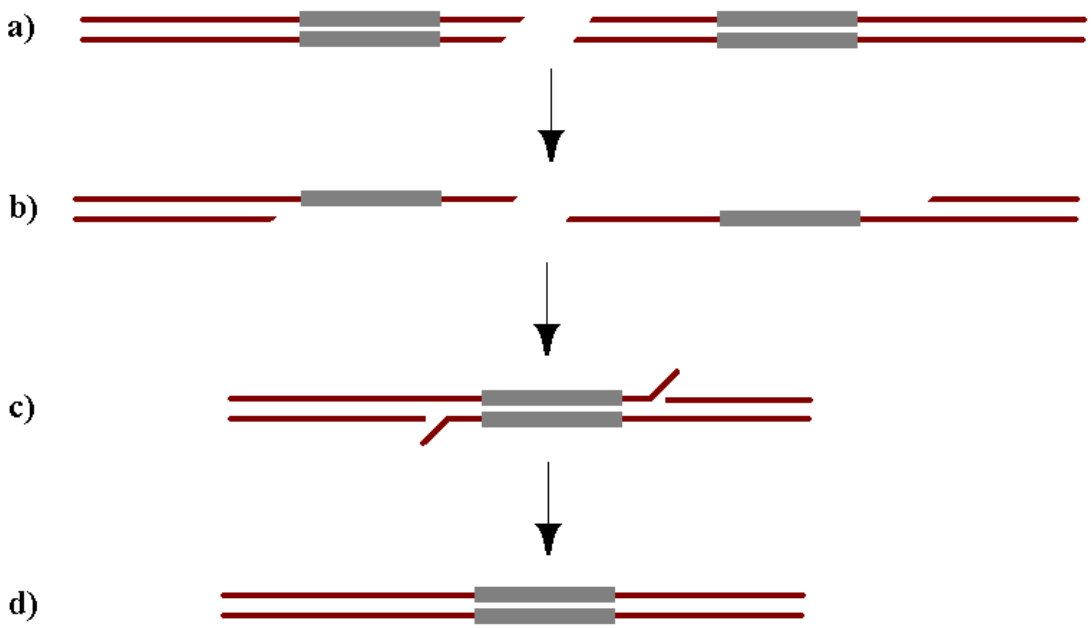

Figure 1.1 Illustration of single strand annealing (SSA) mechanism. A double strand break (a) is first processed by the 5′→ 3′ trimming by Radiation 1 (Rad 1) and Rad10 to produce a 3′ overhang (b). Rad 52 then facilitates the search and annealing of homologous sequences found on either side of the double strand break (c). FEN 1 then removes the 3′ flap as DNA polymerase δ binds the two strands together to produce (d), a repaired double helix with the deletion of sequence between the two homologous (grey) regions (Masuda-Sasa *et al.*, 2006; Tsukamoto & Ikeda, 1998).

Study of site-induced DSBs has led to the observation of ectopic and T-DNA integrations which have fingerprints of both HR and NHEJ, one process occurring on either side of the integrated DNA fragment. Integration of a DNA molecule by both processes is not readily explainable by the DSBR model which proposes a Holliday junction on both sides of the integrated DNA, and thus are thought to be the product of SDSA repair. SDSA repair using NHEJ on one end only requires one side of the transgene to possess homology to the integration site, and it has been observed that constructs containing homology only at one end integrate only 1/3 less often as those with homology at both ends (Puchta, 2002). This suggests, at least in the somatic cells studied, that SDSA is the main HR pathway used to repair double strand breaks (Puchta, 2005).

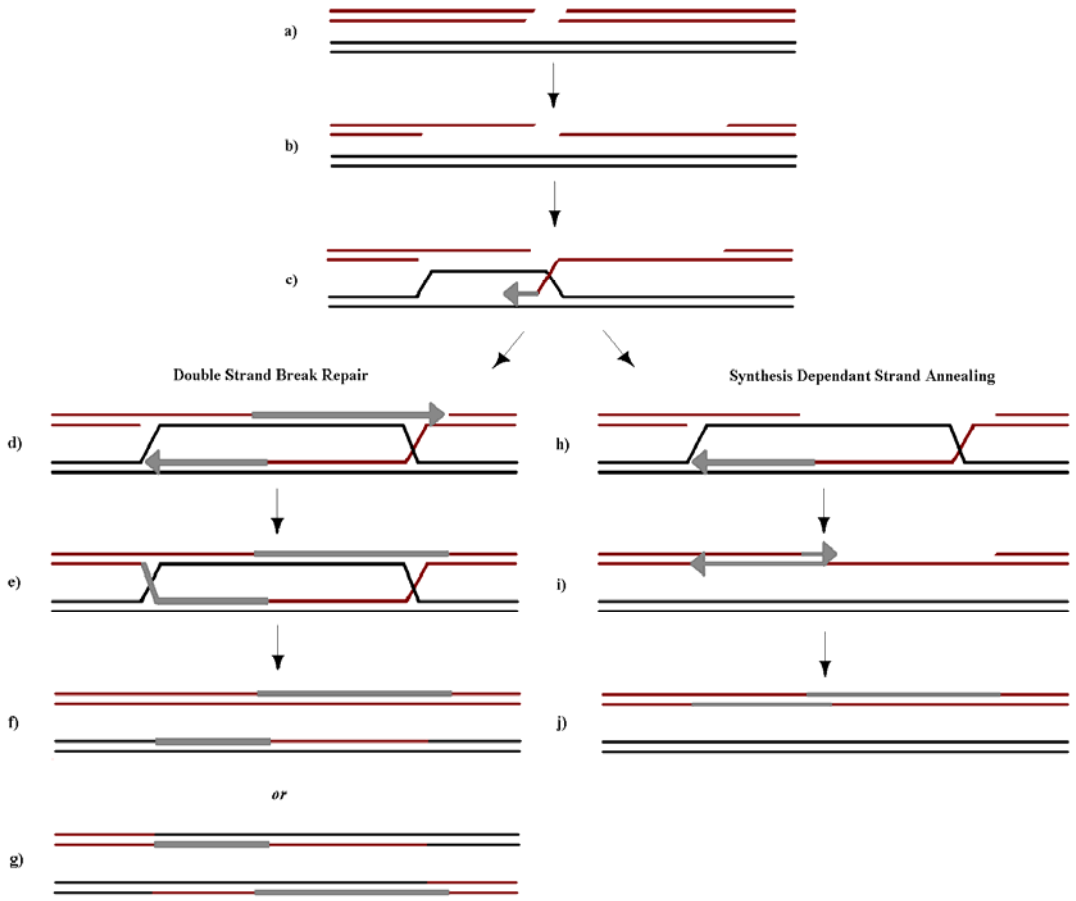

Figure 1.2 Illustration of double strand break repair and synthesis dependant strand annealing mechanisms of HR. After a DSB is created in a strand of DNA (a), exonucleases digest the strands from 5′ to 3′ to produce 3′ overhangs (b). Nucleoprotein filaments and a recombinase then facilitate the invasion of one of the 3′ overhangs into the nearby strand of DNA to form a structure known as a D-loop (c). At this point, in the DSBR model DNA synthesis, indicated in grey, extends the 3′ overhangs using the undamaged DNA strand (black) as a template (d). The invading 3′ strand then reengages its original double helix to produce (e) a double Holiday junction complex. Resolvases can then either resolve the intermediate to produce a non-crossover (f) or cross over (g) recombinants. In the SDSA model, the DNA synthesis extends the invading 3′ overhang using the undamaged DNA strand (black) as a template (h). The invading strand then re-associates with its sister chromatid to produce (i). This sister chromatid then uses the returned strand as a template for its own extension by DNA synthesis. After the completion of SDSA the only the non-crossover product (j) is produced. (Puchta, 2005; Wyman & Kanaar, 2006)

1.2.3 Gene Targeting

1.2.3.1 Advantages to Gene Targeting

Gene targeting is the capacity to direct the integration of a transgene into a specific location within the target genome. The technique has numerous advantages over random integration of the transgene into the genome. Gene targeting can ensure that the introduced gene will be placed within a region of the genome that is transcriptionally active, avoiding the possibility that the transgene will be poorly or erratically transcribed. As well, gene targeting can be used to alter the native expression of endogenous genes by inserting, deleting or modifying promoters and enhancer elements. This is a strong asset to gene targeting as it allows for spatial and temporal control of desired genes. Modification to genes as small as point mutations can be introduced via gene targeting (Muller, 1999). Of great importance as well is the capacity of gene targeting to remove (knockout) a select sequence of DNA without adversely affecting the background genome of that sequence (Lida & Terada, 2004; Puchta, 2002). This is in contrast to certain chemical mutagens used for knockouts which can cause significant background mutation and require numerous rounds of backcrossing to remove (Henikoff & Comai, 2003).

In yeast, where gene targeting is achieved facilely, transgenic DNA with homology to genomic sequences will almost exclusively integrate to the location in the genome which has those sequences (Puchta, 2002). Thus, gene targeting may also be beneficial by being able to reduce the recombinant DNA copy number by limiting the locations in which the introduced DNA can integrate. Transgenes frequently suffer from poor expression, gene silencing and instability as a consequence of their presence in high

numbers (Fu *et al.*, 2000; Hanin & Paszkowski, 2003; Altpeter *et al.*, 2005; Jones, 2005). In yeast, transgenes will integrate almost exclusively into their target site, and thus gene targeting also prevents transgenic DNA from being inserted into native genes, and thus altering the genetic background of the target species (Puchta, 2002). Thus gene targeting provides the capacity to introduce a foreign gene into a system in a very specific and 'clean' fashion at minimal risk to the native genome of the targeted species.

1.2.3.2 Strategies for Gene Targeting in plants

Homologous recombination is so prevalent in lower eukaryotes that gene targeting can be accomplished by simply introducing a transgene which has flanking regions of homology to the desired insertion location into the nucleus of the target species. In higher eukaryotes, however, double strand breaks are only mended by homologous recombination once every 10^3 to 10^6 repair events (Lida & Terada, 2004; Puchta, 2002; Hanin & Paszkowski, 2003; Puchta, 2005). As a result, the predominant NHEJ pathway will incorporate the transgene randomly into the higher eukaryote's genome essentially every time regardless of whether or not the transgene possesses homology to certain areas within that genome.

The majority of the work pursuing gene targeting in plants has focused on shifting the native ratio from NHEJ to HR. To this end, varying degrees of success have been achieved by the overexpression of important genes involved in HR such as *RecA*, *RuvC*, *Rad50* and *Rad54* (Shalev *et al.*, 1999; DeBlock *et al.*, 1997; Reiss *et al.*, 2000; Hanin & Paszkowski, 2003; Tzifira & White, 2005). *Rad 54* is a member of the S*WI2*/*SNF2* superfamily and is involved in chromatin remodeling and strand invasion during HR. It

was found that following yeast *Rad54* cloning into *Arabidopsis*, the amount of gene targeting rose by one to two orders of magnitude (Shaked *et al.,* 2005) even though *Arabidopsis* already has its own, though potentially different in function, ortholog of RAD54. *RecA* (homolog of plant *RAD 51*), acts alone in searching for homology and promoting strand exchange during HR in *E. coli* (Reiss *et al.,* 2000). Overexpression of *RecA* in tobacco has also been shown to increase homologous recombination (Reiss *et al.,* 1996), but its overexpression *in vivo* did not lead to an increase in gene targeting in tobacco (Reiss et *al.,* 2000).

In addition to increasing HR protein expression, decreasing the expression of proteins in NHEJ has also been attempted in order to favor HR. Interestingly, it was found that the *Arabidopsis ku80* mutant was not impaired in its *Agrobacterium* mediated transformation efficiency. The authors, however, also observed that these plants were only slightly incapacitated in their ability to circularize linear plasmids, suggesting that the mutant possesses an unknown mechanism of compensating for the loss of KU80 (Gallego *et al.,* 2003). Conversely, another group which published the same year that *atku80* plants were impaired in *Agrobacterium* transformation (Friesner & Britt, 2003). This study of the latter group, however, was larger in scale than the former, and observed changes in transformation frequencies smaller than the estimated lower range of the former study (Gallego *et al.,* 2003)

The repair mechanism can also be influenced by the application of external factors. Pretreatment of calli with niacinamide prior to biolistic transformation was suggested to produce lower copy numbers in the transgenic plants produced by inhibiting poly(ADP-ribose)polymerase (PARP), which is a promoter of NHEJ (DeBlock *et al.,*

1997). PARP has been shown to bind to DSB and various nuclear acceptor proteins, including histones. It is suggested that PARP promotes NHEJ by transiently modifying chromatin structures (Puchta, 1995). Inhibition of PARP by 3-methoxybenzamide was also shown to promote HR in *Arabidopsis* and tobacco (Puchta, 1995). The pretreatment with niacinamide has since been patented for it's potential promoting HR in transformed cells (United States Patent 6074876).

Altering the repair systems integral to maintaining genomic stability can produce numerous undesirable phenotypes (Lida & Terada, 2004). For example, *atku80* plants are severely impaired in their resistance to radiation exposure (Gallego *et al.,* 2003). As an alternative, there are systems developed which can achieve some of the goals of gene targeting without altering the endogenous repair mechanisms of target plant. For example, specially designed zinc-finger nucleases can specifically recognize target sequences, and then induce a DSB at that point. The zinc fingers can be designed to recognize tracks up to 24 bps, thus making it sufficiently selective for any genome smaller then 2.8×10^{14} bps (Lloyd *et al.,* 2005). In plants, NHEJ will be the predominant repair mechanism that repairs breaks induced by the zinc finger nucleases, and will often leave insertions or deletions of several nucleotides cleanly knocking-out the gene function (Lloyd *et al.,* 2005; Tzifira & White, 2005). It has been suggested that genes could be introduced at these targeted locations as well (Tzifira & White, 2005; Porteus & Carroll, 2005) as demonstrated in human (Moehle *et al.,* 2007 and Drosophilia (Beumer *et al.,* 2006).

Quite similar is the use of a rare cutting endonuclease, I-*Sce*I, which can create DSB at target locations. These target sequences recognized by I-*Sce*I, however, are

dispersed randomly through the genome and thus are inutile for gene knock-outs (Li *et al.,* 2007, Puchta, 1995). However, as DSB are rate limiting in recombinant DNA integration, if the I-*Sce*I restriction sites are in desirable locations in the target species, induced cleavage of I-*Sce*I sites could be used to encourage a gene to insert within a desired locus or at least outside of important endogenous sequences.

Lastly, the Cre-*lox* system has also been shown to be effective at directing transgenes to a specific locus. The bacteriophage P1 protein Cre binds to a 38 base pair DNA *lox* sequences and catalyzes a recombination between them while maintaining perfect fidelity of the sequence (Baer & Bode, 2001). Using this process for site directed transformation in plants first involves cloning in a construct containing a promoter and a downstream *lox* target via conventional means. Once a plant is found to have the construct integrated in a suitable location, a second transgene with a *lox* sequence and a promoterless selection marker can be transformed facilely into the chromosomal *lox* target site. The Cre-*lox* system has also been employed to remove undesirable selection markers after the introduction of transgenes by flanking the marker with *lox* sequences. After the induction of the Cre, the marker is excised from the genome and the flanking sequences are ligated together (Gilbertson, 2003). Despite their advantages, however, the Cre/lox and the I-*Sce*I facilitated DNA introduction fall short of being considered true gene targeting mechanisms as they either require prior integration of insertion sites or are not capable of targeting any location within the genome. Thus, although powerful tools are being developed to aid in the successful production of transgenic plants, more work needs to be done to elevate plant gene targeting to the status seen in yeast and animals.

1.3 Somatic Embryogenesis

1.3.1 Somatic Embryogenesis

Somatic embryogenesis (SE) is the process of utilizing the intrinsic totipotency of non-germ cells to regenerate full plants (Sahrawat *et al*., 2003). This process can either proceed by developing pre-embryonic cells into somatic embryos, or by de-differentiate developed somatic tissue into an unorganized mass of dividing cells, termed calli, from which somatic embryos can arise (Chawla, 2002, Rout *et al.,* 2006). These processes are referred to as direct and indirect somatic embryogenesis, respectively. In either case, it appears that somatic embryos will be produced only from small minority of pre-embryongenic determined or induced embryonic determined cells, making the resulting plant the progeny of one sole cell or a small cluster of cells (Mathew & Philip, 2003; Chawla, 2002; Jimenez, 2001). This property of somatic embryogenesis is believed to allow for the production of full grown non-chimeric plants from the mass of transformed and untransformed cells produced by the majority of DNA introduction techniques (Nhut *et al*., 2006). Remarkably, it is often the difficulty in regenerating transformed cells and not the introduction of transgenic material that is the limiting factor in producing transgenic plants (Takumi & Shimada, 1997)

Inducing somatic embryogenesis often only requires applying a single exogenous signal to a suitable explant. A frequently used is auxins such as NAA or 2, 4-D, but can also be initiated with other plant hormones or even via certain macromolecules (Chugh & Khurana, 2002; Jimenez, 2001). This signal leads to cessation of the existing gene expression pattern of the cell and the commencement of an 'embryonic program' (Jimenez, 2001). Expression assays have revealed that the induction and development of

somatic embryogenesis only entails the production of 10-25% new genes. These genes involved in somatic embryogenesis include heat shock proteins, house keeping genes, mitogen activated protein kinases (MAPKs), hormone responsive genes and G-proteins (Racusen & Schiavone, 1990; Nato *et al*., 2000; Chugh & Khurana, 2002; Chugh & Eudes, 2007).

After this initial alteration in expression pattern which is unique to somatic embryogenesis, the maturation of the embryo proceeds analogously to zygotic embryogenesis with only minor differences (Mathew & Philip, 2003; Racusen & Schiavone, 1990). The first step in embryogenesis involves the establishment of a longitudinal axis via cell division along the transverse plane. In zygotic cells this cell division is asymmetrical, where the apical cell produced is smaller than the basal cell (Dodeman *et al.*, 1997), as opposed to somatic embryogenesis which can produce equal sized cells (Mathew & Philip, 2003). In either scenario, the progeny of the apical cell will produce the aerial shoot system of the plant, and the basal cell the subterranean root system (Leyser & Day, 2003). After this initial division, several more rounds of cell division occur to produce a compact globular embryo. The globular embryo cells are thick walled and small as no volume is gained throughout these divisions (Chapman *et al.*, 2000). As the globular embryo develops, auxin influx and efflux proteins polarize on cell surfaces in such a fashion that they create pathways which push the auxin towards the base of the embryo (Leyser & Day, 2003). Auxin is known to promote cellular division (Chawla, 2002) and its focusing at the base of the embryo will eventually produce the root apical meristem (RAM) (Bassuner *et al.*, 2006). In tissue culture it may be necessary to remove the somatic embryos from the high auxin containing medium

after the induction phase of somatic embryogenesis as these levels can impede the focusing of the auxin and formation of the RAM (Bassuner *et al.*, 2006). It has been observed that somatic embryos often have very weak connections to the mother calli, which may help them focus the auxin at the root pole (Bassuner *et al.*, 2006; Mathew & Philip, 2003). It has also been suggested that culturing explants in liquid, as opposed to solid media, reduces the embryo's dependency upon the mother tissue and thus explains the improved embryo yields often associated with this technique (Nhut *et al.*, 2006; Sakhanokho *et al.,* 2001). It is noteworthy that somatic embryos often have difficulty producing normal shoot apical meristems (SAMs) as well, which may also be a product of over exposure to hormones in the induction media (Dodeman *et al.,* 1997). On the whole, however, somatic embryogenesis follows a nearly identical developmental process to that of zygotic embryogenesis, progressing from the globular to heart, torpedo and finally cotyledonary stage (Figure 1.3), or to scutellar and coleoptilar in the case of monocots. It would then be typical for the somatic embryos to bypass the desiccation and seed formation process and germinate directly. If so desired, however, some techniques are available to produce so called 'synthetic seeds' (Jimenez *et al.,* 2001; Ipecki & Gozukirmizi, 2003)

Somatic embryogenesis excels beyond other forms of regeneration such as organogenesis in that within a single step it produces a vascular system, functional meristem and a root/shoot axis (Bassuner *et al.*, 2006). Moreover, it is possible to add a second round of embryogenesis to the procedure to further improve yield. This is done by transferring the globular embryos from the initial induction medium onto one high in cytokinin concentration. Cytokines promote radial over axial growth, which when

applied to primary embryos leads to numerous secondary embryos budding from the lateral sides of the primary embryo (Eudes *et al*., 2003). In some species, such as bananas and plantains, nearby embryonic determined cells develop together to produce a single embryo, potentially leading to a chimeric plant should the originating cells be heterogeneous for an introduced trait (Lee *et al.,* 1997; Gaj, 2004). Alternatively, a chimeric plant could be produced by the rare occurrence that a cell contained within a multi-cellular embryo is transformed, producing a heterogeneous mix of cells within the embryo (Polito *et al.,* 1989). This being the case, a secondary round of embryogenesis adds the benefit of not only improving the overall yield of explant regeneration, but also removing the possibility that the plants produced through this process are chimeric by dividing the cell lineages of the primary embryo into numerous plants (Eudes *et al*., 2003; Polito *et al.,* 1989).

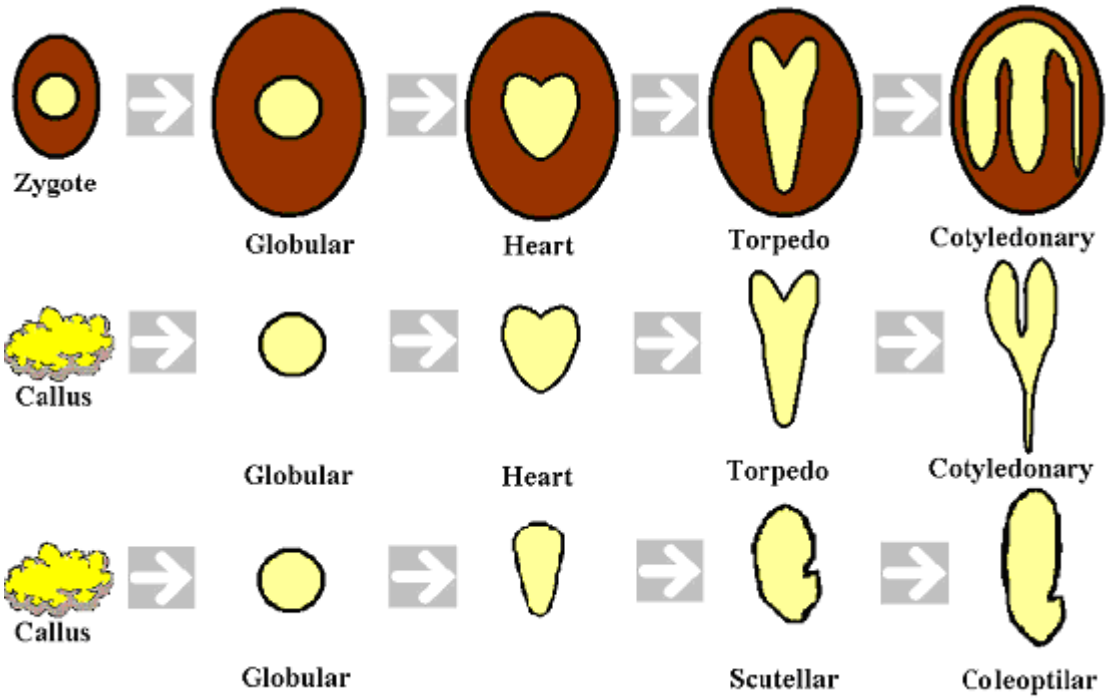

Figure 1.3 Comparison of zygotic and somatic embryogenesis in dicotyledons and monocotyledons. (a) Surrounded by the endosperm (brown) the polarity axis of the zygotic embryo (light brown) is predetermined by the maternal tissue. The first division along this axis is asymmetrical producing a smaller upper cell which will parent all of the aerial shoot system, and a larger basal cell that will parent the subterranean root system of the plant. After several more divisions the embryo passes through the octant stage, which has 2 layers of 4 cells, and begins to develop a protoderm important for constricting the size of interior cells during the following cell divisions. During the globular phase the embryo reorganizes its cellular membrane auxin efflux proteins (PIN-FORMED) to promote the transport of auxin towards the base of the embryo. The apolar distribution of auxin can be observed from the mid-globular stage on. Focusing of auxin at the base of the embryo is important for proper development of the root apical meristem. In the model shown here where the zygote is a dicot, two cotyledons begin to bud out of the spherical globular embryo to produce the heart shaped embryo and then the torpedo and cotyledonary embryo. At this point a zygotic embryo would then prepare itself for desiccation to complete seed formation. In somatic embryogenesis of dicots (b) and monocots (c), the callus provides no signals to dictate the axis of development to the somatic embryo as is done for the zygotic embryo from the maternal tissue. The somatic embryos do, however, decide upon an axis and progress similarly to the development pattern followed by zygotic embryos. In monocots, only one cotyledon is formed and thus the terms scutellar and coleoptilar are used to describe the later stages of embryo development.

1.3.2 Somatic Embryogenesis in Wheat

Fifteen years after somatic embryogenesis was first demonstrated in *Daucus carota* (carrot) (Steward *et al.,* 1958), a successful protocol to regenerate full plants from somatic wheat cells was developed (Shimida *et al*., 1978). *T. aestivum*, however, is not a model system and to date regenerating plants through somatic embryogenesis has remained an arduous task. There are currently only two tissues commonly used to culture embryos, immature inflorescence (Jones, 2005) and scutellar tissue from immature embryos (Figure 1.4) (Maës *et al*., 1996). Using either tissue necessitates large supplies of donor plants be maintained to provide the embryonic explants, which can be costly and laborious (Bhalla *et al.,* 2006b). Relative to scutellar regeneration, immature inflorescence can be harvested more easily and at a younger age, somewhat reducing this burden. However, immature inflorescence is more cultivar-dependant than scutellar regeneration (Jones, 2005).

Cultivar plays the greatest role in the somatic embryogenesis competency, out-weighing other known factors such as explant source, donor plant conditions and even experimental factors including media composition (Eudes *et al.,* 2003; Popelka *et al*., 2003; Jimenez, 2001; Maës *et al*., 1996; Li *et al*., 2003; Bommineni & Juuhar, 1996). To date, there is little understanding of what makes certain cultivars more responsive to tissue culture then others, requiring a large amount of trial and error to elucidate ideal regeneration conditions for each (Li *et al.,* 2003). Unfortunately, many of the most responsive lines, such as the most commonly used *cv.* Bobwhite, are argonomically inferior (Bhalla *et al.,* 2006b; Li *et al.,* 2003). This cultivar-dependence can lead to a

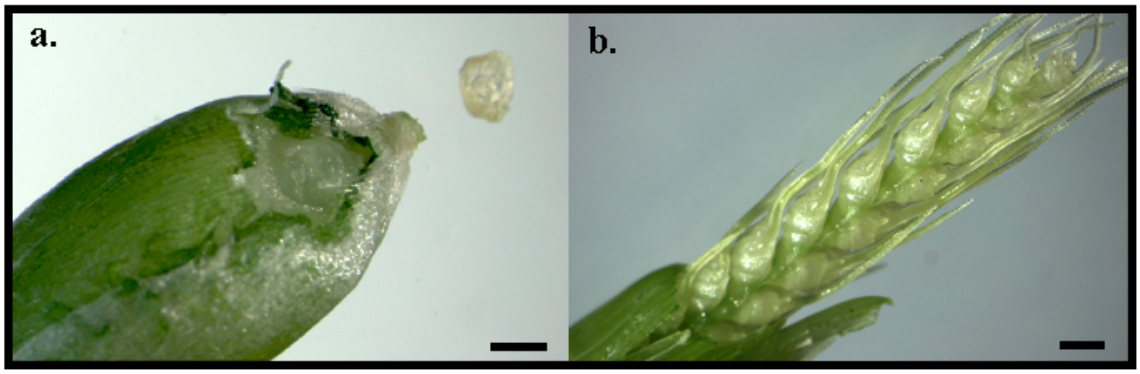

Figure 1.4 Regenerative wheat tissues a. scutellum with germ attached shown within and excised from whole seed b. immature inflorescence. Scale bar indicates 1 mm. Both scutellar (1.5mm) and immature inflorescence (10mm) tissues are shown at proper harvesting age for somatic embryogenesis tissue culture.

dangerous lack of genetic diversity in the transgenic lines available. Extensive backcrossing can be done to introduce a transgene into a more desirable cultivar, but is costly and does not expand the genetic diversity of the crop (Sakhanokho *et al.,* 2001).

1.3.3 Influence of Salts on Somatic Embryogenesis

Although the properties of a cultivar cannot be altered to make it less recalcitrant to somatic embryogenesis, the constituents of the media which induce and support this process can be altered. For example, it has been shown that various methods of increasing the cytosolic levels of calcium are beneficial to SE (Racusen & Schiavone, 1990; Chugh & Khurana, 2002; Malabadi & Staden, 2006). As well, the plant growth regulator (PGR) ethylene kills embryonic tissue and prevents shoot formation, whereas silver a known antagonist of ethylene, acts as a promoter of SE (Dias & Martins, 1999; Sahrawat *et al.,* 2003). Cupric sulphate has also been shown to promote shoot regeneration (Saharawat *et al.,* 2003), whereas iron EDTA beyond a minimum requirement will prevent it (Kothari *et al.,* 2004).

It has been suggested that ammonium and nitrate levels are capable of promoting somatic embryogenesis as well (He *et al.,* 1989; Grimes & Hodges, 1990; Mordhorst & Lorz, 1993; Jiminez *et al.,* 2001; Kothari *et al.,* 2004). In rice, the relative ratio of ammonium to nitrate affects the sensitivity of immature embryos to auxin, a ratio of 1:1 producing the greatest insensitivity (Grimes & Hodges, 1990). Conversely, in barley microspore culture it was shown that the ratio of the two ions, except when extreme, had no influence upon somatic embryogenesis yields. Here, however, the authors established a link between the total concentration of inorganic nitrogen and the yield of somatic

embryos (Mordhorst & Lorz, 1993). In wheat, Menke-Milczarek & Zimny (2001) were not able to show a strong connection between either ratio or total content of nitrogen species and somatic embryogenesis yield.

Although the effects of salt modification in regeneration media are better understood than the effects of cultivar and have led to significant improvements in yields, the majority of these findings are confined to the explant studied (Shoeb *et al.,* 2001). For many salts, it has been shown that each species, cultivar and even tissue has its own unique preference for different salt concentrations (Maës *et al*., 1996; He *et al.,* 1989). As a result, elucidating the specific concentration of macro- and microsalts which best promote SE often requires a large degree of trial and error (Jimenez, 2001).

1.4 Summary

As transformation of species is reliant upon the capacity to successfully and reproducibly regenerate fertile and non-chimeric plants from transformed tissues, understanding how to control and promote tissue culture of elite crops is of paramount importance. Currently cultivar selection plays a major role in establishing the success of tissue culture in main species. Ideally, techniques to improve somatic embryogenesis will be developed that are independent of genotype, circumventing the need to use highly regenerable but agronomically inferior cultivars.

In addition to comprehending how to best promote the yield of plants from transformed tissues, understanding how best to elevate the quality of transgene integration in these transformed tissues will maximize the value of those plants yielded. *T. aestivum* is of major importance globally both economically and nutritionally, yet

traditional development methods are estimated to fall short of satisfying the increasing demand for this cereal. The introduction of foreign genes and biochemical pathways could provide the capacity to meet the world's growing demand, but remains a very arduous task in wheat. It is therefore necessary to develop techniques to improve the quality of transformation, and the efficient tissue culture of *T. aestivum*.

Chapter 2. Effect of Ammonium Nitrate on Direct somatic embryogenesis of *Triticum aestivum and x Triticosecale*

2.1 Introduction

Globally, 200 million hectares are devoted to the production of *Triticum aestivum* (Jones, 2005). Closer to home, Canadians seeded 8.8 million hectares with wheat in 2007 (Statistics Canada), which accounts for 17.6 percent of Canada's arable land. In the 2005/2006 growing season, Canadian farms produced 11.9 million tonnes of wheat generating 3.5 billion dollars in net income (Canadian Wheat Board). Currently, Canada is the world's second largest exporter of wheat and fifth largest producer (FAO).

In order to maintain its status on the world stage, Canada will have to continue to develop heardier and better yielding wheat varieties. To this end, the introduction of novel transgenes into wheat genomes provides the means to study and develop varieties in ways inaccessible by traditional breeding techniques. The capacity to introduce such transgenes, and thus reap the full rewards of this crop modification technique, is contingent upon being able to reliably regenerate transgenic cells into fertile green plants (Bhalla *et al.,* 2006b; Sahrawat *et al.,* 2003). This process occurs facilely in species such as carrot (*Daucus carota L.*) (Dodeman *et al.,* 1997) and in many ornamental plants (Rout *et al.,* 2006), but remains an arduous task in wheat. Further complicating endeavours to produce more resilient wheat plants is the unpredictable responsiveness of cultivars to tissue culture. To date, there is little understanding of what makes certain cultivars more responsive than others (Li *et al.,* 2003). Superb is a Canadian western red spring (CWRS) developed specifically for western Canadian growing conditions (Alberta

Government). The elite variety is known for its high grain yield, large kernel size, shorter and strong straw and good bread making quality. It also benefits from strong resistance to leaf and stem rust as well as intermediate resistance to loose smut, root rot and bunt (PRRCG *et al.,* 2000; Alberta Government). Six years after its registration in 2000, the CWB announced that Superb had become the most prevalent CWRS seeded. Yet although excellent agronomically, hard red spring cultivars such as Superb are known for their recalcitrance to tissue culture (Maës *et al.,* 1996). To date, very little work has been done to improve Superb regeneration conditions. Improved conditions, however, will be necessary to facilitate the introduction of novel transgenes for the study and improvement of this elite cultivar.

Investigations into the influence of ammonium nitrate upon DNA integration post bombardment suggested that this salt may also beneficially influence the tissue culture of the bombarded explant. Modification of ammonium nitrate levels has been shown to have beneficial effects on somatic embryogenesis in ginseng (Choi *et al.,* 1997), rice (Grimes & Hodges, 1989), finger millet (Kothari *et al.,* 2004) and microspore culture in barley (Mordhorst & Lorz, 1993). It has been suggested that the ratio of ammonium to nitrate in the regeneration medium can alter the sensitivity of the explant to auxin levels (Grimes & Hodges, 1989).

Investigated here is the effect of ammonium nitrate concentration upon somatic embryogenesis in Superb. The effects of ammonium nitrate where then also studied in *cv.* Fielder (*Triticum aestivum*, soft wheat) and *cv.* AC Alta (X *Triticosecale*) to assess whether this effect is cultivar or species specific.

2.2 Materials and Methods

2.2.1 Growth Conditions

Triticum aestivum (cv. Fielder & Superb) and X *Triticosecale* (cv. AC Alta) were grown in 19 hours light (250 µM/min) at 15˚C and 5 hours dark at 12˚C. Soil used was a mixture of Sunshine® peat moss (SUN GRO Horticulture BC, CAN) and Terra Lite 2000 vermiculite (W. R. Grace & Co. ON. CAN). At approximately the five leaf stage plants were treated with the fungicide Tilt® (Syngenta AB, CAN) (2.5 ml/L soil) and the pesticide Intercept (Bayer Crop Science Inc. AB, CAN) (0.004 g/L soil) and every fourteen days were fertilized with a 20-20-20 fertilizer (nitrogen-phosphorus-potassium, 200 ppm).

2.2.2 Tissue Culture

Immature scutella were isolated as in Eudes *et al.,* (2003). Immature caryopses were sterilized with 70% ethanol (30 seconds) and 10% (v/v) bleach (10 minutes) before three 1 minute washes in sterile water. The epidermis of the caryopses was torn using a modified size 11 scalpel blade to reveal the immature scutella and attached germs. The modified scalpel blade, having an induced hook the size of a germ, was used to delicately remove this tissue from the immature scutellum in place in the caryopses before the scutellum was plated germ side down onto the modified DSEM media. Control DSEM media contained 1x ($[NH_4NO_3]$ = 2.06 mM, $[KNO_3]$ = 18.79 mM), whereas modified DSEM contained either 1.5x ($[NH_4NO_3]$ = 31.28 mM, $[KNO_3]$ = 0.00 mM), or 3x ($[NH_4NO_3]$ = 62.55 mM, $[KNO_3]$ = 0.00 mM) concentrations of inorganic nitrates. 1.5x and 3x mediums were prepared with ammonium nitrate being the sole source of nitrate to

remain consistent to previous work done by Kovalchuk (work unpublished). 1x control medium was left as published by Eudes *et al.,* (2003) to comparison to published regeneration rates. As a result of leaving the control as such, ammonium concentration does not increase in linear fashion as nitrate does. 1.5x and 3x refers to relative nitrate concentration, not total inorganic nitrogen content.

After 14 days of incubation in the dark at 23˚C, which was sufficient for primary embryo production, each embryo was individually excised from surrounding callus tissue using a scalpel and transferred to SEM medium (containing 1x nitrate concentrations) using a dissecting microscope. Transferred primary embryos were spaced equally apart on the surface of the SEM media. The number of embryos transferred were then scored and allowed to develop for 7 days on SEM before the number of responding and un-responding embryos were counted. Embryos were scored as 'responding' if they had begun producing a small shoot. Embryos were scored as dying if they did not produce a shoot and were beginning to brown. Embryos which were neither dying nor producing a shoot at the end of the 7 day period were given more time to produce either phenotype before being scored.

2.2.3 Statistical Evaluation of Results

The experiment was conducted according to a factorial design. The number of primary embryos and responding embryos produced by the cereal lines and ammonium nitrate concentrations was analyzed using the Statistical Analysis Software package Version 6.0.9 (SAS Institute Inc. NC, USA). Significant mean differences were determined based on the LSD method at the $P \leq 0.05$ confidence level. Residuals were

checked for normalcy and outlying data points were identified and removed using the univariate procedure.

2.3 RESULTS

2.3.1 Primary Embryo Production

The production of primary somatic embryos on the control and experimental DSEM mediums had a large standard deviation (Table 2.1). The use of ANOVA mixed data analysis was capable of removing this random variation and showed both cereal line ($P < 0.0001$) and nitrate concentration ($P = 0.0003$) had significant effects upon the induction of somatic embryos. Furthermore, there was a significant interaction observed between cereal line and nitrate exposure ($P = 0.0084$), indicating that each cereal line responds differently to varying nitrate exposures.

Table 2.1 Average number of primary embryos per scutellum, ± standard deviation, recovered from several cereal lines after 14 days incubation on modified DSEM.

	Superb		***AC Alta***		***Fielder***	
	n	***average***	***n***	***average***	***n***	***average***
1x	734	2.96 ± 1.51^{d}	916	3.73 ± 1.85^{e}	1680	$7.77 \pm 2.09^{b,c}$
1.5x	1122	$4.56 \pm 2.14^{d,e}$	532	2.22 ± 0.87^{d}	1601	7.37 ± 2.04^{b}
3x	1502	6.68 ± 3.16^{c}	698	$2.85 \pm 1.18^{d,e}$	1890	9.45 ± 2.33^{a}

*Values associated with different letters are statically different from each other.

'*n*' indicates the total number of embryos excised from the callus material

The cereal line Fielder produced significantly more primary embryos on the control and the two experimental media when compared to the two other cereal lines. Increasing the nitrate concentration by three fold produced a significant gain of 21% over the control, as well as a significant increase of 28% over the 1.5x to 3 fold range. The 1.5 fold treatment had no effect.

The triticale AC Alta scutella produced the next most primary embryos on the control medium after Fielder. AC Alta control produced only 3.73 (48%) as many primary embryos as the Fielder control. Increasing the nitrate concentration in the DSEM media to 1.5 fold further reduced this yield significantly to 2.22 embryos per scutellum. Increasing the nitrate concentration from 1.5 fold to 3 fold over the control levels recovered this loss to produce 2.85 embryos per scutellum, which was statistically insignificant from the control and the 1.5x medium.

Superb produced the fewest primary embryos on the control medium with 2.96 embryos per scutellum cultured. Superb, however, produced the best response to increased nitrate concentration by producing 125% more embryos on the 3 fold nitrate medium. At this level, the amount of primary embryos produced was statistically identical to that of Fielder control.

2.3.2 Primary Embryo Transition

Both cereal line ($P = 0.0004$) and level of nitrate exposure ($P = 0.0039$) significantly influence the percentage of primary embryos capable of producing plantlets once transferred from the DSEM mediums to SEM (Table 2.2). Here, however, there was no interaction observed between the two variables ($P = 0.45$).

Analysis of individual treatments showed no difference in the percentage of responding embryos between the different treatments in Superb and Fielder (Table 2.2). In the triticale AC Alta, there was a significant reduction in percentage of primary embryos capable of producing plantlets from 65% observed in the control to 46% in the 1.5 fold nitrate treatment. This loss however, was recovered by further increasing the nitrate concentration to 3 fold, which was statistically not different from the 1x control.

Although the control produced similar results, Fielder was significantly more productive than AC Alta at both the 1.5 and 3 fold exposure level, being 50 and 21% higher respectively. There was no difference observed at any of the nitrate exposure levels between Fielder and Superb.

The net number of responding embryos in relation to the number of primary embryos produced by each cultivar at the different nitrate exposure levels is displayed in Figure 2.1. It was observed that both cereal line ($P < 0.0001$) and nitrate exposure ($P = 0.005$) affected the number of responding embryos yielded. As well, there was an interaction between the line and nitrate concentration assayed ($P = 0.0049$).

Table 2.2 Percentage of transferred primary somatic embryos, ± standard one deviation, surviving on SEM after 7 days incubation.

	Superb	***AC Alta***	***Fielder***
1x	69.84 ± 15.86[a,b]	65.65 ± 6.97[a,b]	74.33 ± 11.43[a]
1.5x	57.35 ± 17.52[b]	45.77 ± 16.51[c]	69.16 ± 15.33[a,b]
3x	63.60 ± 13.49[a,b]	58.98 ± 14.83[b]	71.69 ± 10.64[a]

*Values associated with different letters are statically different from each other.

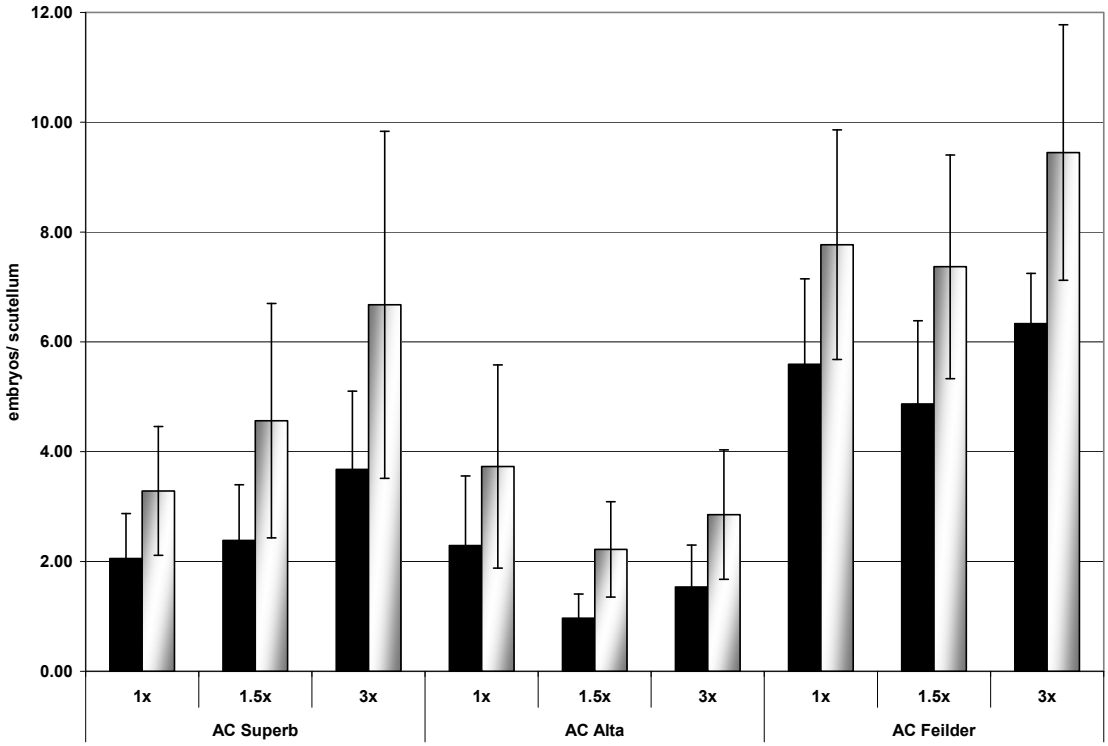

Figure 2.1 Number of primary embryos (white) and responding embryos (black) from several cereal lines after 14 days incubation on modified DSEM (embryos) and 7 days incubation on regular SEM (plantlet producing embryos). Error bars indicate one standard deviation.

Fielder produced the most responding embryos across all three ammonium nitrate treatments (Table 2.3). The 1x control did not differ significantly from either the 1.5 or 3 fold nitrate treatments. These two treatments, however, were significantly different from each other, improving by 30% over the 1.5 to 3 fold range.

AC Alta and Superb produced significantly fewer responding embryos than Fielder with 2.29 and 1.85 responding embryos per scutellum cultured, respectively. Exposure to the 1.5 fold nitrate significantly reduced the number of responding embryos in AC Alta. In contrast, this loss was regained in the 3 fold where there was no difference observed from the control. The Superb showed no change from the control if the nitrate level was increased by 1.5 fold, but did if the nitrate was increased to 3 fold. Here, the number of embryos capable of producing plantlets on SEM rose by 99% over the control.

Table 2.3 Average number of responding embryos produced per scutellum± one standard deviation, after 14 days incubation on modified DSEM and 7 days incubation on SEM, producing embryos, from several different cereal lines.

	Superb		***AC Alta***		***Fielder***	
	n	***Superb***	***n***	***average***	***n***	***average***
1x	458	$1.85 \pm 1.00^{d,e}$	562	$2.29 \pm 1.27^{d,e}$	1209	$5.59 \pm 1.56^{a,b}$
1.5x	589	2.38 ± 1.01^{d}	231	0.97 ± 0.44^{f}	1096	4.87 ± 1.52^{b}
3x	827	3.68 ± 1.43^{c}	372	$1.53 \pm 0.77^{e,f}$	1266	6.33 ± 0.92^{a}

*Values associated with different letters are statically different from each other.

'*n*' indicates the total number of plantlet

2.4 Discussion

2.4.1 Primary Embryo Formation

Antecedent study into the affects of ammonium nitrate upon transgene uptake suggested higher concentrations of the salt might increase the yield of embryos produced from somatic embryogenesis. The goal of this study was to investigate whether or not ammonium nitrate could affect tissue culture of wheat embryos. Fielder, which has published regeneration rates (Eudes *et al.,* 2003), was chosen as a benchmark for the variety the increased plant regeneration was noticed in, Superb. The triticale AC Alta was also included to explore the possibility that this promotion of somatic embryogenesis is not confined to the *T. aestivum* species.

The Fielder on control medium produced 5.59 responding embryos per scutellum cultured. In 2003 Eudes *et al*. (2003) reported being capable of regenerating 8.73 plants per scutellum dissected. The work by Eudes *et al.,* (2003), however, reflects a primary round of embryogenesis followed by another secondary round, will reliably produce at least two secondary embryos per primary embryo cultured. Thus had the number of responding secondary embryos been fully cultured and recorded in these studies, it can be reliably estimated that they would have achieved or even surpassed the yield reported by Eudes *et al.* (2003). Extrapolating from the work by Eudes *et al.* (2003), it can be concluded that the reduced number of primary embryos produced by Superb and AC Alta is due to intrinsic properties of theirs and not due to an outside source.

This study found that the number of primary embryos formed during induction of direct somatic embryogenesis could be increased through increasing the ammonium nitrate exposure in both the soft and the hard wheat lines tested. This finding in hard

wheat is of particular importance as these cereals are known to be very difficult to regenerate compared to their soft wheat counterparts (Maës *et al.,* 1996). Here, though, it was shown that this initial recalcitrance to tissue culture could be overcome in Superb by increasing the ammonium nitrate exposure to the point where it performs equally to the soft wheat Fielder control. Often it is the case, that due to the unique sensitivity of a cultivar to hormone and salt concentrations, ideal yields require the fine tuning of each of these compounds for each cultivar approached (Jimenez, 2001). The modest gain in Fielder is not without importance as well, as it is shows even a cereal line already considered a model for wheat regeneration (Zale *et al.*, 2004) can be induced to produce somatic embryos by simple nitrate manipulation in the induction medium.

Although ammonium nitrate levels have been shown to be important for induction of somatic embryogenesis in several species (Smith & Kriorian, 1989; Grimes & Hodges, 1990; Thompson & von Aderkas, 1992; Mordhorst & Lorz, 1993; Choi *et al.,* 1998; Mondal *et al.,* 2001; Kothari *et al.,* 2004), little work has been done elucidating how the salt affects plant regeneration in wheat. Menke-Milczarek & Zimny (2001) found that both ammonium and nitrate were necessary for the regeneration of *Triticum aestivum cv.* Grana, a polish feed wheat. The authors were unable to establish a link between ammonium to nitrate ratio, or their total concentrations, to the efficiency of somatic embryogenesis. Their analysis of efficiency, however, was based on the percentage of calli that were regenerative, and not the number of primary embryos produced as was the case in this work. This, or potentially the effect of genotype could explain the discrepancy in results. Here it was shown that there is interdependence between nitrate

exposure and the rate of somatic embryogenesis, thus it is quite possible that the Menke-Milczarek group used a cultivar less sensitive to inorganic nitrogen manipulation.

It has been shown in rice (Grimes & Hodges, 1990) that the relative balance of inorganic ammonium to nitrate has greater influence on tissue culture than the absolute concentrations. Conversely, in barley microspore culture it was observed that the total concentration of nitrates affected tissue culture and the ratio of ammonium to nitrate did not (Mordhorst & Lorz, 1993). In this study, the relative ratio of ammonium to nitrate was altered from 1:10 in the control to 1:1 in the 1.5 and 3 fold nitrate mediums. This was done to remain consistent with earlier work done in investigating the effects of ammonium nitrate upon biolistic transformation efficiency. Thus it is possible that modification of ammonium to nitrate ratio was responsible for the increased production of somatic embryos observed in the wheat lines. In both these instances, however, the 1.5 fold exposure to nitrate which had an equivalent change in ammonium to nitrate ratio from the control, did not differ significantly in embryo production from the control. Thus it appears that the rate of embryo production does rely upon the total ammonium nitrate content to some extent.

2.4.2 Embryo Transition

The number of responding embryos produced is a product of both the number of primary embryos produced during the induction process and the number of those embryos capable of transitioning from an embryo to a plantlet. The performance of the primary embryos transferred to SEM media was followed to observe whether or not the pretreatment with increased ammonium nitrate would affect the embryos' capacity to

develop beyond the embryo stage. It has been shown in numerous systems that a large number of somatic embryos do not always yield a large number of regenerated plants (Gaj, 2004). The results here show that higher ammonium nitrate exposure can reduce the percentage of primary embryos capable of surviving and producing a shoot once transferred to SEM independently of the influence of cultivar. As a result of the decreased capacity to regenerate, the gains observed in Fielder primary embryo production were less extreme. There was, however, still a significant 30% improvement over the 1.5 fold nitrate medium. In the case of Superb, increasing the amount of nitrate by 1.5 fold did not significantly affect the number of responsive embryos cultured, but increasing the nitrate exposure by 3-fold did produce a significant improvement of nearly 2-fold. Increasing the nitrate exposure over this range produced 126% increase in the number of primary embryos produced. Thus although increased salts still produces greater yields, there was a slight reduction in the gains in Superb as well as in Fielder.

The number of primary embryos capable of producing the beginnings of small shoots once transferred to SEM media ranged between 46 and 74 percent (Table 2.2). These transition rates could be explained by several factors. Firstly, these cereal lines may intrinsically have less than ideal capacity to convert from a somatic embryo into a plantlet. Numerous other species are produce many somatic embryos and yet suffer from low regeneration rates as they are impaired in this capacity (Gaj, 2004). Secondly, this reduced capacity to regenerate could be exacerbated by the meticulous nature where each primary embryo was individually removed from the calli. Doing so required using a scalpel to cut calli material away from the surface of the primary embryo, which may damage the embryos to a greater extent than common excision procedures. Calli material

requires the high auxin content of the induction mediums to survive, and thus in experiments where the focus is not specifically to track the number of somatic embryos transferred off the induction media or their progress, some of this material can be left associated with the transferred embryo. As a result, the majority of experiments would be able to excise the somatic embryos without incurring the amount of damage to embryos as was done here. It should be noted that the use of a selection marker would also allow somatic embryos to be transferred off the induction media whilst still in close proximity to each other as the selection media will eliminate the non-transgenic plants in addition to any calli transferred.

The aforementioned stresses would have been consistent throughout the experiment. Nitrate exposure was also observed to have a significant effect upon the number of embryos capable of converting into a plant. Two, non-preclusive explanations of this effect could be that the salt stress which induced greater somatic embryogenesis rates reduced the overall health of the embryos making them less capable to survive once transferred of the induction media, or conversely the sudden transfer from an elevated level of nitrate in the DSEM back to the control level of nitrate in the SEM could have induced some stress. In support of the prior explanation, it was observed in previous experiments that exposure to media containing 5x and 10x ammonium nitrate concentrations where lethal to excised scutella. To evaluate the second explanation, a study involving modified SEM as well as the DSEM could elucidate if there is a heath effect associated with rapid changes in nitrate concentrations.

Potentially, modifying the ammonium nitrate concentration in the SEM medium could increase the number of plants regenerated as this medium supports a secondary

round of embryogenesis. It has been suggested, however, that ammonium nitrate levels affect somatic embryogenesis by modifying the explants' sensitivity to auxin (Grimes & Hodges, 1990; Kothari *et al.,* 2004), of which there is none in the SEM. The results of such an experiment would be quite valuable to shed light how these regulators of somatic embryogenesis work together.

2.4 Conclusion

This study found that Superb, as is the case for other hard wheat cultivars, is significantly recalcitrant to induction to direct somatic embryogenesis. As a result, endeavors aimed at transforming this elite red spring which require tissue regeneration would be extremely hindered. Here, however, it was shown that simple modification of the nitrate sources in the somatic embryogenesis induction media can greatly reduce this intrinsic resistance to tissue culture. The results also showed that the increased ammonium nitrate exposure was capable of reducing the capacity of the primary embryos to develop plantlets on unmodified SEM media. As a consequence of this influence, the modest gain observed in the soft wheat Fielder primary embryo production was not realized in responding embryos. Superb remained significantly better than the control in this regard in spite of the reduced capacity to respond on the SEM. This stress negated the gains observed in Fielder but not in Superb, which was improved here by nearly 100%.

Chapter 3. Effect of Ammonium Nitrate on Biolistic Transformation of *Triticum aestivum cv.* Superb

3.1 Introduction

The introduction of novel transgenes into crops has produced numerous high profile successes. Monsanto, the developer of transgenic corn resistant to the European corn borer, made 2.8 billion dollars US in the 2007 fiscal year selling corn seed (Monsanto). Ten years after the transgenic corn's approval in 1995, Monsanto's annual profits have risen from 8 million to 5.4 billion dollars US (Fortune 500). In addition to economic success, the advent of Golden Rice which was specifically developed to feed malnourished countries is a well-renown example of utilizing transgenics for humanitarian endeavors (Ye *et al.,* 2000).

Although highly desirable, introducing foreign genes into plants systems can be very costly and time consuming, particularly in recalcitrant crops such as *Triticum aestivum*. A broad range of transformation techniques including microinjection, transformation via protoplast (PEG & calcium phosphate), electroporation (Altpeter *et al*., 2005), vortexing with DNA and silicon carbide bristles, pollen tube mediated introduction (Bhalla, 2006a) and even laser mediated uptake (Jones, 2005) have all been shown to be ineffective or irreproducible in wheat. *Agrobacterium* mediated gene transfer is used routinely, but suffers from low yields as wheat is not a native host of the pathogen. As a result, super-virulent strains of *Agrobacterium* or agronomically inferior but susceptible wheat lines must be used for transformation. Often this limits *Agrobacterium* mediated transformation to the transgenic improvement of agronomically

inferior cereal lines. As an alternative, microparticle bombardment is relatively genotype independent (Takumi & Shimada, 1997; Jones, 2005). Bombardment is also advantageous as it has no need for plasmid backbone, thereby allowing only cassettes to be introduced into the genome. Plasmid backbones, which are necessary for *Agrobacterium* transformation, have been shown to promote transgene rearrangements as well as transgene silencing if integrated (Fu *et al.,* 2000). Microparticle bombardment is also well suited for the introduction of polygenic traits as numerous constructs can be loaded onto a bullet and introduced simultaneously (Sahrawat *et al*., 2003; Halpin *et al.*, 2005). This process can also target mitochondrial and chloroplast DNA (Altpeter *et al.*, 2005), and introduce DNA fragments as large as YACs and BACs (Taylor & Fauquet, 2002). Despite these advantages, however, *Agrobacterium* remains popular in wheat transformation as it avoids the disadvantages associated with microparticle transformation. Gene guns are expensive and their operation requires greater skill than *Agrobacterium* transformation (Bhalla, 2006a). As well, microparticle bombardment is thought to be prone to producing high copy numbers as well as the insertion of fragmented and unstable transgenes (Hu *et al*., 2003; Jones, 2005) which can be avoided by the use of *Agrobacterium*.

As the introduction of foreign genes into the wheat system is reliant upon using either of the less than ideal delivery systems, the setback of lack of gene targeting within plant systems is greatly exacerbated. In yeast, homologous recombination is the predominant DSB repair mechanism, which ensures that introduced recombinant DNA will be integrated based upon common homology between the transgene and the genomic target location at a high success rate. Conversely, in plants non-homologous

recombination repairs 10^3 to 10^6 double strand breaks to every one repaired by homologous recombination (Puchta, 2002; Hanin & Paszkowski, 2003; Lida & Terada, 2004; Puchta, 2005). As a result, introduced transgenes are predominantly integrated at random into the plant genome by NHEJ regardless of how they are designed (Puchta, 2002; Taylor & Fauquet, 2002).

Potentially, being able to promote homologous recombination in plant cells could increase the efficiency of transformation or improve transgene selectivity for integration sites. Recent discoveries in ammonium containing compounds may allow for the temporary up-regulation of HR pathways without the introduction of exogenous DNA repair genes. Studies with ammonium sulfate have shown that the salt can up-regulate the activity of hRAD51 by up to 4 fold *in vitro* (Sigurdsson *et al.*, 2001; Liu *et al.*, 2004; Ristic *et al*., 2005). A high ammonium sulfate concentration was also shown to produce a RAD51-ssDNA complex having much more open morphology which may facilitate the search for homology by making the ssDNA available for duplexing with the unbroken DNA (Liu *et al.,* 2004). Working with ammonium nitrate, the Kovalchuk group found that tissue exposed to higher levels of the salt would reconstitute a homologous recombination substrate *in vivo* at an elevated rate (data not published). Furthermore, they showed that exposure to the higher levels of ammonium nitrate did not increase or decrease the number of DSBs observed in the plant tissue, suggesting that the salt exposure was altering the balance between NHEJ and HR in the cell. The purpose of the study here was to investigate the effect of increased ammonium nitrate levels upon the efficiency and quality of DNA integration delivered by microparticle bombardment in the wheat system.

3.2 Materials and Methods

3.2.1 Growth Conditions

Triticum aestivum (*cv.* Superb) was grown in 19 hours light (250 µM/min) at 15 ˚C and 5 hours dark at 12 ˚C. At approximately the five leaf stage plants were treated with the fungicide Tilt (2.5 ml/L soil; Bayer Environmental Science, Calgary AB.) and the pesticide Intercept (0.004 g/L soil; CiBa-Geigy, Mississauga, ON.) and every fourteen days were fertilized with 20-20-20 (nitrogen-phosphorus-potassium, 200ppm).

3.2.2 Construct Generation

The *BAR* cassette used for bombardment was produced by high fidelity PCR (TaKaRa) amplification of the pMB4 plasmid using pMB4+ 5′-CGTCAACATGGTGGAGCA-3′ and pMB4- 5′-CATTAGCAAGGCCGGAAAC-3′ primers. The *LUCIFERASE* DNA was produced by high fidelity PCR amplification (TAKARA BIO INC. Shiga, Japan) using pGN35SLUC as a template and 35SLUC+ 5′-AACAGCTATGACCATGATTACGC-3′ and 35SLUC- 5′-TAAAACGACGGCCAGTGC-3′ as primers. After amplification, both cassettes were then precipitated in acetate and 100% ethanol before being resuspended in sterile water. To produce the *ANTHOCYANIN* cassette, *E. coli* DH5α carrying the pLtp1CB plasmid (Doshi *et al.,* 2006) was incubated overnight in a INNOVA^{tm} 4400 incubator (New Brunswick Scientific Co., Inc. NJ, USA) (37˚C, 180 RPM) until an OD of 2.00 was reached. The pLtp1CB plasmid was then isolated by GenElute^{tm} High Performance (HP) Plasmid Maxiprep Kit (SIGMA, ON, CAN) and then serially digested with *BamHI* (New England Biolabs, MA, USA) and *SphI* (NEB), respectively, according to manufacturer's

instructions. The cassette was then isolated by gel electrophoresis and recovered using the Wizard® SV (PROMEGA, WI, USA) gel extraction kit.

3.2.3 Bullet Preparation & Bombardment

Bullets were prepared according to the Helios^{TM} Gene Gun System Instruction Manual (Bio-Rad Laboratories, 1996) using either 50mg gold (INBIO GOLD, VIC, AUS) (1 micron) with 25 µg DNA (approx. 1.25 mg gold & 0.625 µg DNA /bullet) or 25mg gold with 12.5 µg DNA (approx. 0.625 mg gold & 0.3125 µg DNA /bullet). Each bullet contained equal moles of the BAR, LUCIFERASE and ANTHOCYANIN cassette. Concentrations of each cassette for bullet preparation were determined by ImageJ (National Institute of Health, http://rsb.info.nih.gov/ij/) analysis of electrophoresis gels. Forty eight hours after dissection, scutella from Superb were pooled into the center of the Petri dish and bombarded using a Helios^{TM} gene gun (Bio-Rad Laboratories Ltd. ON, CAN) at 160 PSI. Pooled scutella were then separated 3-12 hours post bombardment as a safe guard against microorganismal contamination.

3.2.4 Tissue Culture

Scutella were excised and cultured as per Eudes (*et al.*, 2003) except that the quantity of inorganic nitrate sources. In the initial embryo inducing DSEM medium was modified to 1x ($[NH_4NO_3]$ = 2.06mM, $[KNO_3]$ = 18.79mM), 1.5x ($[NH_4NO_3]$ = 31.28mM, $[KNO_3]$ = 0.00mM), and 3x ($[NH_4NO_3]$ = 62.55mM, $[KNO_3]$ = 0.00mM) concentrations. 1.5x and 3x mediums were prepared with ammonium nitrate being the sole source of nitrate to remain consistent to antecedent work done be Kovalchuk

(unpublished). 1x control medium was left as published by Eudes (*et al.,* 2003) to allow comparison with published regeneration rates. As a result, ammonium concentration does not increase in linear fashion from 1x to 3x as nitrate does. 1.5x and 3x refers to relative nitrate concentration, not total inorganic nitrogen content. Germs were removed from immature scutella approximately 1.5- 2 mm in size using a modified size 11 scalpel blade with the aid of a dissecting scope before the scutellum explants were plated on the modified DSEM media. For each trial, scutella were dissected for each medium on the same day using the same pool of wheat to eliminate any potential influence from the mother plants between treatments. During regeneration, plants were transferred directly from the GEM media to the rooting medium without the use of the MS_{reg} medium specified by Eudes *et al*. (2003).

3.2.5 Screening

Tissue was excised from the T_0 wheat plants at approximately the three leaf stage and frozen in liquid nitrogen. Genomic DNA was then extracted from this tissue using DNeasy Plant MiniKit (QIAGEN, ON, CAN) to be used for PCR screening. Positive T_0 plants were identified using specific *BAR* (5′- AGTCGACCGTGTACGTCTCC-3′, 5′-GAAGTCCAGCTGCCAGAAAC-3′), *LUCIFERASE* (5′-TCAAAGAGGCGAACTGTGTG-3′, 5′-GGTGTTGGAGCAAGATGGAT-3′) and *ANTHOCYANIN* (5′-AACCCGTCAGCATACGAAAC-3′, 5′-ATTTCCTCGCAGAGGCTGTA-3′) primers.

3.2.6 Statistical Evaluation of Results

Transformation efficiency was calculated based on the number of PCR-positive plants identified versus the number of scutellum dissected. Integration ratio was calculated base on number of PCR-positive plants identified versus the number of DNA samples assayed. Statistical evaluation of the data was based on log-linear analysis of the data performed using the Statistic Analysis Software package Version 6.0.9 (SAS Institute Inc. NC, USA). Significant differences were determined using the Least squares means method at the $P = 0.005$ confidence level.

3.3 Results

3.3.1 Transformation Efficiency

Initial microparticle bombardments of Superb were performed using bullets comprised of 1.25mg gold and 0.625µg DNA. Five replicates, of approximately 25 scutella, were successful in producing regenerated plants. The control (1x), 1.5 x and 3 fold nitrate treatments produced 5, 42 and 67 plants, respectively. Of these plants, 4, 29 and 50 plants were of sufficient health and size to permit tissue extraction.

Two out of five of the control plants tested positive for the BAR construct (Figure 3.1). The 1.5 fold nitrate treatment produced two plants PCR-positive for the BAR construct as well 4 luciferase and 9 anthocyanin PCR-positive plants. The three-fold nitrate exposure treatment yielded 11 BAR, 1 luciferase and 2 anthocyanin plants PCR-positive for the recombinant DNA constructs (Table 3.1).

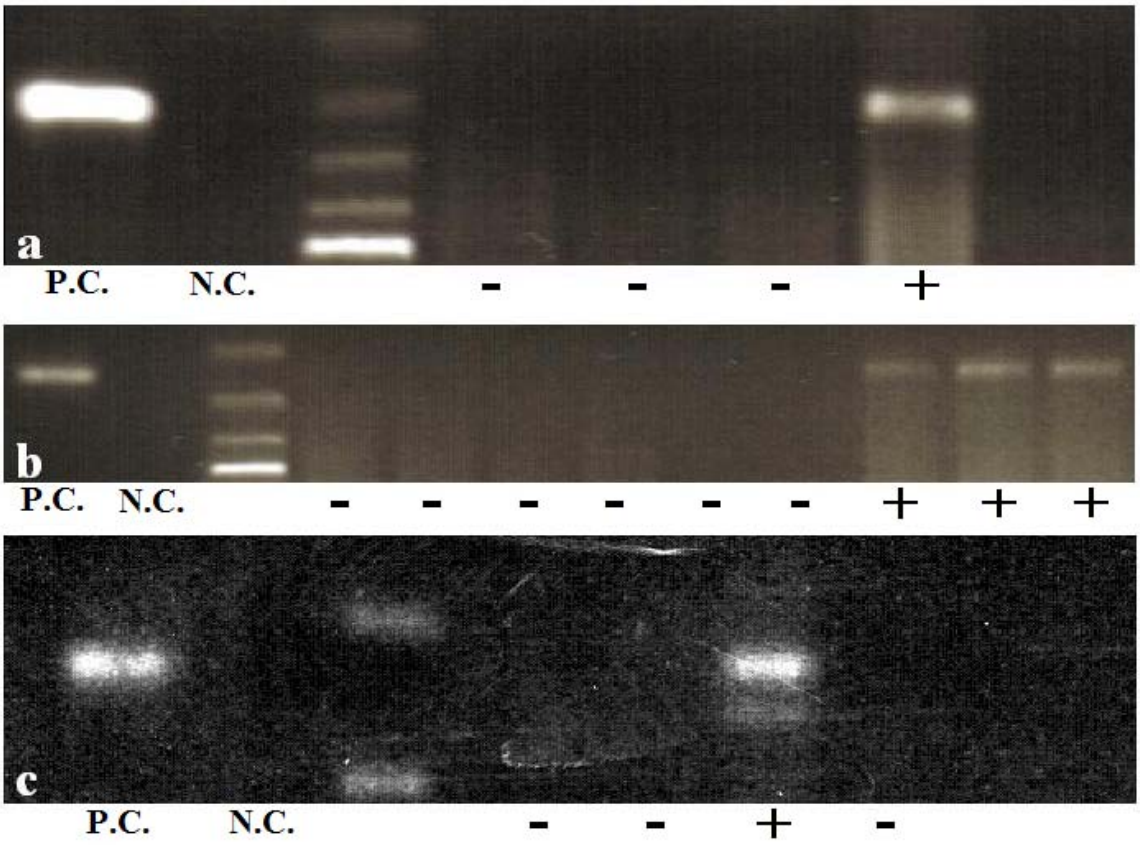

Figure 3.1 PCR analysis of regenerated plants for (a) anthocyanin, (b) bar and (c) luciferase. P.C. indicates positive control and N. C. indicates negative control. + indicates regenerated plants positive for assayed construct, - indicates regenerated plants negative for assayed construct.

The 1x control had a transformation efficiency of 1.7%, measured by transgenic plants produced per scutellum bombarded. The 1.5 fold nitrate treatment had an efficiency of 13% and the 3x nitrate treatment 12%.

The latter transformations were preformed using bullets comprised of 0.625 mg gold and 0.313 µg DNA. This series of transformations showed transient expression of the anthocyanin construct post-bombardment (Figure 3.1). For this series of transformations, 450 scutella were bombarded on the control media. Two hundred and eighty four scutella were bombarded on 1.5 fold nitrate media and two hundred and fifty eight on the 3 fold nitrate media. The control produced 42 regenerated plants, whereas the 1.5 and 3 fold nitrate treatments produced 57 and 27 respectively. Thirty eight of the control plants were of sufficient biomass to allow for tissue extraction. DNA was extracted from 50 of the 1.5 fold nitrate treatment and 22 of the 3-fold treatment plants.

The control plant produced one plant PCR positive for the anthocyanin construct. The 1.5-fold nitrate treatment produced one PCR positive plant for the anthocyanin construct as well. The 3-fold nitrate treatment produced two plants which were PCR positive for the anthocyanin construct.

The control for the second series of bombardments had a transformation efficiency of 0.2%. The 1.5 fold nitrate treatment had an efficiency of 0.4% and the 3-fold nitrate treatment had 0.8%.

Both the gold-DNA composition of the microparticle bullets ($P = 0.001$), and the amount of nitrate used in the induction media ($P = 0.0018$) were found to significantly affect the number of transgenic plants produced per scutella bombarded. Significant

differences could be detected between the control and the 1.5-fold nitrate treatment (P = 0.0057) as well as between the control and 3-fold nitrate treatment (P = 0.0078). There was no significant difference observed between the 1.5- and 3-fold nitrate treatments (P = 0.8811).

Table 3.1 Number of wheat (*cv.* Superb) plants PCR positive for co-bombarded constructs.

	n	BAR	LUCIFERASE	ANTHOCYANIN	Total
1x	5	2	0	0	2
1.5x	29	2	4	9	15
3x	50	11	1	2	14

*Bullets used for this data contained 1.25mg gold and 0.625µg DNA per bullet.

'*n*' indicates the number of plants assayed for construct integration.

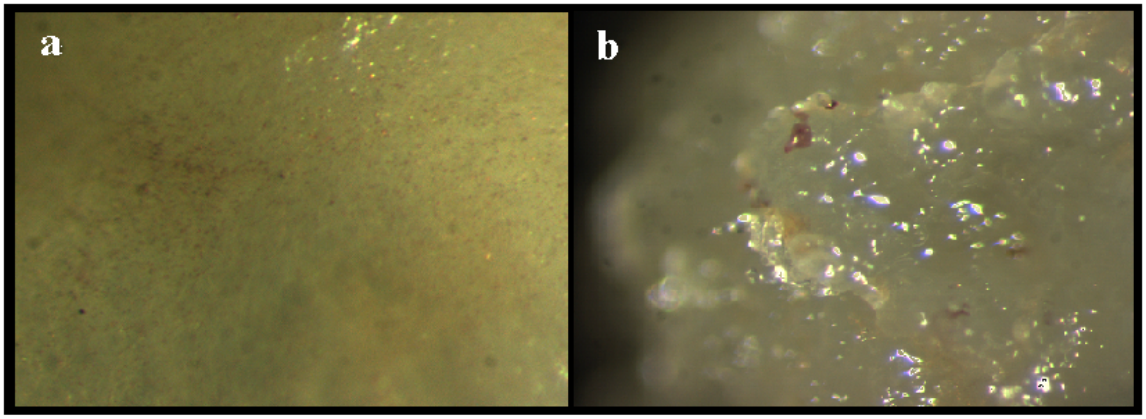

Figure 3.2 Anthocyanin expression in *cv.* Superb calli (a) three days (100x magnification) and (b) eight days (50x magnification) post-biolistic transformation on control medium.

Table 3.2 Number of wheat (*cv.* Superb) plants PCR positive for bombarded constructs.

	n	BAR	LUCIFERASE	ANTHOCYANIN	Total
1x	38	0	0	1	1
1.5x	50	0	0	1	1
3x	22	0	0	2	2

*Bullets used for this data 0.625mg gold and 0.313µg DNA per bullet.

'*n*' indicates the number of plants assayed for construct integration.

3.3.2 Integration Rate

Transformation of Superb scutella with bullets comprised of 1.25mg gold and 0.625µg DNA yielded five testable plants, two of which were PCR positive for the one of the three recombinant DNA constructs bombarded (Table 3.3). Bombardment with the bullets containing half as much gold yielded one PCR positive plant from the 38 assayed. The 1.5-fold nitrate exposure generated 52% and 2% transgenic plants after bombardment with bullets containing the higher and lower gold and DNA content. The 3-fold nitrate treatment generated 26% and 9.1% transgenic plants with these bullets, respectively.

The composition of the bullets used for transformation was found to affect the integration rate of the bombarded wheat plants ($P < 0.0001$). No significant differences were observed in the integration rate in response to the different ammonium nitrate exposures ($P = 0.3857$).

Table 3.3 Biolistic transformation integration rates for Superb.

treatment	*n* [a]	gold | DNA [b]		transgenic	integration rate [c]
1x	5	1.25	0.63	2	40
	38	0.63	0.32	1	2.6
1.5x	29	1.25	0.63	15	52
	50	0.63	0.32	1	2.0
3x	50	1.25	0.63	13	26
	22	0.63	0.32	2	9.1

a) Number of regenerated plants tested for recombinant DNA insertion.

b) Compositions of bullets used for microparticle bombardment. Values for gold and DNA are in mg and µg respectively.

c) Percentage of regenerated plants PCR positive for one of the three transgenic DNA constructs.

3.4 Discussion

The goal of this study was to investigate the possible effects of nitrate upon biolistic transformation in wheat. Antecedent work by the Kovalchuk group (unpublished) suggested that exposure to nitrate could up-regulate homologous recombination without inducing double strand breaks. During this group's work, ammonium was used as a counter ion to nitrate. Thus it was deemed important here to modify the nitrate content within the induction medium using ammonium as counter ion. The control induction media was left as described by Eudes *et al.* (2003), having two sources of nitrate (KNO_3 & NH_4NO_3), to retain a link to published results. As a result, the concentration of ammonium did not increase in a linear fashion as the nitrate did. Substitution of the potassium for another counter ion would have no effect on the overall concentration of potassium as potassium hydroxide is used in far excess to pH the induction medium.

Prior to biolistic transformation and several weeks post bombardment, immature scutella were incubated on DSEM for 48 hours. Modification of the DSEM medium's nitrate content was thereby capable of pre-treating immature scutellar tissue with elevated nitrate levels two days prior to transformation in addition to maintaining high nitrate levels throughout the period introduced recombinant DNA could potentially integrate. As the following regeneration mediums are used after the potential recombinant DNA integration window, no need was found to deviate further from established protocols and modify their ammonium nitrate contents. These media were left as described in Eudes *et al.* (2003).

After the forty eight hour pretreatment with increased nitrate, the wheat scutella were initially transformed using bullets carrying a large quantity of gold microcarriers, which maximized the amount of recombinant DNA that could be introduced into the scutellar cells. The benefit of using greater quantities of microcarriers for transformation is counter acted by the greater damage induced to the target tissue during the bombardment process (Rasco-Guant *et al.,* 1999). No reports of transformations carried out using an equivalent number of microcarriers per shot could be found, but bombardments are routinely practiced in the Eudes lab using bullets prepared in the same manner. Here, with the control medium producing only 5 plants, it was shown that the stress of transformation using these bullets superseded the advantages of introducing the maximum amount of recombinant DNA.

As the yield of regenerated plants per explant bombarded with the high levels of gold was insufficient to support investigations into the integration rate and quality of recombinant DNA insertion, the amount of gold used per bombardment was reduced to a level shown to facilitate plant regeneration (Doshi *et al.,* 2006 & 2007; Eudes *et al.,* 2001). More plants were produced with these bombardments as less repetitions failed to produce regenerated plants. As well, expression of the anthocyanin cassette, under the control of an embryo specific promoter, was observed in these repetitions post bombardment whereas it had not been noted when bullets of greater gold content had been employed. Two possible explanations for this lack of expression could be either that the expression was masked by the significant yellowing of the necrotic tissue induced by bombardment with higher gold quantities, or that the tissue bombarded with less gold were healthier and thus more capable of transiently expressing the anthocyanin

construct. In either event, decreased transient gene expression is a documented symptom of tissue injured by microparticle bombardment (Janna *et al.,* 2006), suggesting that the appearance of anthocyanin expression does signify that transformation with the bullets comprised of less gold microcarriers were less stressful to the target tissue than their higher gold counterparts.

By introducing a second variable, the uses of two different gold quantities for transformation of the wheat scutellar tissue allowed for the use of log linear analysis of the transformation rates and integration rates produced by the modified nitrate media. Log linear analysis is a variant of the chi square test that has been shown to be a very powerful tool with which to identify statistically significant results missed by other statistical tests (Decarlo *et al.,* 2000). This statistical analysis revealed that increasing the nitrate concentration of the induction media used to induce somatic embryogenesis in scutellar tissue by 50% can significantly improve biolistic transformation efficiency, as measured by number of transgenic plants produced per explant bombarded.

Transformation efficiency, as measured here, is a product of the regeneration efficiency of the bombarded tissue as well as the rate of integration which affects the number of the regenerated plants which posses the introduced transgene. Here, it was observed that increasing the nitrate concentration over a three-fold range did not visibly increase the percentage of regenerated plants which were PCR-positive for one of the introduced transgenes, suggesting that the improved transformation yields were a result of improved tissue culture. On the whole of the experiment, 0.08 plants were produced per bombarded scutellum on the control DSEM, where as the 1.5- and 3-fold treatments produced 0.23 and 0.24 plants per scutellum bombarded, respectively. These data are

consistent with the findings in chapter two which showed that increasing the amount of ammonium nitrate in the DSEM induction media can beneficially increase the number of primary embryos produced, thus increasing the number of plants which can be potentially regenerated.

The data in chapter two demonstrated somatic embryogenesis is affected by increased exposure to ammonium nitrate without the stress of biolistic transformation. The improved somatic embryogenesis was achieved by an increase in the number of primary embryos produced from the scutellum explants. Here, however, with the added stress of microparticle bombardment, it is possible that the inclusion of high salt levels in the induction media may beneficially aid in the development of somatic embryos via additional mechanisms. The microcarriers used for biolistic transformation commonly damage cells by perforating cells allowing cytoplasm to escape. As a preventive measure, explants can be exposed to various compounds prior to transformation which induce slight plasmolysis, which inhibits the loss of cytoplasm to the environment (Vain *et al.,* 1993; Jones, 2005). Ammonium nitrate is unlikely to act directly in preventing osmotic shock induced by microparticle bombardment as it flows freely from the environment to cytoplasm and thus does not have the capacity to draw water from the cell. However, in *Arabidopsis*, general salt stress response up-regulates many genes involved in osmotic shock response (Ma *et al.,* 2006). As potassium nitrate is the most prevalent salt in the media described by Eudes *et al.* (2003), increasing nitrate levels by 1.5- and 3-fold greatly increases the total amount of salts contained within the media. Increasing the total nitrate content of the DSEM medium by three-fold resulted in a two-fold increase in the total amount of salt in this medium. As no compound was used in

this study to protect the Superb scutella from osmotic shock induced during transformation, it is possible that increasing the salt concentration of the media preemptively prepared the scutella for osmotic shock, thus increasing their competency to withstand the stress. Thus, although it is likely that that increase in nitrate levels in the induction media promoted the development of primary embryos, it is also possible that this effect was added to by reduction in the amount of stress incurred by the primary embryos during transformation.

As no selection was used in this experiment, comparing the number of plants regenerated which possessed one of the introduced transgenes to the total number of plants assayed reflects the rate at which DNA was taken up by the cell and integrated into the genome. Note that plants regenerated through tissue culture are often weak and fragile. As a result, some regenerated plants produced seeds and died before producing sufficient biomass to be assayed for construct integration. Modification of the ammonium nitrate content of the induction medium showed no significant increase in percentage of regenerated plants PCR-positive for one of the introduced constructs. The integration rate was also comparable to that reported by Doshi *et al.* (2007) when similar gold quantities were used for transformation. This apparent lack of relationship could either be due to the two variables having no interdependency, or conversely not enough transgenic events were produced to illustrate this relationship. The lack of a visible increase in integration rate is consistent with the work of the Kovalchuk group which demonstrated that ammonium nitrate does not alter the overall rate of DSB repair. This study here cannot, however, rule out the possibility that overall rate of integration was not influenced by the concentration of nitrates.

Currently it is believed that the ratio of double strand break repaired by homologous recombination versus non-homologous end joining is in the order of 10^{-2} to 10^{-6} (Puchta, 2002; Hanin & Paszkowski, 2003; Lida & Terada, 2004; Puchta, 2005;). As a result, even large increases in the rate of HR would be largely masked by the number of transgenes integrated into the genome by NHEJ. By enlarge, however, the value of increasing the rate of HR is not improving the integration rate of transgenes, but to have the transgenes integrated in a simpler and more targeted fashion then occurs naturally. Investigating such improvements would require analysis of each integration event to decipher which of the two repair mechanisms integrated the transgene into the genome (Windels *et al.,* 2003).

3.5 Conclusion

Superb is an elite cultivar of hard wheat that excels in the growth conditions of the Canadian prairies. Here, it was demonstrated that the exposure of Superb scutellar tissue to elevated levels of ammonium nitrate can increase the biolistic transformation efficiency of this cereal, making it more suitable as a candidate for transgenic improvement. Nitrate modification of the induction medium used to initiate somatic embryogenesis is an economical, facile and expedient method of increasing biolistic transformation efficiency. These gains were achieved by improved tissue culture of the bombarded explants, suggesting that transformation techniques for other elite cereal lines could potentially be improved via optimising the ammonium nitrate content in the somatic embryogenesis induction media to that particular species.

Summary

Microparticle bombardment introduces foreign genes into a system via metal microcarriers propelled at high speeds into the targeted tissue. Once transgenes have been delivered into the targeted cells, it is believed that the DSB repair machinery of the targeted cells integrate the foreign DNA into their genomes. Although this technology has been shown to be relatively genotype independent, it is reliant upon the capacity to successfully regenerate fertile, non-chimeric plants from the targeted tissue. Direct somatic embryogenesis creates a full vascular system, functional meristem and a root/shoot axis in a single step. This tissue culture practice has been shown to be high yielding in some species such as carrot, but difficult to apply efficiently in other crops such as wheat.

Optimization of ammonium and nitrate contents of tissue somatic induction mediums has been shown to be beneficial in several cereal crops such as rice and barley. Here it was shown that the number of primary somatic embryos regenerated from scutellar tissue can be increased by two-fold in the agronomically superior Superb through ammonium nitrate modification of the induction medium. It was also shown that the salt induces a stress upon the primary embryos, inhibiting their capacity to transition into shoot producing embryos. Irrespective of this inhibition, the number of somatic embryos capable of producing shoots increased by almost two-fold when the nitrate concentration was increased by three-fold.

Antecedent work suggested that varying the amount of ammonium nitrate that a tissue is exposed to may alter the balance between the HR and NHEJ double strand break repair mechanisms. After biolistic transformation of Superb scutellum, an increase in

transformation efficiency was observed. As there was no apparent increase the in percentage of transgenic plants per plant regenerated, it is apparent that this increase is a result of improved tissue culture of bombarded tissues.

This work has shown a two-fold improvement in direct somatic embryogenesis in an agronomically elite cultivar via an inexpensive, rapid and facile method and demonstrated its impact directly in its application to microparticle bombardment.

Future Directions

Modification of the ammonium nitrate content in the DSEM medium proved to increase the number of primary embryos induced by direct somatic embryogenesis in the elite hard wheat cultivar Superb. Wheat cultivars have been shown to require hormones and macromolecules in unique concentrations to best respond to tissue culture. Thus, the exact concentrations which have best promoted the tissue culture of Superb are likely not to apply directly to the tissue culture of other cereal lines. Having produced a two-fold increase in the yield of direct somatic embryogenesis via a facile, inexpensive and expedient manner, however, demonstrates the value of optimizing the inorganic nitrogen species in induction media for other recalcitrant cultivars.

Ammonium nitrate was also suggested to alter the balance between HR and NHEJ *in vivo*. Having shown the effects of the salt on somatic embryogenesis with and without the stress of microparticle bombardment, study can now be focused on how recombinant DNA integration is affected by increased nitrate exposure. This study was incapable of addressing this issue as it did not produce enough transgenic plants to allow for the comparison of integration methods between trials. It may be possible to investigate this possibility in a less laborious manner by studying integration events within a tissue and not through plants regenerated from this tissue. This could potentially be accomplished by generating plants carrying a truncated non-functional visual reporter gene. This line could be then bombarded with a construct possessing the same reporter gene truncated at the opposite end, which possesses regions of homology to the genomic gene. Thus, the reporter gene would only become functional when the bombarded construct is integrated in a target manner to the complimentary reporter gene site. As a control, it would be

necessary to link the non-functional recombinant DNA reporter gene to another visual reporter gene, which would demonstrate that all of the cells in the tissue which have integrated the recombinant DNA construct through both targeted and random integration mechanisms. As thousands of cells can be expected to have the constructs integrated after a single bombardment, overlays of the expression of the two visual markers would quickly show large numbers of integration events and the efficiency of their targeting. As a result, this approach would be a very valuable tool to progressing forward in comprehending how ammonium nitrate and other compounds can beneficially impact transgene integration.

References

Alberta Government *October 16th 2007* http://www.agric.gov.ab.ca/app21/rtw/index.jsp

Altpeter, F.; Baisakh, N.; Beachy, R.; Bock, R.; Capell, T.; Christou, P.; Daniell, H.; Datta, K.; Datta, S.; Dix, P. J.; Fauquet, C.; Huang, N.; Kohli, A.; Mooibroek, H.; Nicholson, L.; Nguyen, T. T.; Nugent, G.; Raemakers, K.; Romano, A.; Somers, D. A.; Stoger, E.; Taylor, N.; Visser, R. (2005) Particle bombardment and the genetic enhancement of crops: myths and realities *Molecular Breeding* 15:305-327

Baer, A.; Bode, J. (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes *Current Opinion in Biotechnology* 12:473-480

Bassuner, B. M.; Lam, R.; Lukowitz, W.; Yeung, E. C. (2006) Auxin and root initiation in somatic embryos of Arabidopsis *Plant Cell Reporter*: 1-11

Beumer, K.; Bhattacharyya, G.; Bibikova, M.; Trautman, J. K.; Carrol, D. (2006) Efficient gene targeting in Drosophilia with zinc-finger nucleases *Genetics* 172: 2391-2403

Bhalla, P. L (2006a) Genetic engineering of wheat- current challenges and opportunities Trends in Biotechnology 24(7):305-311

Bhalla, P. L.; Ottenhof, H. H.; Singh, M. B. (2006b) Wheat transformation – and update of recent progress *Euphytica* 149:353-366

Bommineni, V. R.; Jauhar, P. P. (1996) Regeneration of plantlets through isolated scutellum culture of durum wheat *Plant Science* 116:197-203

Britt, A. B. (1996) DNA damage and repair in plants *Annual Review of Plant Physiology and Plant Molecular Biology* 47:75-100

Canadian Wheat Board *October 16th 2007* http://www.cwb.ca/public/en/

Chapman, A.; Blervacq, A.-S.; Hendriks, T.; Slomianny, C.; Vasseur, J.; Hilbert, J.-L. (2000) Cell wall differentiation during early somatic embryogenesis in plants. II. Ultrastructural study and pectin immunolocalization on chicory embryos Canadian Journal of Botany 78:824-831

Chawla, H. S. (2002) Introduction to plant biotechnology second edition *Science Publishers, Inc.* 383-386

Choi, Y.-E.; Yang, D.–C.; Choi, K.-T. (1998) Induction of somatic embryos by macrosalt stress from mature zygotic embryos of *Panax ginseng Plant Cell, Tissue Culture and Organ Culture* 52:117-181

Chugh, A.; Khurana, P. (2002) Gene expression during somatic embryogenesis- recent advances *Current Science* 83(6):715-729

Chugh, A.; Eudes, F. (2007) Isolated microspores in cereals: aspects and prospects *Recent Advances in Plant Biotechnology and its Applications*

CIMMYT international maize and wheat improvement center *November 24, 2007* http://www.cimmyt.cgiar.org/research/economics/map/facts_trends/wft9596/htm/wft9596part2.htm

DeBlock, M.; Debrouwer, D.; Moens, T. (1997) The development of a nuclear male sterility system in wheat. Expression of the *barnase* gene under the control of tapetum specific promoters *Theoretical and Applied Genetics* 95:125-131

Decarlo, T. E.; Laczniak, R. N.; Azevedo, K. A.; Ramaswami, S. N. (2000) On the log-linear analysis of multiple response data *Marketing letters* 11(4): 349-361

Dias, J. S.; Martins, M. G. (1999) Effect of silver nitrate on anther culture embryo production of different *Brassica oleracea* morphotypes *Scientia Horticulturae* 82 :299-307

Dodeman, V. L.; Ducreux, G.; Kreis, M. (1997) Zygotic embryogenesis versus somatic embryogenesis *Journal of Experimental Botany* 48(313): 1493-1509

Doshi, K. M.; Eudes, F.; Laroche, A.; Gaudet, D. (2006) Transient embryo-specific expression of anthocyanin in wheat *In Vitro Cellular and Developmental Biology-Plant* 00:1-8

Doshi, K. M.; Eudes, F.; Laroche, A.; Gaudet, D (2007) Anthocyanin expression in marker free transgenic wheat and triticale embryos *In Vitro Cellular and Development Biology – Plant* In Press http://www.springerlink.com/content/vq7468n4353mg784/

Eudes, F.; Acharya, S.; Laroche, A.; Selinger, L. B.; Cheng, K.-J. (2003) A novel method to introduce direct somatic embryos, secondary embryogenesis and regeneration of fertile cereal plants *Plant Cell, Tissue and Organ Culture* 73:147-157

Eudes, F.; Laroche, A.; Surya, A. N. (2001) Process for inducing direct somatic embryogenesis in immature scutella cells of pooideae, and rapidly regenerating fertile plants. *US patent 6,995,016*

FAO- Food and Agriculture Organization of the United Nations *October 16th 2007* http://www.fao.org/

Fortune 500 *November 15, 2007* http://money.cnn.com/magazines/fortune/fortune500_archive/snapshots/2005/3779.html

Friesner, J.; Britt, A. B. (2003) Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation defective in t-DNA integration *The Plant Journal* 34:427-440

Fu, X.; Duc, L. T.; Fontana, S.; Bong, B. B.; Tinjuangjun, P.; Sudhakar, D.; Twyman, R. M.; Christou, P.; Kohli, A. (2000) Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns *Transgenic Research* 9:11-19

Gaj, M. D. (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh *Plant Growth Regulation* 43: 27-47

Gallego, M. E.; Bleuyard, J.-Y.; Daoudal-Cotterell, S.; Jallut, N.;White, C. I. (2003) Ku80 plays a role in non-homologous recombination but it not required for t-DNA integration in Arabidopsis *The Plant Journal* 35:557-565

Gilbertson, L (2005) Cre-lox recombination: Cre-activated tools for plant biotechnology *Trends in Biotechnology* 21(12): 550-555

Grimes, H. D.; Hodges, T. K. (1990) The inorganic NO_3^- : NH_4^+ ratio influences plant regeneration and auxin sensitivity in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.) *Journal of Plant Physiology* 136:362-367

Hanin, M.; Paszkowski, J. (2003) Plant genome modification by homologous recombination *Current Opinion in Plant Biology* 6:157-162

Haplin, C. (2005) Gene stacking in transgenic plants- the challenge for 21st century plant biotechnology Plant Biotechnology Journal 3:141-155

He, D. G.; Yang, Y. M.; Scott, K. J. (1989) The effect of macroelements in the induction of embryogenic callus from immature embryos of wheat (Triticum aestivum L.) *Plant Science* 64:251-258

Hu. T.; Metz, S.; chay, C. ; Zhou, H. P. ; Biest, N. ; Chen, G.; Chen, M.; Feng, X.; Radionenko, M.; Lu, F.; Fry, J. (2003) Agrobacterium-mediated large-scale transformation of wheat (Triticum aestivum L.) using glyphosate selection *Plant Cell Report* 21:1010-1019

Helenius, E.; Boije, M.; Niklander-Teeri, V.; Palva, E. T.; Terri, T. H. (2000) Gene delivery into intact plants using the Helios^{tm} Gene Gun *Plant Molecular Biology Reporter* 18: 287a-287l

Henikoff, S.; Comai, L (2003) Single-nucleotide muations for plant functional genomics *Annual Review in Plant Biology* 54:373-401

Ipekci, Z.; Gozukirmizi, N. (2003) Direct somatic embryogenesis and synthetic seed production from Paulownia elongate *Plant Cell Reports* 22:16-24

Janakiraman, V.; Steinau, M.; McCoy, S. B.; Trick, H. N. (2002) Recent advances in wheat transformation *In Vitro Cellular and Developmental Biology –Plant* 38:404-414

Janna, A. O.; Maziah, M.; Ahmad Parveez, G. K. A.; Saleh, K. (2006) Factors affecting delivery and transient expression of ß-glucuronidase gene in *Dendrobium* Sonia protocorm-like-body *African Journal of Biotechnology* 5(2): 88-94

Jones, H. D. (2005) Wheat transformation: current technology and applications to grain development and composition *Journal of Cereal Science* 41:137-147

Jimenez, J. M. (2001) Regulation of in vitro somatic embryogenesis with emphasis on the role of endogenous hormones *Revista Brasileira de Fisiologia Vegetal* 13(2):196-223

Kim, S. –I.; Gelvin, S. B. (2007) Genome-wide analysis of Agrobacterium T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions *The Plant Journal* 51: 779-791

König, A.; Cockburn, A.; Crevel, R. W. R.; Debruyne, E.; grafstroem, R.; Hammerling, U.; Kimber, I.; Knudsen, I.; Kuiper, H. A.; Peijnenburg, A. A. C. M.; Penninks, A. H.; Poulsen, M.; Schauzu, M.; Wal, J. M. (2004) Assessment of the safety of foods derived from genetically modified (GM) crops *Food and Chemical Toxicology* 42:1047-1088

Kothari, S. L.; Agarwal, K.; Kumar, S. (2004) Inorganic nutrient manipulation for highly improved in vitro plant regeneration in finger millet- *Elusine coracana* (L.) gaertn. *In Vitro Cellular and Developmental Biology –Plant* 40:515-519

Lee, K. S.; Zapata-Arias, F. J.; Brunner, H.; Afza, R. (1997) Histology of somatic embryo initiation and organogenesis from rhizome explants of *Musa* spp. *Plant Cell, Tissue and Organ Culture* 51:1-8

León, E.; Marín, S.; Barro, F. (2006) Improvement of in vitro culture response of elite wheat cultivars by selecting the source spike, the scutellum size and the temperature for the induction of embryogenesis *Plant Breeding* 125: 580-583

Leyser, O.; Day, S. (2003) Mechanisms in plant development *Blackwell publishing*

Li, J.; Hsia, A. –P.; Schnable, P. S. (2007) Recent advances in plant recombination *Current Opinion in Plant Biology* 10:131-135

Li, W.; Ding, C.-H.; Hu, Z.; Lu, W.; Guo, G. –Q. (2003) Relationship between tissue culture and agronomic traits of spring wheat *Plant Science* 164:1079-1085

Lida, S.; Terada, R. (2004) A tale of two integrations, transgene and T-DNA: gene targeting by homologous recombination in rice *Current Opinion in Biotechnology* 15:132-138

Liu, Y.; Stasiak, A. Z.; Masson, J.-Y.; Mcllwaith, M. J.; Stasiak, A.; West, S. C. (2004) Conformational changes modulate the activity of human RAD51 protein *Journal of Molecular Biology* 337:817-827

Lloyd, A.; Plaisier, C. L.; Carroll, D.; Drews, G. N. (2005) Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis Proceedings of the National Academy of Science* 102(6): 2232-2237

Ma, S.; Gong, Q.; Bohnert, H. J. (2006) Dissecting salt stress pathways *Journal of Experimental Botany* 57(5): 1097-1107

Maës, O. C.; Chibbar, R. N.; Caswell, K.; Leung, N.; Kartha, K. K. (1996) Somatic embryogenesis from isolated scutella of wheat: effects of physical, physiological and genetic factors *Plant Science* 121:75-84

Malabadi, R. B.; Staden, J. V. (2006) Cold enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium *South African Journal of Botany* 72(4): 613-618

Masuda-Sasa, T.; Polaczek, P.; Campbell, J. L. (2006) Single strand annealing and ATP-independant strand exchange activities of yeast and human DNA2 possible role in okazaki fragment muturation *The Journal of Biological chemistry* 281(50): 38555-38564

Mathew, M. M.; Philip, V. J. (2003) Somatic embryogenesis versus zygotic embryogenesis in *Ensete superbum Plant Cell, Tissue and Organ Culture* 72:267-275

Menke-Milczarek, I.; Zimny, J. (2001) NH_4^+ and NO_3^- requirements for wheat somatic embryogenesis *Acta Physiologiae Plantarum* 23(1): 37-42

Moehle, E. A.; Rock, J. M.; Lee, Y. –L.; Jouvenot, Y.; DeKelver, R. C.; Gregory, P. D.; Urnov, F. D.; Holmes, M. C. (2007) Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases *Proceedings of the National Acamedy of Science* 104(9): 3055-3060

Mondal, T. K.; Bhattacharya, A.; Ahuja, P. S. (2001) Induction of synchronous secondary somatic embryogenesis in *Camellia sinesis* (L.) O. Kuntze *Journal of Plant Physiology* 158: 945-951

Monsanto *November 15, 2007*
http://monsanto.mediaroom.com/index.php?s=43&item=542

Mordhorst, A. P.; Lorz, H. (1993) Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspores are influenced by the amount and composition of nitrogen sources in culture media *Journal of Plant Physiology* 142:485-492

Muller, U. (1999) Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis *Mechanisms of Development* 82: 3-21

Nato, A.; Fresneau, C.; Mouralimova, N.; De Buyser, J.; Lavergne, D.; Henry, Y. (2000) Expression of auxin and light-regulated arrestin-like proteins, G proteins and nucleoside diphosphate kinase during induction and development of wheat somatic embryos *Plant Physiology and Biochemistry* 38(6):483-490

Nhut, D. T.; Hanh, N. T. M.; Tuan, P. Q.; Nguyet, L. T. M.; Tram, N. T. H.; Chinh, N. C.; Nguyen, N. H.; Vinh, D. N. (2006) Liquid culture as a positive condition of induce and enhance quality and quantity of somatic embryogenesis of *Lilium longiflorum Scientia Horticulturae* 110:93-97

Polito, V. S.; McGranahan, G.; Pinney, K.; Leslie, C. (1989) Origin of somatic embryos from repetitively embryogenic cultures of walnut (*Juglans, regia* L.): implications for *Agrobacterium*-mediated transformation *Plant Cell Reports* 8:219-221

Popelka, J. C.; Xu, J.; Altpeter, F. (2003) Generation of rye (*Secale cereale* L.) plants with low transgene copy number after biolistic gene transfer and production of instantly marker free transgenic rye Transgenic Research 12:587-596

Porteus, M. H.; Carroll, D. (2005) Gene targeting using zing finger nucleases *Nature Biotechnology* 23(8): 967-973

PRRCG- Prairie Registration Recommending Committee for Grain (2000) Wheat, rye & triticale subcommittee report *Chair: Graf, R. J.; Secretary: Fox, S. L.*

Puchta, H. (1995) Introduction of intrachromosomal homologous recombination in whole plants *The Plant Journal* 7(2): 203-210

Puchta, H. (2002) Gene replacement by homologous recombination in plants *Plant Molecular Biology* 48:173-182

Puchta, H. (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution *Journal of Experimental Botany* 56(409): 1-14

Racusen, R. H.; Schiavone, F. M. (1990) Positional cues and differential gene expression in somatic embryos of higher plants *Cell Differentiation and Development* 30:159-169

Rasco-Gaunt, S.; Riley, A.; Barcelo, P.; Lazzeri, P. A. (1999) Analysis of particle bombardment parameters to optimize DNA delivery into wheat tissues *Plant Cell Reporter* 19:118-127

Ray, A.; Langer, M. (2002) Homologous recombination: ends as the means *Trends in Plant Science* 7(10): 435-440

Reiss, B.; Klemm, M.; Kosak, H.; Scell, J. (1996) RecA protein stimulates homologous recombination in plants *Proceedings of the National Academy of Sciences USA* 93:3094-3098

Reiss, B.; Schubert, I.; Kopchen, K.; Wendeler, E.; Schell, J.; Puchta, H. (2000) RecA stimulates sister chromatid exchange and the fidelity of double-strand break repair, but not gene targeting, in plants transformed by *Agrobacterium Proceedings of the National Academy of Sciences USA* 97:3358-3363

Reynolds, M. P.; van Ginkel, M.; Ribaut, J-M. (2000) Avenues for genetic modification of radiation use efficiency in wheat *Journal of Experimental Botany* 51:459-473

Ristic, D.; Modesti, M.; Van der Heijden, T.; Noort, J.; Dekker, C.; Kanaar, R.; Wyman, C. (2005) Human Rad 51 filaments on double- and single-stranded DNA: correlating regular and irregular forms with recombination function *Nucleic Acids Research* 33(10):3292-3302

Rout, G. R.; Mohapatra, A.; Jain, S. M. (2006) Tissue culture of ornamental pot plants: a critical review on present scenario and future prospects *Biotechnology Advances* 24(6): 531-560

Sahrawat, A. K.; Becker, D.; Lütticke, S.; Lörz (2003) Genetic improvement of wheat via alien gene transfer, an assessment *Plant Science* 165: 1147-1168

Sakhanokho, H. F.; Zipf, A.; Rajasekaran, K.; Saha, S.; Sharma, G. (2001) Induction of highly embryogenic calli and plant regeneration in upland (*Gossypium hirsutum* L.) and pima (*Gossypium barbadense* L.) cotton *Crop Science* 41:1235-1240

Shaked, H.; Melamed-Bessudo, C.; Levy, A. A. (2005) High frequency gene targeting in Arabidopsis plants expressing the yeast RAD54 gene *National Academy of Sciences USA* 102:12265-12269

Shalev, G.; Sitrit, Y.; Avivi-Ragolski, N.; Lichtenstein, C.; Levy, A. A. (1999) Stimulation of homologous recombination in plants by expression of the bacterial resolvase *ruvC National Academy of Sciences USA* 96: 7398-7402

Shimida, T. (1978) Plant regeneration from the callus induced from wheat embryos, *Japanese Journal of Genetics* 53:371-375

Shoeb, F.; Yadav, J. S.; Bajaj, S.; Rajam, M. V. (2001) Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of indica rice *Plant Science* 160:1229-1235

Sigurdsson, S.; Trujillo, K.; Song, B. W.; Stratton, S.; Sung, P. (2001) Basis for avid homologous DNA strand exchange by human Rad 51 and RPA *Journal of Biological Chemistry* 276(12):8798-8806

Smith, D. L.; Kriorian, A. D. (1989) Release of somatic embryongenic potential from excised zygotic embryos of carrot and maintenance of proembryonic cultures in hormone-free medium *American Journal of Botany* 76(12):1832-1843

Somers, D. A.; Makarevitch, I. (2004) Transgene integration in plants: poking or patching holes in promiscuous genomes? *Current Opinion in Biotechnology* 15:126-131

Statistics Canada *October 16th 2007* http://www.statcanada.com/

Steward, F. C.; Mapes, M. O.; Mears, K. (1958) Growth and organized development of cultured cells *American Journal of Botany* 45:705-708

Sugawara, N.; Ira, G.; Haber, J. E. (2000) DNA length dependence of the single-strand annealing pathway and the role of Saccharomyces cerevisiae RAD59 in double-strand break repair *Molecular and Cellular Biology* 20(14): 5300-5309

Takumi, S.; Shimada, T. (1997) Variation in transformation frequencies among six common wheat cultivars through particle bombardment of scutellar tissues *Genes and Genetic Systems* 72:63-69

Taylor, N. J.; Fauquet, C. M. (2002) Microparticle bombardment as a tool in plant science and agricultural biotechnology *DNA and Cell Biology* 21:963-977

Thompson, R. G.; von Aderkas, P. (1992) Somatic embryogenesis and plant regeneration from immature embryos of western larch *Plant Cell Reports* 11:379-385

Tsukamoto, Y.; Ikeda, H. (1998) Double-strand break repair mediated by DNA end-joining *Genes to Cells* 3:135-144

Tzifira, T.; Frankman, L. R.; Vaidya, M.; Citovsky, V. (2003) Site-specific integration of Agrobacterium tumefaciens T-DNA via double-stranded intermediates *Plant Physiology* 133: 1011-1023

Tzfira, T.; White, C. (2005) Towards targeted mutagenesis and gene replacement in plants *Trends in Biotechnology* 23(12):567-569

Vain, P.; McMullen, M. D.; Finer, J. J. (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize *Plant Cell Reports* 12: 84-88

Windels, P.; De Buck, S.; Van Bockstaele, V.; De Loose, M.; Depicker, A. (2003) T-DNA integration in Arabidopsis chromosomes. Presence and origin of filler DNA sequences *Plant Physiology* 113: 2061-2069

Wyman, C.; Kanaar, R. (2006) DNA double-strand break repair: all's well that ends well *Annual Review of Genetics* 40:363-383

Ye, X.; Al-Babili, S.; Klöti, A.; Zhang, J.; Lucca, P.; Beyer, P.; Potrykus, I. (2000) Engineering the provitamin A (-carotene) biosynthesis pathway into (carotenoid-free) ride endosperm *Science* 287(5451):303-305

Zale, J. M.; Borchardt-Wier, H.; Kidwell, K. K.; Steber, C. M. (2004) Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes *Plant Cell, Tissue and Organ Culture* 76:277-281

Appendices

Appendix 1 Components of direct somatic embryogenesis mediums used described by Eudes *et al.* (2003).

Media components (mg • L^{-1})	DSEM medium	SEM medium	GEM medium	Rooting medium
Salts				
$CaCl_2 • 2H_2O$	440	440	440	440
$MgSO_4 • 7H_2O$	370	370	370	370
NH_4NO_3	165	165	165	1650
KH_2PO_4	170	170	170	170
KNO_3	1900	1900	1900	1900
$MnSO_4 • 4H_2O$	22	22	22	22
$CoCl_2 • 6H_2O$	0.2	0.2	0.2	0.2
H_3BO_3	6.2	6.2	6.2	6.2
$ZnSO_4 •.7H_2O$	8.6	8.6	8.6	8.6
$CuSO_4 •5 H_2O$	0.15	0.15	0.15	0.15
$NaMoO_4 • 2H_2O$	0.25	0.25	0.25	0.25
KI	-	-	-	0.83
$FeSO_4 • 7H_2O$	27.85	27.85	27.85	27.85
$NaEDTA • 2H_2O$	37.23	37.23	37.23	37.23
Carbohydrates				
Maltose	15000	15000	15000	-
Sucrose	5000	5000	5000	10000
Xylose	350	350	350	-
Ribose	350	350	350	-
Myo-inositol	200	200	200	-
Amino acids				
L-Glutamine	750	750	750	-
L-asparagine	60	60	60	-
L-Arginine	30	30	30	-
g-amino butyric acid	80	80	80	-
L-Serine	55	55	55	-
L-Alanine	30	30	30	-
L-Cysteine	10	10	10	-
L-Leucine	10	10	10	-
L-Isoleucine	10	10	10	-
L-Proline	10	10	10	-
L-Lysine	10	10	10	-
L-Phenylalanine	5	5	5	-
L-Tryptophan	5	5	5	-
L-Methionine	5	5	5	-
L-Valine	5	5	5	-
L-Histidine	2.5	2.5	2.5	-
L-Threonine	2.5	2.5	2.5	-

L-Glycine	2.5	2.5	2.5	-
Plant growth regulators				
Spermine	1	1	1	-
Spermidine	4	4	4	-
2,4-D	1.75	-	-	-
Phenyl acetic acid	3	1.5	-	0.5
Benzyl amino purine	0.5	25	-	-
Vitamins				
Pyridoxine.HCl	1	1	1	-
Thiamine.HCl	1	1	1	-
Pantothenate	0.5	0.5	0.5	-
Nicotinic acid	1	1	1	-
Riboflavin	0.2	0.2	0.2	-
Folic acid	0.2	0.2	0.2	-
Biotin	0.2	0.2	0.2	-
Betaine chloride	7.9	7.9	7.9	-
Choline.HCl	10	10	10	-
Ascorbic acid	0.4	0.4	0.4	-
Organic acids				
Malic acid	1000	1000	1000	-
Fumaric acid	200	200	200	-
Succinic acid	20	20	20	-
a-Ketoglutaric acid	20	20	20	-
Citric acid	5	5	5	-
Pyruvic acid	5	5	5	-
Agar purified	-	-	-	6000
Gelrite	3000	3000	3000	-

Appendix 2 Number of primary embryos recovered from several cereal lines after 14 days incubation on modified DSEM.

		1	2	3	4	5	6	7	8	9	10
Superb	**1x**	x	46	70	49	66	71	110	116	84	122
	1.5x	60	51	64	77	123	109	176	100	222	140
	3x	55	96	124	x	110	179	192	228	313	205
AC Alta	**1x**	18	36	45	107	104	134	83	152	147	90
	1.5x	31	20	41	67	57	62	70	92	47	45
	3x	36	23	52	78	93	93	62	120	55	86
Fielders	**1x**	213	159	247	221	188	205	154	130	163	x
	1.5x	227	119	157	221	199	209	166	176	127	x
	3x	285	x	311	296	245	224	164	161	204	x

Appendix 3 Number of plantlet producing embryos, from several different cereal lines after 14 days incubation on modified DSEM and 7 days incubation SEM

		1	2	3	4	5	6	7	8	9	10
Superb	**1x**	x	36	31	26	44	56	66	92	47	60
	1.5x	44	37	24	39	80	59	110	87	56	53
	3x	36	54	77	x	72	126	123	143	107	89
AC Alta	**1x**	8	19	14	69	67	92	54	86	90	63
	1.5x	23	4	28	28	19	26	40	32	16	15
	3x	10	14	37	33	28	58	46	68	31	47
Fielder	**1x**	150	119	175	168	146	127	138	102	84	x
	1.5x	138	55	83	107	145	169	145	156	98	x
	3x	195	x	158	174	177	155	128	132	147	x

Appendix 4 Percentage of primary somatic embryos transferred from modified DSEM to unmodified SEM capable of surviving and producing plantlets

		1	2	3	4	5	6	7	8	9	10
Superb	**1x**	x	81.82	46.97	63.41	75.86	76.71	64.08	79.31	56.63	53.57
	1.5x	78.57	68.52	40.68	51.32	67.80	60.20	66.67	75.65	26.79	37.32
	3x	75.00	56.84	65.81	x	65.45	75.90	78.34	69.42	41.15	44.50
AC Alta	**1x**	57.14	76.00	53.85	64.49	73.63	70.23	69.23	61.43	66.18	64.29
	1.5x	69.70	22.22	73.68	43.75	38.00	41.27	59.70	38.10	37.21	34.09
	3x	30.30	70.00	75.51	56.90	36.84	67.44	71.88	63.55	60.78	56.63
Fielder	**1x**	75.38	79.33	74.79	76.02	80.66	62.56	92.00	76.69	51.53	x
	1.5x	66.99	48.67	56.85	48.42	69.38	78.97	86.31	89.66	77.17	x
	3x	73.86	x	53.20	58.78	75.64	73.81	83.12	83.02	72.06	x

Appendix 5 soil free mixture used for mother plants growth

Soil free mix was made in batches of 320 L. Batches included 107 L sphagnum peat moss, 110 L medium horticultural grade vermiculite, 1 kg calcium carbonate flour, 1.5 kg 18-6-12 Osmocote (Southern Agricultural Insecticides, Inc.), 1.2 kg phosphate 21P, 20 g 'fritted' trace elements, 15 g of 13.2 % (W/W) chelated iron and 7 g of 14 % (W/W) chelated zinc(The Scotts Co.).