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Assessment of wheat x maize doubled haploid technology for genetic improvement of New Zealand wheat

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at Lincoln University, Canterbury, New Zealand

By Anna Wendy Campbell

Lincoln University, 1999.
New Zealand must continue to produce superior wheat cultivars to retain competitive advantage within New Zealand and contribute in terms of global wheat breeding. Doubled haploid (DH) technology allows the production of homozygous wheat lines in a single generation. The integration of DH technology into New Zealand wheat breeding and genetics programmes has the potential to reduce the breeding time of new cultivars and improve our understanding of agronomically important genetic traits. The main goal of this thesis was to increase the efficiency of the development of genetically improved New Zealand wheat cultivars by inducing direct homozygosity through wheat x maize crosses.

To achieve this goal, a number of objectives needed to be met. The first of these was to develop a method of wheat DH production through wheat x maize crosses using New Zealand germplasm. A number of New Zealand wheat cultivars were crossed with different maize genotypes. A successful method for producing wheat DHs was achieved with all genotypes. Several factors were identified as being important in the efficiency of wheat DH production. These included: seasonal constraints, time of embryo excision, method of auxin application and media composition. Of these factors, seasonal constraints were the major limitation in the use of the wheat x maize method for producing DHs in New Zealand.

The second objective was to determine optimal environmental conditions for the production of wheat DHs through wheat x maize crosses. New Zealand wheat cultivars were grown in a glasshouse until they reached the booting stage when they were transferred to differing temperature and light intensity conditions. Results showed that both temperature and light intensity significantly affected haploid embryo numbers. In a further examination, it was found that light intensity acted at the level of pollen tube
growth. This was a maternal plant effect and measurements of electron transport rate and quantum yield showed that photosynthesis may influence the maternal plant in such ways as to promote and/or inhibit pollen tube growth in wheat x maize crosses.

The third objective was to evaluate whether wheat DHs produced through the wheat x maize method are ‘normal’ and genetically stable. DHs were made from homozygous wheat cultivars and compared with their parent cultivar in a field trial. Results showed that a small amount of aberrant genetic variation was introduced into DH lines. It was concluded that the variation was minimal and unlikely to have an effect in breeding programmes, verifying the use of wheat x maize crosses over anther culture.

The fourth objective was to determine where in a wheat breeding programme DHs could be used for maximum genetic gain. A model was tested which integrated DH and marker assisted selection (MAS) into a breeding programme. When genetic variation of the MAS selected DH plants was compared to the genetic variation of an unselected F₃ population, five of the six measured traits were normally distributed and had no significant loss of genetic variation. The sixth trait, height, was bimodally distributed and further analysis of the data showed that the inheritance of major gene/s and factors such as linkage and/or epistasis may have been involved in its distribution. Overall the model was shown to be a valid breeding tool, allowing a breeder to estimate minimum DH population sizes required for minimal loss of genetic variation.

Results from the research outlined in this thesis indicate that DH technology can be used to reduce the time involved in producing new wheat cultivars and for producing useful populations for genetical studies.

Key words: Triticum aestivum, Zea mays, doubled haploids, breeding, genetics.
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1: General introduction

Wheat is the most widely grown and consumed food crop in the world (Vasil and Anderson, 1997). Intensive breeding in modern times has led to the adaption of wheat to a wide range of ecological conditions (temperate, subtropical, and tropical). Even in Asia, where rice has historically been the dominant crop, wheat is fast becoming a major crop. For example, in 1994, Asia produced 217 million tons of wheat compared with a combined harvest of 209 million tons in the USA, Canada, Europe and Mexico (Vasil and Anderson, 1997). In addition to wheat being grown in countries not traditionally associated with wheat growing, over the past 40 years the introduction of new cultivars and improved husbandry has led to major increases in global wheat yields (2% increase annually from 1961-1994) (Braun et al., 1998). Currently, global wheat production is 560 million tonnes per year (Braun et al., 1998). It has been predicted that with the increasing world population, the annual global demand for wheat will increase to 1050 million tonnes by the year 2020 (annual increase of 2.6 %) (Kronstad, 1998). However, recent figures show yield increases are beginning to slow (from 3% 1977-1985 to 1.6% from 1986-1995) (Braun et al, 1998). Considerable research in the past decade has been devoted to novel techniques and methodologies in wheat biotechnology. It is hoped that the use of these, in combination with conventional breeding methods, will be the key to increases in global wheat yields into the next millennium.

The introductory chapter of this thesis provides a review of past and current research into both wheat genetics and breeding, with a focus on breeding of New Zealand wheats and doubled haploid (DH) technology. The chapter finishes with the overall goal and objectives of this thesis, based on the literature reviewed.

1.2: Wheat genetics

1.2.1: The wheat genome

Classification of wheat began with the work of Linnaeus in 1753. The turning point for
A major advance in the classification of wheat occurred in 1918 when Sakamura reported the chromosome number for each wheat type (Briggle, 1980). It was recognised that wheats fell into three natural groups based on chromosome number: diploids \((2n = 14)\), tetraploids \((2n = 28)\) and hexaploids \((2n = 42)\). The ploidy level of wheat is determined by the presence/absence of three genomes: 'A', 'B' and/or 'D'. The two genomes of tetraploid wheat \((AABB)\) are different but homeologous, each with seven pairs of chromosomes. In hexaploid wheat, an additional genome \((DD)\) exists, again with seven pairs of chromosomes. The 'D' genome was the last to be incorporated into hexaploid wheat and is the least modified genome (Yen et al., 1996).

Most of the wheat grown throughout the world is *Triticum aestivum*, which is hexaploid and commonly referred to as bread wheat. Other *Triticum* species of consequence are tetraploid *T. durum* (used for pasta) and hexaploid *T. compactum* (used for biscuits). All three are products of natural hybridisation with ancestral types. For example, *T. aestivum* was derived from hybridisation between tetraploid *T. turgidum* \((AABB)\) and a genetic accession of seven-chromosome wildgrass *Aegilops tauschii* spp. *stangulata* \((DD)\) (Dvorak et al., 1998). The 'A' genome of *T. turgidum* is thought to have come from *T. monococcum*, spp. *urarta* (Friebe and Gill, 1996) whilst the origin of the 'B' genome is less clear (Fig. 1.1).
Fig. 1.1: A pictorial history of hexaploid wheat ancestry (adapted from Singh, 1993).

The genetics of bread wheat is complex because of its polyploidy. Although bread wheat is hexaploid and each locus is theoretically present six times, wheat behaves cytologically as a diploid, with recombination occurring between homologous chromosome pairs only. Alleles on homeologous chromosomes generally segregate independently of each other. However, the presence of alleles on one chromosome can affect the phenotypic expression of segregation at a corresponding homeoallelic locus leading to complex segregational patterns and epistatic effects. As it is difficult to analyse the effects of component genes, the description and location of such genes lags behind diploid species such as barley (Snape, 1987).
Most of the 'major' wheat genes which have been identified and located are those affecting qualitative variation (e.g., dwarfing, spike morphology and grain colour) (Law et al., 1987). In the 1960s and 1970s the identification and localisation of such genes was enhanced by both conventional and aneuploid methods of analyses. Conventional analyses of major genes showed that when contrasting genotypes were crossed, major genes segregated into discrete phenotypic classes. Once genes were recognised through this segregation pattern, wheat aneuploids, of which there are a complete range, were used to locate the genes on chromosomes and show the role of certain chromosomes in the development of wheat as a whole (Borojević, 1990).

Although the use of conventional breeding methods and aneuploidy have been useful in establishing the genetic effects of major genes, they are of little use in understanding the genetic variation which is of most interest to wheat breeders. This genetic variation is known as quantitative variation. Quantitative variation is caused by the combined actions of 'minor' genes which convey a specific genotype. Such quantitative traits include yield, height, many processing qualities and many types of durable disease resistance. Analysis of the genetical basis of quantitative trait loci (QTL) is complex. This is because it is difficult, if not impossible, to combine all the 'best' alleles, at all segregating loci, into a single genotype and then identify that genotype (Sorrels and Wilson, 1997). Recognition of the 'best' genotype is further complicated by environmental influences on genotypes. Dominance effects, epistatic effects and linkage effects can all alter the mean expression of a QTL within a population.

Domesticated bread wheat has a narrow gene pool with low polymorphism, thus future progress by conventional selection methods is expected to be slow, eventually reaching a plateau (Sorrels and Wilson, 1997). The integration of novel techniques and methods into wheat breeding programmes is necessary to facilitate continued and accelerated progress in producing new wheat lines. In the 1970s and 1980s, development of electrophoretic methods allowed the visualisation of genetic differences of isozymes and grain storage proteins, but the levels of polymorphism were too low to provide comprehensive genetic maps (Snape, 1998). In the last decade, the development of molecular techniques has allowed the construction of detailed genetic maps of wheat for further elucidation of gene function, expression and regulation. Genetic maps are made by finding polymorphic
molecular markers which are used to identify desired genes. Linkage analysis can then be used to define the genetic distances between the polymorphic loci. One of the main tools used in wheat and other species to find these polymorphisms is restriction fragment length polymorphisms (RFLP) (Van Deynzel et al., 1995). However, RFLP analysis has had limited success in wheat as it is significantly less polymorphic than other cereal species such as maize, rice or barley (Helenjaris et al., 1986). In addition to this, many markers located have been found to be clustered in centromeric regions of the genetic map (Devos and Gale, 1993). These markers are mostly linked to homeologous loci, while the less populated distal regions contain most of the non-homeologous loci. Current mapping efforts are attempting to fill the less populated regions (Devos and Gale, 1993). Other PCR-based techniques, including microsatellites and amplified fragment length polymorphisms (AFLPs), are now showing promise in finding polymorphisms in the wheat genome for more detailed mapping and eventually sequencing of loci important in the control of QTL (Mohan et al., 1997). Random-amplified polymorphic DNAs (RAPDs) have also been used, but only to a limited extent as repeatability can be uncertain (Brennan et al., 1996).

There is also tremendous potential in the use of 'comparative genetic mapping' for improvements in our understanding of wheat genetics (Snape et al., 1996, Cadalen et al., 1997). The genetics of wheat are now clearly linked to the genetics of other cereal species, in particular, barley and rye (Linde-Laursen et al., 1997, Snape, 1998). Thus, it is possible to make comparative major gene and QTL analysis across genera. This has been useful for locating genes associated with dwarfing, vernalisation, flowering time and photoperiodism (Börner et al., 1998, Sarma et al., 1998, Snape et al., 1998).

1.3: Wheat breeding

Few attempts were made to improve varieties of wheat crops until the early years of the 19th century. Crops grown at this time consisted of a wide variety of 'land-races' each of which had evolved in its particular area, mainly through natural selection (Bell, 1987). Wheat hybrids were made in the 18th and 19th century, before the rediscovery of Mendel’s laws, by a number of workers, including Sherriff and later Hallet (Bell, 1987). However improvements through hybridisation at this stage were limited as many hybrids were made
to create variation from which to select, rather than with clearly defined objectives in mind. The beginning of the 20th century saw the rediscovery of Mendel's laws of segregation as well as Johanssen's discovery of the concept of pure lines. These discoveries have formed the basis of modern wheat breeding (Lupton, 1987).

Wheat is now known to be a naturally self-pollinating crop which leads to homozygosity (pure lines). Some cross-pollination can occur and this can vary from effectively zero to over 3%, but usually occurs at less than 1% (Griffin, 1987). Maximum variation in a wheat breeding programme is obtained by purposeful hybridisation. There are three basic methods used in wheat breeding: the bulk method, the pedigree method and the backcrossing method. These methods are now being increasingly combined with tissue culture techniques (such as doubled haploid technology) and molecular information (such as marker assisted selection) to further improve wheat varieties and speed up the breeding process.

1.3.1: Bulk-population breeding

Bulk breeding relies on natural selection to shift gene frequencies. Superior lines are often produced because many characteristics that contribute to the survival of the wild species are associated with the productivity of the cultivated species. In bulk population breeding, seeds from all F1 plants are grown in bulk to produce enough seeds for the F2 generation. The F2 generation is then planted in a plot large enough to accommodate several hundred or even several thousand plants. Seeds are harvested from all plants and samples are re-sown in bulk again in following generations (generally not all seeds are sown due to space constraints). By the F6 generation 96% homozygosity has occurred for all traits and individual selection of prospective phenotypes which possess desirable recombinations of traits from their parents can be started. Selection is based on progeny performances for the F7 generation and later generations (Fig. 1.2).

The advantage of using bulk-population breeding is that a large number of cross combinations can be grown at low cost with low input into selection and observation by the breeder. However, bulk-population breeding is limited in that valuable genotypes can
be lost due to difficulties of recognition in a large bulk. To counter this, modifications of bulk-population breeding are now used which include negative or positive selection in the segregating generations.
Crossing of selected parents

$F_1$ Bulk sowing of $F_1$ seeds

$F_2$ Bulk sowing of $F_2$ plants

$F_3 F_4$ Procedure repeated

$F_5$ Best plants selected

$F_6$ Sowing of $F_5$ selected plants

$F_7$ Seeds from best rows/lines sown in a preliminary field trial

$F_8$ Best lines sown in comparative yield trials with a standard (std)

$F_9$ Best lines further tested in comparative yield trials, in official trials for registration

Multiplication

**Fig. 1.2:** Diagram of a bulk selection scheme (adapted from Borojević, 1990).

### 1.3.2: Single seed descent

Single seed descent (SSD) is a modification of the bulk population system. The main
Advantage of SSD is in the acceleration of selection and the production of new lines (Choo et al., 1982, Borojevic, 1990). Once a cross is made, single seeds of each plant are randomly sampled in subsequent generations. As only single seeds are used, the segregating generations can be grown in the glasshouse allowing 2-3 generations per year. However, like individual genebank accessions, SSD relies on the assumption that every individual genotype has an equal opportunity of being selected for subsequent plantings and is of equal reproductive fitness when compared with other genotypes (Cross and Wallace, 1994). Seeds do not have equal reproductive rates and different environments will favour some individuals over others (Cross and Wallace, 1994). Therefore, during SSD, certain genotypes may be favoured whilst others are lost due to selection pressures. The examination of cereal lines produced through SSD have shown that a portion of genotypes are lost when only a single seed per plant is sampled and skewed segregation ratios can result (Borojevic, 1990, Snape et al, 1992, Bjørnstad et al, 1993a).

1.3.2: Pedigree method

In the pedigree method, superior types are selected in segregating generations and a record is maintained of all parent-progeny relationships. Selection begins in the F2 generation. At this stage progeny are highly heterozygous and a large number are screened on an individual plant basis. By the F3 and F4 generations, many loci will have become homozygous and family characteristics begin to appear. There is still a lot of heterozygosity between the families, so selection is for the best plants in the best families. By the F5 and F6 generation, most families are homozygous at most loci, therefore, selection within families is no longer effective. The pedigree record at this stage is used to eliminate all but one member of such closely related families.

It has been shown that the chance of obtaining a superior genotype is higher in the pedigree method than bulk selection methods, as selection is based on genotype as well as phenotype (Borojevic, 1990). The main disadvantage of the pedigree method is that it is both time and labour intensive. As well, the effects of heterosis and dominance can mask the genotypic value of a plant for many generations. The pedigree method is used more frequently in self-pollinated crops than bulk methods, because of the greater chance of selecting superior genotypes (Borojevic, 1990). However, a recent comparison of bulk,
selected-bulk and pedigree selection schemes showed that although the pedigree selection scheme produced plants with higher yields than the other schemes, factors such as disease resistance were similar in all schemes (Singh et al., 1998). When other factors were considered, such as land use and labour costs, the selected bulk scheme was found to be superior to the other schemes. The authors proposed that any selection scheme that shifts population frequencies towards desirable phenotypes should result in higher frequencies of superior genotypes. Furthermore, they concluded that the selection of parents is more important than the type of selection scheme imposed upon segregating populations (Singh et al., 1998).

Fig. 1.3: Diagram of a pedigree selection scheme (adapted from Borojević, 1990).
1.3.4: Backcross breeding

Backcross breeding provides a precise way of improving varieties that excel in a large number of attributes but are deficient in a few characteristics. An illustration of the use of backcrossing in a breeding programme would be if a cultivar had superior characteristics, but was susceptible to a new disease strain. In a breeding programme the susceptible cultivar (recurrent parent A) would be crossed with a germplasm accession resistant to the disease (parent B). The procedures of crossing and selection would differ according to whether the disease trait was inherited in a dominant or recessive manner. If the disease resistant gene was dominant, then after the first cross all progeny would be heterozygous and resistant to the disease. The progeny would then be backcrossed to the recurrent parent A. The progenies of the backcross would segregate for 50% resistant plants ($Rr$) and 50% susceptible plants ($rr$). Once resistance was confirmed, the procedure would be repeated up to six times. Lines would then be selected by the pedigree method and tested for yield performance and resistance. If the trait of interest was recessive, intermediate progeny testing generations are required to identify the homozygous recessive genotype, so overall a longer time period is required to reach final homozygosity.

Backcrossing is generally not used exclusively in a wheat breeding programme, rather it is a tool used in combination with pedigree breeding schemes. The main disadvantage of backcrossing is that in the time taken to backcross a cultivar, new and improved lines may be developed. As well, although selection is successful if a trait is controlled by one or two major genes, it is less efficient if the desired trait is quantitative, or if it is linked with a negative trait.

1.3.5: Marker assisted selection

With the advent of improved genetic maps of wheat there is potential for the use of molecular markers for selection purposes when the markers are linked to genes of interest. Molecular markers are unaffected by the conditions in which the plant is grown and are detectable at all stages of growth. For markers to be used efficiently, they must be close to the gene of interest (1 cM or less) to minimise recombination (Mohan et al., 1997,
Edwards and Page, 1994; Lande and Thompson, 1990). If markers are close, linkage drag can also be decreased, which reduces the chance of introducing undesirable genes with the gene of interest. Molecular markers are also potentially valuable for use in the acceleration of backcrossing, pyramiding genes, identification of hybrids, selection for resistance to pests and diseases not present in the environment (quarantine traits) and analysis of alien chromosome segments (Langridge and Chalmers, 1998). However, a number of problems exist which need to be countered before molecular markers can be fully implemented in a breeding programme.

i) Although several markers may be found to be linked to a trait in one cross, the markers may not exist in other crosses. Using current screening methods, it would be inconceivable to have to identify markers for all crosses in a breeding programme.

ii) The sheer complexity of some quantitative traits may confound the use of markers to select for particular genotypes. For example, Parker et al., (1996) evaluated RFLP markers for flour protein content and flour colour in field trials over 1995 and 1996. They found several markers linked to flour colour which were common over all data sets. However, of the several markers found for protein content, none were found to be common to more than one set of data and there was a poor correlation within the replicates for protein content. This shows the effect of the environment on protein content and indicates the effect of multiple genes for the trait. However, when selecting for traits such as protein content, these problems exist for all selection methods, marker assisted selection (MAS) should help in resolving some of these problems.

iii) Another limitation of MAS in wheat breeding programmes has been the volume of early generation lines (Howes et al., 1998). As well, selected plants must be retested in subsequent segregating generations.

The development of PCR technology has led to a more efficient means of screening populations than previously used RFLP and isozyme technology and future technological improvements should allow rapid screening of large breeding populations. The combination of MAS with DH technology will prevent the need for re-screening as marked
genes are fixed in a single generation. The logistics of combining larger numbers of genes using MAS requires different breeding strategies, depending on the number of genes involved and the mode of inheritance of the genes. Over the next few years the evaluation of different strategies will be important for implementation of MAS into breeding programmes at maximum efficiency.

1.4: Wheat breeding in New Zealand

1.4.1: Historical perspective

Captain James Cook brought wheat into New Zealand in 1769. The first record of an attempt to grow wheat in New Zealand was in 1813 (Bushuk, 1995). Maori Chief Ruatara was the first wheat miller and is also thought to be the first successful wheat grower in New Zealand. He imported a pair of mill stones and from the resultant flour baked the first wholly New Zealand bread in 1814 (Ireland, 1979).

Wheat growing in New Zealand was not widespread until the arrival of settlers to Otago in 1848 and Canterbury in 1850. The open country of these provinces suited the plough and the availability of coastal transport facilitated trade (Hilgendorf, 1938). By 1851 over 3000 acres of wheat was grown in New Zealand and by 1857, due to the favourable climate and fertile soil, more wheat was produced than was required for the local market. As a result, much of the locally produced wheat was exported to New South Wales, Australia. A peak in wheat production of 270 000 tons occurred in 1891/92. After this time, problems began to develop with disease, lack of uniformity in cultivars, poor agronomic performance and milling quality (Bushuk, 1995). By the early 1900s local production had dropped so much that in some years Australian flour needed to be imported to make up for the short fall. As a result of the dissatisfaction with the cultivars available, a wheat improvement programme for the development of new cultivars was established at Lincoln College (now Lincoln University), Canterbury. The cultivars grown at this time were those best adapted of the European lines introduced by pioneer colonisers. The first hybrid crosses for use in wheat breeding in New Zealand were made in 1923 (Smith, 1979). From these early crosses, "Cross-7" was released in 1934. This cultivar quickly
displaced other wheats as it had improved adaption to New Zealand growing conditions and superior milling and baking properties.

Price controls were introduced during World War One to encourage domestic wheat production. However, these controls were abandoned in the early 1920s, as New Zealand wheat quality was so poor, bakers were unable to make commercially acceptable bread. Under the system of state control, millers were denied the right to specify their quality requirements or to pay price differentials based on quality (Bushuk, 1995). Therefore, wheat growers based their choice of cultivars on yield and agronomic characters, rather than quality characters.

To encourage domestic production again, a sliding scale of import duty was introduced in 1928 (Bushuk, 1995). However, this sliding scale did not address the price changes as supplies of wheat fluctuated throughout the year causing price fluctuations. A ‘wheat pool’ was organised by farmers in an attempt to stabilise prices. By 1932 most farmers and millers were operating by this system and a wheat marketing company was formed to handle the sole marketing and selling of wheat crops to millers. Bakers were not covered by any overall sector group and millers and bakers were often in conflict. The Wheat Council (established 1936) was an organisation set up to represent all three sectors of the industry; growers, millers and bakers (Bushuk, 1995). The Wheat Council was the sole trader of wheat and flour in New Zealand and also the sole importer in the event of a shortage of domestic wheat.

The Wheat Council succeeded in stabilising prices and operations in each of the three sectors. However, they had no responsibility for the quality of wheat or flour and failed to decrease the amount of imported wheat entering the country. The local market was producing only 22% of that required because of the poor quality of the wheat. This led to a commission of enquiry into the industry which resulted in the establishment of the Wheat Board in 1965 (Bushuk, 1995). The Wheat Board introduced wheat quality as a criterion of value to the millers and the bakers. Wheat quality was judged on several criteria, including a minimum acceptable bake score. However there was no premium paid for improved quality over the minimum required. As a result, there was no real incentive to maximise wheat quality either by breeding or management (Bushuk, 1995).
The continuing poor quality of New Zealand wheat meant that when price controls on bread were eventually abolished in 1980, bakers started using expensive additives (such as gluten and modified fats) to improve the quality. Consequently, bread prices increased dramatically and this was used as a lever to convince the Wheat Board to improve the quality of New Zealand milling wheats (Bushuk, 1995). Although an improvement resulted from splitting the milling grade into three price categories, these adjustments did not resolve all of the difficulties of the milling and baking industries. In 1987 the Wheat Board was dis-established and the entire wheat industry was deregulated. Millers were now free to negotiate their own price, set their own specification level and contract wheat from any source, be it domestic or foreign (Bushuk, 1995). Thus, the wheat industry structure and dynamics have changed dramatically in New Zealand. The industry is now led by bakers and millers and is characterised by the quality of the end product. This has meant that both breeders and growers have had to respond quickly to ‘market signals’, resulting in reduced plantings but increased numbers of specialised cultivars to meet market demands.

1.4.2: Current wheat breeding programme in New Zealand

The only full wheat breeding programme in New Zealand is run by the New Zealand Institute for Crop & Food Research. The programme is based on the traditional pedigree breeding system (Griffin et al., 1996). Cooperating breeding programmes are run for bread, biscuit, feed, coloured, snack, durum and other specialty wheats.

From hybrid crosses, segregating generations are grown and selected. The number of lines retained decreases over each generation and increasingly complex tests are applied to the reduced number of lines. These tests range from agronomic measurements in early generations, such as maturity and yield, to more complex tests in later generations, such as test baking and measurements of dough strength (Griffin et al., 1996). Generally, the wheat lines are evaluated on site for the first seven years and are then moved onto regional trials for another two generations. The ACE (arable cultivar evaluation) committee, which represents breeders, producers and processors, then manages line evaluation for another three years before the new cultivar is eligible for full listing in the ACE comparative table. Details of all wheat lines are retained throughout the three years of testing and advanced
lines are fed back into the crossing stage at the beginning of the breeding programme. As another source of genetic variation for the crossing programme, wheat germplasm is exchanged with approximately 25 breeders in more than 15 countries (Griffin et al., 1996). On occasions the imported material proves to have commercial potential in its own right and is developed for cultivar release following the same system as above. Two examples of this are Otane and Kotuku. These cultivars were re-selected from lines originally from CYMMIT (Mexico) and Plant Breeding International (UK) respectively. Although the New Zealand Institute for Crop & Food Research runs the only full breeding programme within New Zealand, other private companies are involved in the evaluation of imported material at different generations.

The formation of the ACE committee has ensured that processing sectors are involved in breeding programmes. ACE is largely funded by the Foundation for Arable Research (FAR), (the arable farmers’ organisation which controls their production levy funds) and the New Zealand Flour Millers Association (NZFMA). Breeding companies can also receive significant funding directly from such industry groups as well as other private processing companies. Any commercialisation of a new cultivar is taken after consultation with all appropriate industry groups, particularly the processing sector (Griffin et al., 1996).

1.5: Doubled haploids in cereal breeding and genetics

The natural occurrence of haploid plants has been found in a wide variety of plant species (their existence first reported in 1921) (Raina, 1997). However, research into haploid plants was limited by their low frequency within species. In the past two decades, developments of in vitro culture techniques have enabled the production of large numbers of haploid plants, particularly in cereals (Kasha et al., 1990). Of these techniques, anther culture and chromosome elimination in intergeneric crosses have been the most widely used. The production of DHs has been an important development in cereal breeding, because, after chromosome doubling to recover fertility, the recovery of homozygous lines can be achieved in a single generation (Pickering and Devaux, 1992). This can significantly reduce the time taken before advanced comparative trials can be made and
Anther culture exploits the fact that a certain proportion of pollen grains \textit{in situ} are embryogenic (Gilpin, 1996). These pollen grains can develop into embryos only when they are placed on artificial medium. The process of anther culture begins in the selection of primary cereal spikes which contain anthers with pollen at the mid-late uninucleate stage of development (Sunderland, 1974, Dunwell, 1985, Mitchell, \textit{et al.}, 1992). The stage of pollen development is very important as minor deviations can lead to major decreases in yield (Dunwell, 1985). Anthers are then dissected and cultured on nutrient media where embryo-like structures may form directly from the pollen grains (Wan and Widholm, 1993).

Anther culture is used in many cereal breeding programmes and is more cost-effective than intergeneric crosses in the production of DHs (Snape \textit{et al.}, 1986a, Snape and Parker, 1987). However, there are limitations in using anther culture technology, the main being the poor response of many genotypes. To counter the genetic effect, components of the system have been investigated. These include: growth environment of donor plants, modification of the medium components, changing the physical state of the medium (liquid or solid), cold pretreatment of anthers before culturing, increasing the incubation temperature during the first few days of culture and subjecting anther explants to gamma irradiation (Dunwell, 1985, Kasha \textit{et al.}, 1990, Ling \textit{et al.}, 1991). A further disadvantage of anther culture in barley and wheat breeding is in the high rates of albino plants. In some wheat genotypes the frequency of albinism has been found to range from 20-50\% with a mean of 30\% of all regenerated plants (Marsolaris, 1989). \textit{In vitro} genotypic selection can also occur in anther cultures which can skew segregating ratios of the resulting DH populations (Snape, \textit{et al.}, 1992, Bjørnstad \textit{et al.}, 1993b). In New Zealand the application of anther culture to the wheat breeding programme has also been limited because of these problems (Cooper and Griffin, 1988).

Haploid plants produced through isolated microspore culture are derived from either
pollen grains (Gustafson et al., 1995) or ovaries (Mejza et al., 1993). To produce haploid plants from pollen grains, the pollen grains can be shed using the natural dehiscence of anthers (Wei, 1982) or they can be mechanically isolated by using a blender and separating them through centrifugation (Patel et al., 1994). Many of the same factors are important in the success of isolated microspore culture as those in anther culture. These include genotype of parent plants, stage of microspore development, components of the culture medium and incubation conditions (Dunwell, 1985, Luckett and Darvey, 1992). There are advantages in the use of isolated microspore culture over anther culture in that it avoids the effect of anther tissue and competitive effects within the anther (Pickering and Devaux, 1992). Further advantages are in the potential regeneration of larger numbers of haploid plants without the interference of somatic callus (Pickering and Devaux, 1992, Darvey, 1998a). Culture of pollen microspores is not as efficient as anther culture procedures because of the low yield of green pollen plants (Pickering and Devaux, 1992). In addition to this, isolated microspore culture of wheat pollen has proved to be more difficult than of other cereals (Gustafson et al., 1995).

It is possible to recover haploid plants by culturing maternally derived haploid tissue. Successful culture of ovaries in cereal haploid production involves an estimate of the stage of development of the embryo sac by reference to the stage of pollen development (Dunwell, 1985). The optimal stage of development of the embryo sac is when it is almost mature which corresponds to microspores having two nuclei in pollen grains (Pickering and Devaux, 1992). Other factors important in the successful culture of ovaries include cold pretreatment, components of the culture medium and dorsal orientation of the ovaries on the medium (Pickering and Devaux, 1992). Procedures for ovary culture are complex and numbers of regenerants produced are low. For these reasons its use has been limited in cereal breeding (Mejza et al., 1993).
**Fig. 1.4:** Protocols for wheat anther culture and microspore culture (adapted from Luckett and Darvey, 1992).

1.5.2: **Interspecific crosses**

1.5.2.1: *Hordeum vulgare x Hordeum bulbosum*

*Hordeum bulbosum* is a perennial outcrossing species found in the Mediterranean region and thought to be closely related to *H. vulgare* (Pickering and Devaux, 1992). Hybrids between *H. vulgare* and *H. bulbosum* have been successfully produced since Kuckuck first reported obtaining a plant from the cross in 1934 (Pickering and Devaux, 1992). The intention of making the cross was to introgress desirable genes, such as disease resistance and winter hardiness, from the wild species (*H. bulbosum*) into barley (*H. vulgare*). Later, Kasha and Kao (1970) reported that in the cross, elimination of the *H. bulbosum* (pollen donor) chromosomes took place after fertilisation and resulted in haploid barley embryo formation. Subsequently, this cross has been used in barley breeding programmes to produce DH lines from early generation breeding material (Coles, 1986, Smart, 1990). Haploid production efficiency with this method is determined by a number of factors and their complex interactions, some of which will be discussed.

The success of the *H. vulgare x H. bulbosum* cross varies with the maternal genotype (*H.*
vulgare) used, but not with the genotype of pollen used (Pickering and Rennie, 1990). Chromosomes are not immediately eliminated in H. vulgare x H. bulbosum crosses and temperature has been shown to affect the rate of chromosome elimination (Pickering, 1985). The rate of elimination of the H. bulbosum genome increases at temperatures above 20 °C (more haploids, fewer hybrids), while chromosomes are more likely to be retained at temperatures below 17.5 °C (more hybrids, fewer haploids) (Pickering, 1985).

Seed set from the H. vulgare x H. bulbosum cross is usually very high in crossable genotypes (approximately 80%) (Chen and Hayes, 1991). However, reduced seed set occurs in some H. vulgare genotypes crossed with H. bulbosum (Pickering and Devaux, 1992). Pickering (1983) found in cultivar ‘Vada’ that poor seed setting character was controlled by a single dominant gene located on chromosome seven. Although pollen tubes successfully penetrated the stigma branches in this cross, during subsequent growth the pollen tube tips inflamed and burst and were arrested in the stylar transmitting tract (Pickering, 1981). Incompatibility responses in different genotypes are not necessarily controlled by the same factors, for example, incompatibility in cultivar ‘Agneta’ was found to be strongly influenced by environmental factors and not dominantly inherited (Bjørnstad, 1986). The problem of incompatibility has been overcome in H. vulgare x H. bulbosum crosses to some extent, by screening and selecting among different H. bulbosum genotypes (Pickering, 1980).

The application of PGRs, has been the major advance in improving longevity of developing seeds of H. vulgare x H. bulbosum crosses (Pickering and Devaux, 1992). At the time of embryo rescue (about 2 weeks after pollination), caryopses are small, shrunken and beginning to turn yellow. A pre-pollination (Chen and Hayes, 1991) or post-pollination (Pickering and Wallace, 1994) spray with gibberellic acid (GA₃) and 2,4-dichlorophenoxyacetic acid (2,4-D) has been shown to stimulate embryo growth in most crosses, increasing the efficiency of haploid production.

1.5.2.2: Wheat x H. bulbosum

Chromosome elimination and the production of haploids from the wheat x H. bulbosum cross was first observed by Barclay in 1975 (Sitch and Snape, 1987a). The use of this
cross to produce DHs has limited value for the majority of wheat genotypes, due to the action of Kr genes (Inagaki and Snape, 1982, Inagaki, 1986, Sitch and Snape, 1987b). The Kr genes of wheat regulate its crossability with relatives and other grass species. They act by preventing pollen tube penetration of the ovary wall, severely limiting the frequency of fertilisation. The frequency of fertilisation in crossable wheat and H. bulbosum combinations has been improved by the application of GA$_3$, 10 min after pollination (Sitch and Snape, 1987b). Temperature has also been shown to influence the success of the wheat x H. bulbosum cross in crossable genotypes (Sitch and Snape, 1987b). It is thought that higher temperatures increase the rate of cell division and stimulate rapid chromosome elimination, allowing a stable chromosome number to be reached earlier (Sitch and Snape, 1987b). However, manipulation of PGR treatments and environmental temperature was found to have little effect on the fertilisation frequencies of non-crossable wheat genotypes (Sitch and Snape, 1987b).

1.5.2.3: Wheat x maize

Maize pollen was first found to germinate on wheat stigmas and achieve fertilisation in 1984 (Zenkteler and Nitsche, 1984). The first haploid plants were produced from a wheat and maize cross in 1986 (Laurie and Bennett, 1986) and as yet, there are no wheat varieties which have been found to be incompatible with maize pollen. Since wheat and maize were found to cross, many wide crosses have been made successfully. These include crossing wheat with pearl millet (Ahmad and Comeau, 1990, Laurie, 1989a), sorghum (Laurie and Bennett, 1988a, Ohkawa et al., 1992) and teosinte (Ushiyama et al., 1991) as well as crossing maize with barley (Laurie et al., 1990) and rye (Laurie et al., 1990).

Laurie and Bennett (1990) investigated the fate of maize pollen after wheat x maize crosses were made. They found that in 80% of florets, pollen germinated on the stigma and one or more pollen tubes reached the embryo sac. In about 2% of florets the maize pollen tube severely damaged the wheat gametes, whilst in 34% the pollen tube entered the embryo sac but failed to release the sperm nuclei. In 16% the sperm nuclei were released but failed to fuse with the wheat gametes. Fertilisation occurred in the remaining 28% of florets. This is a remarkably high frequency in view of the taxonomic distance between the two species. However, in florets where fertilisation had occurred it was relatively rare to find both an embryo and an endosperm.
Low embryo viability is the major cause of low haploid production in wheat x maize crosses. The treatment of florets post-pollination with PGRs has been shown to increase the number of viable haploid embryos. However, unlike the *H. vulgare* x *H. bulbosum* cross, the application of GA<sub>3</sub> pre or post-pollination does not have an enhancement effect, rather, its application is detrimental in the production of haploid embryos (Laurie and Bennett, 1989a). Suenaga and Nakajima (1989) found that a post-pollination injection of 2,4-D into wheat internodes increased the number of viable embryos produced. However, if the 2,4-D was applied pre-pollination, no increase in the number of viable embryos occurred. It has now been well established that post-pollination application of synthetic auxins, such as 2,4-D and Dicamba, is beneficial in increasing the numbers of viable embryos (Guo *et al*., 1994, Pienaar and Lesch, 1994). The inclusion of silver nitrate (an inhibitor of ethylene action) with the auxin treatment, increased embryo survival in some genotype crosses of durum wheat x maize (O'Donoughue and Bennett, 1994, Inagaki *et al*., 1998a). Auxins can promote ethylene production (Taiz and Zeiger, 1998) and it has been postulated that the ethylene produced in response to exogenously applied auxins decreased with the inclusion of silver nitrate, resulting in increased embryo survival (O'Donoughue and Bennett, 1994).

In contrast to other interspecific cereal crosses, the effect of temperature on embryo recovery in wheat x maize crosses has not been explored. However, differences have been reported in seed survival between spring, autumn and summer experiments (Pienaar and Lesch, 1994). As well, O’Donoughue and Bennett (1994) showed that the environment influenced durum wheat embryo survival in a genotypically dependent manner.

1.5.2.4: Chromosome doubling

Haploid plants are infertile as sexual fertility depends upon meiotic division of the diploid chromosome number. To regain fertility, the number of wheat chromosomes need to be doubled following haploid embryo rescue and seedling formation. To do this, haploid plants are usually treated with antimitotic agent, colchicine. Seedlings are cleaned and placed in aerated colchicine (500 mgL<sup>-1</sup> colchicine, 10% DMSO) for five hours. The treated seedlings are then washed in tap water and transferred to pots containing fresh potting mix. This procedure is generally very successful, with only a few low vigour plants dying. Suenaga (1994) reported a 97.3% doubling efficiency using colchicine on
haploid wheat plants.

Colchicine is an extremely toxic chemical and other doubling agents are currently being tested as colchicine replacements (Snape, 1998). Hassawi and Liang (1991) evaluated three antimitotic agents at two different duration times (colchicine, trifluralin and oryzalin) for their effects on the production of doubled haploids from anther-derived calli of wheat cultivars. Of the three, they found colchicine was the most effective in doubling chromosome number. No significant differences were observed between concentrations of any of the antimitotic agents. Attempts are also being made to double chromosome numbers in vitro by the inclusion of colchicine for one day in the initial embryo rescue medium (Darvey, 1998b).

1.5.3: The use of doubled haploids in genetical studies

Genotypes and genetic ratios of DH populations are equivalent to the ratios found in gametes. Because of this there are several advantages in using DH populations over conventionally used F2 populations for genetical studies (Pauls, 1996).

i) DH populations have fewer genotypic classes and larger differences between the classes than F2 populations. Therefore, much smaller DH populations are required to observe all possible phenotypes from a cross (Fig. 1.5). This is particularly useful in mapping studies.

ii) Traits which are conveyed by recessive alleles are not masked by dominant alleles.

iii) As DHs are fixed, they can be used for analysis in following generations without losing gene combinations of interest (Hu, 1996, Pauls, 1996).
In barley genetics, DH populations have been used to locate genes to major linkage groups (Thomas et al., 1984), to examine the effects of linkage and pleiotropy on traits (Bjørnstad, 1987, Bjørnstad and Aastveit, 1990, Kjær et al., 1990) and to study the theoretical effects of particular genes on quantitatively inherited characters (Snape et al., 1984). Studies such as these show the potential for the use of DH populations in similar studies of wheat genetics.

1.5.4: Agronomic performance of doubled haploid lines

To be used in a breeding programme, DH plants have to be genetically stable with no aberrant genetic variation arising during the process. Therefore, it is important to determine if any genetic variation is introduced during the production of DH lines. Snape et al. (1988) tested barley and wheat DH populations (produced through H. vulgare crosses) for the presence of introduced genetic variation. In barley, they found no variation between DHs, produced from pure lines, for a range of quantitative characters, suggesting an absence of any introduced variation. In wheat, aberrant variation was detected for ear emergence time, plant height and yield. However, the original self-seeded wheat stocks were shown to contain allelic variation for some of the characters investigated. The type and range of variation was similar to that reported from studies of
somaclonal variation from immature embryos and gametoclonal variation from anther culture. It was suggested that the colchicine treatment could be responsible for the variation exhibited in some plants (Snape et al., 1988).

Similarly, Laurie and Snape (1990) assessed the agronomic performance of wheat doubled haploid lines derived from wheat x maize crosses. They compared the performance of various DH lines of Chinese Spring, Hope, and lines of a single chromosome substitution of Chinese Spring and their respective parents under field conditions. No significant variation was detected in either population of Chinese Spring DH lines and neither population differed significantly from its parent. The Hope DH lines differed significantly for tiller biomass, spikelet number per ear, ear grain weight and 50-grain weight. These lines also showed significant variation in ear emergence time, but it was thought that this was possibly due to genetic heterogeneity in the parent stock.

As mentioned earlier, one of the main disadvantages of anther culture in producing wheat DHs is that aberrant genetic variation can be introduced during the callus stage. Of the fertile regenerated plants produced through anther culture 30% have been shown to be cytogenetically abnormal in the number and structure of their chromosomes (de Buyser et al., 1985). In a comparative study of the production of barley DHs by the H. bulbosum and anther culture methods, significant differences were shown in the two groups of DH plants and the control line 'Berenice' (San and Demarly, 1984). It was found that those plants regenerated from interspecific crosses and embryo culture were genetically closer to Berenice than those regenerated from anther culture. As well, Kisana et al. (1993), in comparing wheat DHs produced through anther culture and wheat x maize crosses, found that anther-derived plants were cytologically unstable, whereas all the plants regenerated from wheat x maize crosses were stable. In contrast, in other experiments, where the extent of variation from intergeneric cross methods and anther culture methods were compared, there were no significant differences in agronomic characters between the methods of DH production (Henry et al., 1988, Bjørnstad et al., 1993b).
1.5.5: Integrating doubled haploids into wheat breeding programmes

In October 1985, the first wheat DH cultivar derived through anther culture, ‘Florin’, was released. Florin was obtained from only 64 green plants produced from an F1 population (de Buyser et al., 1987). The Chinese cultivar ‘Jinghua’ was also released as a product of anther culture from a three-way cross in 1986 and was selected from only 46 plantlets (Picard et al., 1990). Both these cultivars had superior characteristics compared with those of their parents. Results such as these indicate that superior DH lines can be produced from very small populations. However, many other studies show that a more detailed analysis of both the filial generation from which DHs are made and the size of the DH population produced, are important for successful implementation in cereal breeding programmes (Snape and Simpson, 1981, Snape et al., 1986b, Yonezawa et al., 1987, Iyamabo and Hayes, 1995, Howes et al., 1998).

1.5.5.1: Filial Generation from which doubled haploids are made

In barley breeding, most of the DHs used originate from an F1 cross (Snape and Simpson, 1981, Choo et al., 1985). It has been shown that if homozygosity is induced at this early stage, there is limited opportunity for recombination events which create potentially useful genetic variation for breeders. Snape and Simpson (1981) examined the theoretical and practical effects of linkage on traits of DH lines derived from F1, F2, F3, and intermated F2 (S3) generations. In their study, they found increased variation in later generations for spike emergence time, height, grain number/spike, and spikes/plant. They surmised that a significant gain in the genetic variation of these traits could arise from delaying the production of DHs to the F2 generation, but there was little to be gained from delaying it further to the F3 generation. However two traits, grain weight/plant and 250 grain weight, had decreased variation in later generations, showing that there is a risk element involved and that delaying an extra generation may break down desirable coupling linkages. Thus, it would seem the optimal filial generation for producing DHs is the F2 generation. This allows for the breakup of repulsion linkages and creates increased opportunity to generate new allelic configurations at unlinked loci (Snape and Simpson, 1981, Choo et al., 1985, Patel et al., 1985, Yonezawa et al., 1987). In contrast, Iyamabo and Hayes (1995) quantified the effects of an additional round of recombination when comparing F1 and F2 derived barley DH lines and showed the additional round of recombination did not lead to
large performance differences between the two populations. Studies such as this have not been reported with wheat DHs populations, but the complexity of the wheat genome compared with diploid barley may make the effects of factors such as epistasis more pronounced.

1.5.5.2: Doubled haploid population size

As mentioned earlier, the population size the French cultivar ‘Florin’ was selected from (64 plants) was small when compared with the size of the F2 population from which a breeder would normally select (2000-3000 plants). It would seem unlikely that the genetic variation derived from a population of 64 would be enough from which to select a superior line, especially as those lines were made from the F1 generation. Jansen (1992) examined theoretically the problem of how to retain all desired genotypes by one or more plants in a population of F1 derived DHs. Minimum population sizes increased according to how many unlinked loci were to be fixed, for example, to be sure ($p<0.05$) of fixing two unlinked loci, 16 DHs would be needed. To obtain five unlinked loci, a population of 203 would be needed. In the presence of linkage, minimum population sizes would have to increase (Yonezawa et al., 1987 Jansen, 1992) or alternatively, the production of DHs could be delayed a generation.

1.5.5.3: Comparison of doubled haploid technology to conventional breeding methods

Few studies have been made to compare DH derived lines directly to conventionally derived lines. Patel et al. (1985) showed that conventionally derived F4 barley lines were superior to F1-derived DH lines and attributed this to additional rounds of recombination. In comparing the DH method to the bulk method of breeding barley, Song et al. (1978) found DH populations had a lower average yield, but more variation between lines and that the two methods were equally effective in terms of the average yield of the ten best lines. In a computer simulation study the success of DH methods, compared with pedigree methods, was dependent on the size of the F2 population (Walsh, 1974). Another study showed that pedigree selection in barley breeding was more effective in increasing the frequency of superior genotypes for characters with high or moderate heritability, but becomes less effective for characters with low heritability (Choo et al., 1985). Yonezawa et al. (1987) established in a simulation study that DH breeding was superior or equal to conventional breeding, when certain conditions were met. These were: i) a relatively small
number of loci (10 or less) are involved with the breeding objective concerned, ii) desirable genes are recessive to undesirable ones at most of the segregating loci and iii) the genes are not strongly linked.

Few such studies have been reported for wheat breeding. In one study the best lines produced through DH technology-anther culture (three lines), bulk breeding methods (seven lines), pedigree breeding methods (six lines) and SSD (two lines) were compared (Picard et al., 1990). It was found that the means of the DH lines were not significantly different to the bulk and pedigree bred lines. The SSD lines were found to be significantly lower yielding than other lines. In recent studies 65-97 DH lines (produced by wide-crossing) and 110 SSD lines were compared to pedigree selected plants at the F₆ generation which were produced from an original F₂ population of 1500 plants (Inagaki, 1998, Inagaki et al., 1998b). It was found that when the parental varieties were closely related in their pedigrees, no significant differences in grain yield were found among any of the populations. In two crosses with low coefficients of parentage and large progeny variation, grain yield of selected DH lines was significantly lower than grain yield of SSD and pedigree selected lines (Inagaki, 1998, Inagaki et al., 1998b).

Practical studies such as these are difficult to undertake as residual heterozygosity, selection or dominance can affect the performance of conventionally bred lines (Bjornstad, 1987). This is especially the case if DH lines are compared with early generation conventionally bred lines. DH lines need to be compared with conventionally bred homozygous lines, rather than lines still heterozygous, to enable a true comparison. However, this would take a number of generations and during this time the selection criterion of the breeder may change.

Fixing homozygosity early in a breeding programme is complex and different breeding strategies will be needed to make maximum use of DH technologies, according to the particular goal of the breeder. As Jansen (1992) showed, the minimum DH population size required to fix unlinked loci increased with the number of loci. This has important implications for the breeding of quantitative traits, of which many would have more than five loci involved. To select superior plants for these traits DH populations would regularly need to exceed 250 DH lines from an F₁ population, a tall order if required for all
crosses in a breeding programme. The integration of DHs with MAS in breeding programmes is being examined increasingly as an area in which both techniques could be used with maximum efficiency (Howes et al., 1998). Overall, if a breeder has clear objectives from a cross, the potential for the use of DH technology in combination with other breeding strategies, is very attractive because of the time reduction in the production of new and improved commercial cultivars.

1.6: Limitations of current wheat breeding and genetics programmes

The New Zealand wheat breeding programme has similar limitations to other wheat breeding programmes throughout the world, in particular, low heritability of economically important traits, the high cost of some phenotype trait selection and the long time-frame of conventional breeding (Brennan et al., 1996). As mentioned earlier, current advances in molecular biology and tissue culture of wheat are making important contributions in overcoming some of these disadvantages. The development of the genetic map of wheat will allow precise methods of genome analysis through the location and manipulation of major genes and QTL controlling important characters. Genetic mapping of wheat quality traits has just been started for New Zealand wheat cultivars (Ahmad and Griffin, 1998). As well, future improvements in genetic engineering technologies may provide the basis for introducing novel traits into wheat.

To retain competitive advantage and produce new wheat cultivars with more efficiency, DHs must become a step in New Zealand wheat breeding programmes. Of the techniques currently used to produce wheat DHs, the wheat x maize cross has the most potential, largely because of the lack of any genotypic constraints. For this thesis it was decided that the initial experimentation would be to examine and establish the wheat x maize method of producing DHs with New Zealand cultivars. Once this was established the next step would be to determine some of the plant and environmental constraints on the methodology and to determine the genetic stability of wheat DHs. The final step would be to investigate at what stage in a wheat breeding programme DHs could be used. With these steps in mind, the following goal and objectives were formulated. The scope and design of this thesis is illustrated in figure 1.6.
1.7: Goal and objectives

1.7.1: Goal

To increase the efficiency of the development of genetically improved New Zealand wheat cultivars by inducing direct homozygosity through wheat x maize crosses.

1.7.2: Objectives

- To develop a method of wheat DH production through wheat x maize crosses, using New Zealand germplasm.

- To determine optimal environmental conditions for the production of wheat DHs through wheat x maize crosses

- To evaluate whether wheat DHs produced through the wheat x maize method are ‘normal’ and genetically stable.

- To determine where DHs could be used for maximum genetic gain in a wheat breeding programme.
The genetic improvement of New Zealand wheat by inducing direct homozygosity through wheat x maize crosses

Development of a wheat x maize method for DH production:

- Use of a range of New Zealand wheat and maize genotypes in differing seasonal conditions.
- Problems encountered with the method are highlighted, in particular, the influence of seasonal factors on haploid embryo recovery.

The influence of environmental factors on wheat maize crosses

- The effect of temperature and light intensity on haploid embryo recovery is examined
- The effect of light intensity on fertilisation and embryos survival is examined

Genetic stability of DH lines evaluated

- Genetic variation of doubled haploid lines made from conventionally bred homozygous parents is compared to the variation seen in the parent population in a field trial

Integration of DHs and MAS into wheat breeding programme

- Model developed and partly tested whereby DH and MAS are integrated into a breeding programme

General thesis discussion and conclusions

Fig. 1.6: A diagram of how each of the thesis objectives relates to the overall aim of the thesis.
2: Production of wheat doubled haploids, via wide crosses, in New Zealand wheat

2.1 Introduction

The current aims of New Zealand wheat breeding are to improve the quality, sustainability and productivity of New Zealand wheats for both domestic and export markets (Griffin et al., 1995). The development of a new homozygous wheat cultivar in New Zealand can take up to 12 years. In other countries the time to reach homozygosity has been reduced considerably through the production of doubled haploids (DHs) (Laurie and Bennett, 1988b, Islam and Shepherd, 1994, Pienaar and Lesch, 1994). The use of DH technology has potential for improving the efficiency of New Zealand wheat breeding programmes and enhancing genetical studies.

Various techniques to produce DHs have been used in wheat, including: anther and microspore culture and the use of intergeneric crosses (Darvey 1994, Patel et al., 1994 and Laurie and Bennett, 1986). Anther culture has been successfully used to produce superior wheat cultivars (de Buyser et al., 1987), but when it was examined using New Zealand wheat cultivars major limitations were found (Cooper and Griffin, 1988). These included: genotypic specificity, low haploid recovery and relatively high rates of albinism. Finally, due to a requirement for callogenesis, gametoclonal variation may occur which is detrimental to the production of homozygous lines (Kisana et al., 1993, Baenziger et al., 1989a, Ekiz and Konzak, 1994).

The production of wheat DHs through wheat x maize crosses is widely used (Laurie and Bennett, 1988b, Pienaar and Lesch, 1994, Islam and Shepherd, 1994). Following fertilisation, maize chromosomes are almost immediately eliminated (Laurie and Bennett, 1989b). Due to the absence of adequate endosperm development, spikes are treated with plant growth regulators (PGRs) which stimulate embryo growth to a stage where they can be rescued on to nutrient medium (12-16 days post-pollination). The resulting haploid seedlings are then treated with colchicine to restore their diploid chromosome number. The final products are anticipated to be fertile “true-breeding” wheat plants.

The main disadvantages of using the wheat x maize system are the low ratio of
embryo/embryoless caryopses produced and the loss of embryo viability. To counter this, research has focussed on the manipulation of PGR treatments and embryo rescue conditions to optimise the efficiency of the technique (Laurie and Bennett 1988b, Suenaga and Nakajima, 1989, Laurie 1989b, Guo et al., 1994, O'Donoughue and Bennett 1994, Pienaar and Lesch 1994).

The aim of this chapter was to establish a wheat x maize method, suitable for New Zealand wheat genotypes and the New Zealand climate, which can be used routinely in a breeding and/or genetics programme.
2.2: Materials and methods

2.2.1: Crossing procedure

Wheat plants were grown in soil mix in 15 cm x 15 cm PB5 Planta bags (five plants per bag) and maize plants were grown in 20 cm x 18 cm plastic containers (one plant per container). The soil mix consisted of three parts shredded bark (Pinus radiata): two parts sand (washed crusher dust), supplemented with 6.4 kgm⁻³ dolomite lime and 3.32 kgm⁻² “Osmocote Plus” (Scotts). During warmer months (November-March) plants were also supplemented with liquid fertiliser “Nitrophoska” one to two times a week. Plants were grown in a glasshouse (heating below 15 °C and ventilation above 22 °C, daylength supplemented to 16 hr when needed with sodium and mercury lights, relative humidity maintained > 60%).

In all experiments wheat spikes were emasculated 1-2 days before anthesis and pollinated using a small paintbrush on the approximate day of anthesis. Five New Zealand wheat cultivars (Otane, Rata, Belfield, Kotuku and Monad) were used in most experiments, with two F₁ wheat hybrids (Rata x Monad and Rata x Otane) used in one experiment. All five cultivars have completely unrelated pedigrees (Table 2.1) and represent a typical selection of genotypes common within the New Zealand bread wheat breeding programme. The experimental unit was considered to be one spike which was usually from the leading tiller of separate plants. A minimum of three spikes (~60-90 florets) was pollinated for each treatment. Crosses were made in all four seasons (winter, spring, summer and autumn) over two years (1995 and 1996). Various maize genotypes (Crop & Food Research, Palmerston North, New Zealand-NZ71, NZ21, A82.2, Leslie Research Centre, Toowoomba, Australia-11775, 11772) and a pearl millet genotype (National Agriculture Research Centre, Yatabe, Japan- NEC7006) were crossed as pollen parents with the five New Zealand wheat cultivars.
Table 2.1: Information on the five wheat cultivars used in experiments

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Year</th>
<th>Origin</th>
<th>Pedigree</th>
<th>Agronomic type</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otane</td>
<td>1984</td>
<td>Mexico, CYMMIT</td>
<td>Tob’s’/Npo//No66/Er a/3/Bd/Gallo</td>
<td>sp;i;a</td>
<td>r;sh;b</td>
</tr>
<tr>
<td>Monad</td>
<td>1991</td>
<td>NZ, Wrightson</td>
<td>Unknown</td>
<td>sp;i;a</td>
<td>r;sg;b</td>
</tr>
<tr>
<td>Kotuku</td>
<td>1993</td>
<td>England, CFR</td>
<td>SW1031.13.2/Sicco</td>
<td>f;i;a</td>
<td>wh;sh;b</td>
</tr>
<tr>
<td>Belfield</td>
<td>1994</td>
<td>The Netherlands, PGG</td>
<td>Kadett///19151///High bury/Kadett</td>
<td>f;i;sa</td>
<td>r;sh;b</td>
</tr>
<tr>
<td>Rata</td>
<td>1995</td>
<td>NZ, CFR</td>
<td>Oroua/Olympic</td>
<td>f;i;a</td>
<td>r;sh;b</td>
</tr>
</tbody>
</table>

Note: Agronomic type: f=facultative, sp=spring, i=intermediate (between 75 cm and 100 cm), s=short (less than 75 cm), sa=semi awned, a=awned. Quality: wh=white grain, r=red grain, p=purple grain, sh=semi-hard grain, sg=soft grain, b=bread baking.

Spikes were treated with PGRs (2,4-D ± Dicamba), one to two days after pollination, at different concentrations (Table 2.3). Two methods of PGR application were used. The first involved injection of the PGR solution (~1-3 mL) at the first internode, with Vaseline applied to prevent leakage, followed by a drop (~250 µL) of solution being placed into each of the floret cups (adapted from Pienaar and Lesch, 1994 and O’Donoughue and Bennett, 1994). The second method was to spray the PGR solution onto wheat spikes, until run-off, one day after pollination (Table 2.2).
Table 2.2: Concentration and application of plant growth regulator solution 1-2 days after pollination

<table>
<thead>
<tr>
<th>PGR Concentration</th>
<th>Method of Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 mgL(^{-1}) 2,4-D, 38 mgL(^{-1}) Dicamba</td>
<td>Injection</td>
</tr>
<tr>
<td>20 mgL(^{-1}) 2,4-D and 40 mgL(^{-1}) Dicamba</td>
<td>Injection</td>
</tr>
<tr>
<td>10 mgL(^{-1}) 2,4-D and 20 mgL(^{-1}) Dicamba</td>
<td>Injection</td>
</tr>
<tr>
<td>100 mgL(^{-1}) 2,4-D</td>
<td>Spray</td>
</tr>
</tbody>
</table>

2.2.2: Haploid embryo rescue

Seeds were dissected 13-16 days after pollination and developing embryos were rescued onto one of three artificial nutrient media.

1) \(\frac{1}{2}\) MS salts (Murashige and Skoog, 1962) supplemented with 20 gL\(^{-1}\) sucrose and 7 gL\(^{-1}\) agar.
2) \(\frac{1}{2}\) MS salts supplemented with 20 gL\(^{-1}\) sucrose and 12 gL\(^{-1}\) agar.
3) B5 salts (Gamborg et al., 1968) supplemented with 20 gL\(^{-1}\) sucrose and 12 gL\(^{-1}\) agar.

Embryos were incubated at 20 to 24 °C (in the dark) until germination and then transferred to light (65 μmol m\(^{-2}\) s\(^{-1}\), photosynthetically active radiation (P.A.R.)), provided with cool white fluorescent lamps, at a 16-hour photoperiod. Haploid plants were transplanted to soil when they were 20-40 mm in height.

2.2.3: Chromosome doubling procedure

Haploid plants were removed from soil mix (see section 2.2.1) when they had produced 3-4 tillers. Plants were then washed and roots and shoots were trimmed. Incisions were made at each of the stem bases using a scalpel blade. The plants were then placed in an aerated colchicine and dimethyl sulphoxide (DMSO) solution (500 mgL\(^{-1}\) colchicine, 10% DMSO) for five hours, at 30 °C, to double chromosome numbers. The treated seedlings were then washed for 5 min in tap water and transferred to pots containing fresh potting mix.
2.2.4: Scanning electron microscope (SEM) procedure

Wheat x maize and wheat x wheat embryos were snap frozen in liquid nitrogen and sputter coated with gold for 5 min at 1.2 kV and 50 mA. Images were viewed on a Leica S440 SEM and photographs taken at 20 KeV with a probe current of 80 pA.

2.2.5: Statistical analysis

All data in this chapter were analysed using Minitab© version ten. ANOVA was used to examine treatment means. All variables were considered to be fixed effects.
2.3: Results and discussion

Doubled haploid plants were obtained successfully from all five cultivars and both F₁ crosses using maize and pearl millet pollen sources. This is consistent with results from other studies which have shown that maize is relatively insensitive to the action of *Kr₁* and *Kr₂* alleles in wheat (Laurie and Bennett, 1987) which act by inhibiting alien pollen tube growth in both wheat × *Hordeum bulbosum* and wheat × rye crosses (Sitch and Snape, 1987b, Laurie 1989b).

2.3.1: Wheat cultivar effects on haploid embryo recovery

In spring, crosses among the five wheat cultivars (Belfield, Otane, Rata, Monad and Kotuku) and three maize lines (11775, A82.2 and NZ71), the PGR solution (75 mgL⁻¹ 2,4-D and 38 mgL⁻¹ Dicamba) was applied by injection. No significant differences were observed in the frequency of haploid embryo recovery between different wheat cultivars (Fig. 2.1). This confirms previous studies showing no differences in haploid embryo numbers among wheat cultivars (Suenaga and Nakajima, 1989, Matzk and Mahn, 1994, Zhang *et al.*, 1996). However, differences in numbers of haploid embryos recovered have been noted among wheat varieties in some other studies (Lefebvre and Devaux, 1996, Morshed *et al.*, 1996). Matzk and Mahn (1994) suggested variation seen in the other studies may be due to environmental influences rather than genotypic effects.
**Fig. 2.1:** The effect of wheat genotype on haploid wheat embryos recovered 13-15 days after pollination with maize (11775). Graphed values are the means for 21-39 spikes, vertical lines represent standard error of the mean. ANOVA probability level: non-significant ($p > 0.05$).

### 2.3.2: Maize pollen effects on haploid embryo recovery

There were no significant differences in the number of haploid embryos recovered or in haploid embryo germination when pollen from five maize sources were compared (Fig. 2.2).
Fig. 2.2: The effect of maize pollen genotype on haploid wheat embryos recovered 13-15 days after pollination. Graphed values are the means for 15-42 spikes (wheat genotypes are pooled due to non-significance), vertical lines represent standard error of the mean. ANOVA probability level: non-significant ($p > 0.05$), maize x wheat interaction non-significant ($p < 0.05$).

Although haploid embryo recovery and germination were not significantly affected by the maize pollen used, we now routinely use Yates “Early Miracle” sweet corn as the pollen donor for all wheat x maize crosses, as it is a cultivar well suited to local growing conditions and grows to an ideal height in the glasshouse. However, Morshedi and Darvey (1995) showed that there were significant differences in the success of haploid recovery and haploid embryo germination with different pollen sources. Zhang et al. (1996) also showed
a maize genotypic effect in a comparison of pollen sources. One maize pollen source (Rp1-A-mutator) produced haploid embryos at an average frequency of 16% (per floret pollinated) compared to another pollen source (Rp1-D-mutator) which produced embryos at an average frequency of only 2.4% (per floret pollinated).

When pearl millet was used as a pollen donor for 10 wheat spikes, haploid embryo recovery was lower (4% of florets pollinated) than when maize pollen was used (25% of florets pollinated). The same pearl millet genotype has been used successfully as a pollen donor in other studies, producing wheat haploid embryos in 27.6% of pollinated florets (Inagaki and Mujeeb-Kazi, 1996). The low haploid embryo recovery in our study may have been due to either the growing conditions for pearl millet or wheat genotype effects.

2.3.3: Seasonal effects on haploid embryo recovery

A total of 71 Rata x Monad and 113 Rata x Otane spikes were crossed with maize pollen between January 1996 and June 1996. A significant variation in haploid embryo numbers per spike was observed over the six month period. When the results of these crosses were grouped into seasons: summer (January and February), autumn (March, April and May) and winter (June), a marked influence of season was apparent (Fig. 2.3). During the following year (1997) the range of temperatures and mean temperatures (taken at 3:00 pm daily) of the middle month of each season were compared. This comparison showed substantial temperature differences between seasons (Table 2.4) which may account for some of the variation in haploid embryo numbers. Factors such as light quality and light intensity may also have had an impact.
Table 2.3: Seasonal temperature ranges and means recorded in the glasshouse during 1997

<table>
<thead>
<tr>
<th>Temperature range</th>
<th>Mean temperature (3:00pm) ± std. dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer (January)</td>
<td>14.5 - 30.5 25.5 ± 3.5</td>
</tr>
<tr>
<td>Autumn (April)</td>
<td>14.5 - 23.0 21.0 ± 1</td>
</tr>
<tr>
<td>Winter (July)</td>
<td>13.0 - 20.0 18.5 ± 1</td>
</tr>
<tr>
<td>Spring (October)</td>
<td>13.5 - 28 22.5 ± 1</td>
</tr>
</tbody>
</table>

Note: Std. Dev. = standard deviation of the mean.

Fig.2.3: The effect of season on haploid wheat embryos recovered 13-15 days after pollination with maize. Graphed values are the means for 12-72 spikes, vertical lines represent standard error of the mean. ANOVA probability level: highly significant ($p < 0.001$).
There are many possible reasons for the influence of the season on haploid embryo recovery which has also been noted in other studies (Matzk and Mahn, 1994, Pienaar and Lesch, 1994, Pienaar and Lesch, 1995, Pienaar et al., 1996). Viability of maize pollen grains can be influenced by the environment in which the parent plants are grown (Barnbás and Rajki, 1976, Neuffer, 1982). Seasonal variation in the glasshouse therefore, could have had an effect on pollen viability and subsequent wheat fertilisation. O'Donoughue and Bennett (1994) showed environmental influence on durum wheat embryo survival in a genotypically dependent manner. They established that embryo recovery from the cultivar "Rampton Rivet" was significantly better in a 20 °C growth room than in an unheated glasshouse, whereas cultivars "Wakona" and "Chinese Spring" were unaffected. This would indicate that seasonal effects seen in this study may also be a result of environmental differences influencing the wheat plant.

2.3.4 Haploid embryo germination

Although no significant difference in haploid embryo numbers was detected among wheat cultivars (section 2.3.1), a significant difference in the number of embryos which germinated successfully to produce haploid seedlings was found among different wheat cultivars (Fig. 2.4).
Fig. 2.4: The effect of wheat genotype on wheat haploid embryo germination in wheat x maize crosses. Graphed values represent the results for a minimum of 20 embryos, vertical lines represent standard error of the mean. ANOVA probability level: highly significant ($p < 0.01$).

Belfield and Kotuku had particularly low germination rates ($< 20\%$ of all embryos). 2,4-D is beneficial to wheat x maize crosses in stimulating embryo development and increasing embryo size (Zhang et al., 1996), in tissue culture however, synthetic auxins are added to promote callogenesis. At the time of dissection Belfield embryos showed signs of callus initiation and looked distorted (Fig. 2.5c and Fig. 2.5d). It is possible that Belfield had increased sensitivity to the added synthetic auxins and this may have contributed to poor embryo germination. Therefore, the applied auxin concentration for Belfield embryos was
decreased from 75 mgL$^{-1}$ 2,4-D and 40 mgL$^{-1}$ Dicamba, to 20 mgL$^{-1}$ 2,4-D and 40 mgL$^{-1}$ Dicamba. This had no effect in improving Belfield embryo germination. When the auxin concentration was further decreased to 10 mgL$^{-1}$ 2,4-D and 20 mgL$^{-1}$ Dicamba, the numbers of Belfield embryos which germinated increased from 15% to 32%.
Fig. 2.5: Scanning electron microscope images of a wheat embryo and haploid embryos produced through wheat x maize crosses. A=Rata wheat embryo 14 days after pollination with wheat, B=Rata haploid wheat embryo 14 days after pollination with maize, C and D=Belfield haploid embryos 14 days after pollination with maize.
The other cultivar which had low germination, Kotuku, had small embryos (<1mm) in the globular stage of development at dissection. Embryos of cultivars which had higher germination rates (Otane, Rata and Monad) were 1-1.5 mm and in the globular-heart transition phase at dissection (Fig. 2.5b). When dissection of Kotuku embryos was delayed (16 days as opposed to 14 days) germination rates improved from 19% to 42%.

Overall, the germination rates of all cultivars were still low (~40% of all embryos). Other researchers have also observed low haploid wheat germination rates in wheat x maize crosses (Inagaki and Tahir, 1990, Lefebvre and Devaux, 1996, Wilson, 1998). Inagaki and Tahir (1990) reported germination rates of 43%, Wilson (1998) reported average germination rates of 48%, ranging from 21-74% in different crosses and Lefebvre and Devaux (1996) reported that 55.7% of haploid embryos failed to establish themselves as plantlets, despite the embryos initially producing enlarged coleoptiles and/or roots. Similarly, embryos which did not germinate in this study appeared to have initiated germination but then became water soaked, brittle and died, a phenomenon known as vitrification or hyperhydricity (Fig. 2.6). Hyperhydricity occurs when leaf lacunae contain water rather than air, resulting in leaves that lack epicuticular wax, have few stomata and become wrinkled (Schloupf et al., 1995). It has been related to explant preparation, explant location in the culture medium, culture medium composition and environmental conditions (Schloupf et al., 1995). In an attempt to improve germination rates, by circumventing the hyperhydricity, the percentage of agar in the tissue culture media was increased from 7 gL⁻¹ to 12 gL⁻¹. Agar was set on a slant so any water resulting from condensation of the media would drain to the bottom of the slant and wheat embryos were placed on the upper middle of the slant. Germination of embryos from two cultivars, Rata and Belfield, were compared on the two agar concentrations. An improvement in the germination frequency of Rata embryos, but not Belfield embryos, occurred (Fig. 2.7).
Fig. 2.6: Belfield haploid embryos 16 days after rescue onto ½ MS nutrient media, supplemented with 0.7 g L\(^{-1}\) agar and 20 g L\(^{-1}\) sucrose. Note: top three embryos have germinated successfully whilst bottom three embryos are showing signs of hyperhydricity.
Fig. 2.7: The effect of agar concentration on the percentage of wheat haploid embryos (produced by wheat x maize crosses) which successfully germinated. Graphed values represent the results for a minimum of 20 embryos.

Given the improvement in numbers of Belfield embryos which germinated at lower PGR concentrations, it was possible that the injection method itself exposed the embryos to auxin for longer than was necessary to promote embryo survival, inhibiting germination. In an attempt to avoid this situation, wheat spikes were sprayed with 100 mg l⁻¹ 2,4-D, which would expose the embryos to the possible detrimental effects of the auxins for a shorter time. Belfield embryos were germinated on 12 gL⁻¹ as above. The use of the auxin spray improved the germination rates of Belfield embryos (from 29% to 76%) but had no significant effect on the germination of Rata embryos (Fig. 2.8).
Fig. 2.8: The effect of method of PGR application percentage of wheat haploid embryos (produced by wheat x maize crosses) which successfully germinated. Graphed values represent the results for a minimum of 20 embryos.

Many previous studies have used media based on ½ MS salts for haploid embryo germination (Inagaki and Mujeeb-Kazi, 1994, Zhang et al., 1996 and Savaskan et al., 1997). However, Morshedi and Darvey (1997) found the use of Gamborg’s B5 salts superior to ½ MS salts. When Rata and Belfield embryos were grown on these two media, no significant differences in germination rate was observed for either cultivar. Kammholz et al. (1996) also found no difference in germination rates of haploid embryos grown on ½ MS media or B5 media although they did find that germination was improved with the addition of cytokinins to the media.
2.3.5: *Colchicine-induced chromosome doubling*

Following colchicine treatment, wheat plants are very susceptible to stress. Initially (during summer) plants were returned to the glasshouse immediately after treatment. The glasshouse conditions at this time of year (Table 2.4) resulted in the death of these plants. Transfer of treated plants to a controlled growth room (day/night temperature of 17/12 °C, light intensity of 250 μmol m⁻² s⁻¹, P.A.R., with a 16 h light photoperiod and a constant humidity of 70 %) for two-three weeks immediately after colchicine treatment increased survival, with ~80% setting seed (Fig. 2.9). At other times of the year colchicine-treated plants survived in the glasshouse to produce seed at a similar rate.
Fig. 2.9: Doubled haploid Otane wheat spike following treatment with colchicine.
2.3.6: Optimised protocol

The combined use of all the optimal factors found to be important in this study (summer environment, days until embryo dissection, 100 mgL\(^{-1}\) 2,4-D spray, ½ MS salts and 12% agar) resulted in improved frequencies of DHs following wheat x maize crosses. The resulting frequencies (Table 2.5) are similar to those reported in previous studies (Inagaki and Tahir, 1990, Amrani et al., 1993, Lefebvre and Devaux, 1996 and Zhang et al., 1996).

Table 2.4: Production of wheat doubled haploids using the modified wheat x maize method

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>No. of spikes</th>
<th>No. of florets</th>
<th>No. of haploid embryos</th>
<th>No. of haploid plants</th>
<th>No. of DHs</th>
<th>DH Plants/spike pollinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otane</td>
<td>9</td>
<td>193</td>
<td>69 (36%)</td>
<td>55 (80%)</td>
<td>43 (78%)</td>
<td>2.4</td>
</tr>
<tr>
<td>Belfield</td>
<td>15</td>
<td>348</td>
<td>82 (24%)</td>
<td>66 (81%)</td>
<td>59 (90%)</td>
<td>1.1</td>
</tr>
<tr>
<td>Rata</td>
<td>6</td>
<td>146</td>
<td>33 (23%)</td>
<td>26 (78%)</td>
<td>22 (85%)</td>
<td>2.5</td>
</tr>
<tr>
<td>Kotuku</td>
<td>14</td>
<td>381</td>
<td>92 (24%)</td>
<td>66 (72%)</td>
<td>50 (75%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Monad</td>
<td>7</td>
<td>221</td>
<td>42 (19%)</td>
<td>37 (89%)</td>
<td>28 (76%)</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>51</strong></td>
<td><strong>1289</strong></td>
<td><strong>318 (25%)</strong></td>
<td><strong>250 (79%)</strong></td>
<td><strong>202 (81%)</strong></td>
<td><strong>1.6</strong></td>
</tr>
</tbody>
</table>

Note: DH=Doubled haploid, No.=number.
2.4: Conclusion

Results have shown that the wheat x maize method for the production of wheat DHs is a promising method for New Zealand wheat breeding. DHs were produced from all seven wheat genotypes used, verifying the use of this method over other genotype specific methods such as anther culture (Cooper and Griffin, 1988) and wheat x *H. bulbosum* crosses (Sitch and Snape, 1987a). However, during the course of this study, some limitations of the method were exposed. Germination rates of haploid embryos varied significantly according to a number of factors. These included time of embryo excision, method of auxin application and media composition. Manipulation of these factors has led to the development of a protocol for producing wheat DHs from New Zealand wheat genotypes which compares favourably with methods developed in other countries.

Seasonal constraints were the major limitation in the use of wheat x maize crosses to produce DHs for New Zealand wheats. The method is limited to spring, summer and autumn (optimal in summer). A number of environmental factors such as photoperiod, temperature, light intensity and light quality could be influencing the production of wheat haploid embryos. Future investigation of these factors is important for optimising the use of DHs for wheat breeding programmes in New Zealand.
3: The effects of temperature and light intensity on embryo numbers in wheat doubled haploid production through wheat x maize crosses

3.1: Introduction

The use of doubled haploid (DH) technology enables homozygosity of wheat lines to be reached in a single generation, making it a useful technique for both wheat breeding and genetical studies. Various methods are available for producing wheat DHs including anther and microspore culture; these methods have been limited due to genotypic specificity (Kisana et al., 1993, Ekiz and Konzak, 1994, Otani and Shimada, 1994). In contrast, wheat x maize crosses are widely used for the production of wheat DHs because no recalcitrant genotypes have been reported (Laurie and Bennett, 1988a, Islam and Shepherd, 1994, Pienaar and Lesch, 1994).

In the wheat x maize cross, maize chromosomes are eliminated within the first few cell cycles following fertilisation (Laurie and Bennett, 1989b). As a result, the endosperm is either absent or highly abnormal and the embryo fails to develop fully. The application of synthetic auxins, such as Dicamba and/or 2,4-D, to pollinated florets, stimulates haploid embryo development to a stage where the embryos can be rescued on to nutrient media (Suenaga and Nakajima, 1989). Further refinements to the technique have been made to increase haploid embryo survival through manipulation of plant growth regulator treatments (PGR) and tissue culture conditions (Suenaga and Nakajima, 1989, O’Donoughue and Bennett, 1994, Pienaar and Lesch, 1994, Pienaar et al., 1996, Morshedi et al., 1996).

Differences in the efficiency of the wheat x maize system have been related to the time of year in which the crosses are made (Pienaar and Lesch, 1994, Pienaar et al., 1996). O’Donoughue and Bennett (1994) reported that the environment influenced durum wheat embryo survival in a genotypically-dependent manner; embryo recovery from the cultivar Rampton Rivet was significantly greater in a 20 °C growth room than in an unheated glasshouse, whereas cultivars Wakona and Chinese Spring were unaffected.

In other wide cereal crosses the effect of temperature has been noted (Pickering and
Morgan, 1985, Sitch and Snape, 1987b, Nkongolo et al., 1991, Molnár-Láng and Sutka, 1994). There are no reports available on the influence of other environmental factors, such as light intensity, light quality and photoperiod on wide cereal crosses.

The aim of this chapter was to investigate the importance of temperature and light intensity on haploid embryo production in wheat x maize crosses for a number of New Zealand wheat cultivars. This information will be useful for optimising glasshouse growing conditions and deciding optimal seasonal conditions for pollination, thus, maximising the efficiency of the system.
3.2: Materials and methods

3.2.1: Crossing and embryo rescue procedure

Wheat spikes were emasculated one to two days before anthesis. At the approximate time of anthesis, florets were pollinated with fresh maize pollen (NZ75) using a small paintbrush. Spikes were treated with synthetic auxins (40 mgL⁻¹ Dicamba and 20 mgL⁻¹ 2,4-D) two to three days after pollination. This plant growth regulator (PGR) solution was injected at the first internode of the spike. Vaseline was used to prevent leakage and a drop was also placed into each of the floret cups. Immature seeds were dissected and counted, 14-20 days after pollination and developing embryos were rescued onto artificial nutrient medium containing \( \frac{1}{2} \) strength MS salts (Murashige and Skoog, 1962) supplemented with 20 gL⁻¹ sucrose and 7 gL⁻¹ agar. Embryos were incubated in darkness at 20 to 24 °C until germination and then transferred to a 16-hour photoperiod (65 μmol m⁻² s⁻¹, P.A.R.).

3.2.2: The effect of temperature on wheat haploid embryo recovery

Five New Zealand wheat cultivars (Otane, Rata, Belfield, Kotuku and Monad) were grown in a glasshouse until they reached the booting stage. They were then transferred to one of three growth cabinets at different temperature regimes (day/night temperatures of 17/12, 22/17 or 27/22 °C) at a constant light intensity of 250 μmol m⁻² s⁻¹, P.A.R., with a 16 h light photoperiod and a constant humidity of 70%. Wheat spikes were emasculated and pollinated with maize pollen (from plants grown in a glasshouse) as described in section 3.2.1. Florets from at least five wheat spikes per cultivar (taken from the leading tiller of different plants) were pollinated (for the numbers see Table 3.1). The wheat was maintained in the growth cabinets until the time of embryo rescue.
Table 3.1: Number of spikes, followed by the number of florets, for each wheat cultivar pollinated with maize under different temperature regimes

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Day/night 17/12 °C</th>
<th>Day/night 22/17 °C</th>
<th>Day/night 27/22 °C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otane</td>
<td>5, 128</td>
<td>7, 147</td>
<td>7, 143</td>
<td>19, 418</td>
</tr>
<tr>
<td>Rata</td>
<td>8, 176</td>
<td>5, 104</td>
<td>6, 102</td>
<td>19, 382</td>
</tr>
<tr>
<td>Belfield</td>
<td>6, 154</td>
<td>6, 114</td>
<td>5, 97</td>
<td>17, 365</td>
</tr>
<tr>
<td>Kotuku</td>
<td>9, 222</td>
<td>7, 133</td>
<td>7, 156</td>
<td>23, 511</td>
</tr>
<tr>
<td>Monad</td>
<td>8, 148</td>
<td>5, 88</td>
<td>6, 132</td>
<td>19, 368</td>
</tr>
</tbody>
</table>

3.2.3: The effect of light intensity on wheat haploid embryo recovery

Six New Zealand cultivars (Otane, Rata, Belfield, Kotuku, Monad and Karamu) and one advanced breeding line (95ST206), were grown in a glasshouse until they reached the booting stage. They were then transferred to one climate controlled growth room at day/night 22/17 °C and a constant humidity of 70%. The plants were grown under three different light intensities (300, 500 or 1000 μmol m⁻² s⁻¹, P.A.R.) created with shading cloth. Wheat spikes were emasculated one to two days prior to anthesis and at the approximate stage of anthesis the wheat was pollinated with maize pollen (from plants grown in a glasshouse) as described in section 3.2.1. Florets from five wheat spikes per cultivar (taken from the leading tiller of different plants) were pollinated (for the numbers see Table 3.2). The wheat was maintained in the growth cabinet until the time of embryo rescue.
Table 3.2: Number of spikes, followed by the number of florets, for each wheat cultivar pollinated with maize under different light intensity regimes

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>300 μmol m² s⁻¹</th>
<th>500 μmol m² s⁻¹</th>
<th>1000 μmol m² s⁻¹</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otane</td>
<td>5, 88</td>
<td>5, 107</td>
<td>5, 121</td>
<td>15, 316</td>
</tr>
<tr>
<td>Rata</td>
<td>5, 118</td>
<td>5, 122</td>
<td>5, 110</td>
<td>15, 350</td>
</tr>
<tr>
<td>Belfield</td>
<td>5, 118</td>
<td>5, 136</td>
<td>5, 116</td>
<td>15, 370</td>
</tr>
<tr>
<td>Kotuku</td>
<td>5, 158</td>
<td>5, 136</td>
<td>5, 146</td>
<td>15, 440</td>
</tr>
<tr>
<td>Monad</td>
<td>5, 143</td>
<td>5, 158</td>
<td>5, 149</td>
<td>15, 411</td>
</tr>
<tr>
<td>Karamu</td>
<td>5, 98</td>
<td>5, 129</td>
<td>5, 111</td>
<td>15, 338</td>
</tr>
<tr>
<td>95ST206</td>
<td>5, 119</td>
<td>5, 126</td>
<td>5, 110</td>
<td>15, 355</td>
</tr>
</tbody>
</table>

3.2.4: Statistical analysis

All data in this chapter were analysed using Minitab© version ten. ANOVA was used to examine treatment means. All variables were considered to be fixed effects.
3.4: Results

Under the three different temperature regimes a highly significant temperature effect on the frequency of wheat haploid embryos occurred (Table 3.3). A significant cultivar effect and a significant cultivar x temperature interaction also occurred for the frequency of wheat haploid embryo numbers (Table 3.3). The optimal temperature for cultivars Otane, Belfield and Monad was day/night 22/17 °C (Fig. 3.1). Kotuku produced a significantly higher frequency of embryos at day/night 17/12 °C.

Table 3.3: Analysis of variance for frequency of haploid embryos recovered 14-20 days after pollination of wheat with maize under different temperature treatments

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>2</td>
<td>965.2</td>
<td>0.008 **</td>
</tr>
<tr>
<td>Cultivar</td>
<td>4</td>
<td>645.2</td>
<td>0.30*</td>
</tr>
<tr>
<td>Temperature x Cultivar</td>
<td>8</td>
<td>357</td>
<td>0.12*</td>
</tr>
<tr>
<td>Error</td>
<td>82</td>
<td>138.5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: df, MS, * and **, represent degrees of freedom, mean square, and significance at 5% and 1% probability levels respectively. Replicate effect non-significant (p < 0.05).
Fig. 3.1: The effect of temperature on haploid embryos recovered 14-20 d after pollination of wheat with maize. Graphed values are the means for 5-9 spikes, vertical lines represent standard error of the means.

Under the three different light intensity regimes (at optimal day/night temperature 22/17 °C) there was a highly significant light intensity effect on the frequency of haploid embryos (Table 3.4). A highly significant cultivar effect and a highly significant cultivar x light intensity interaction also occurred for the frequency of wheat haploid embryo numbers (Table 3.4). The highest frequency of haploid embryos was obtained at 1000 μmol m⁻² s⁻¹ for all cultivars, except Kotuku which showed a similar response over all light intensities (Figure 3.2).
Table 3.4: Analysis of variance for frequency of haploid embryos recovered 14-20 days after pollination of wheat with maize under different light intensity treatments

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity</td>
<td>2</td>
<td>4239.2</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Cultivar</td>
<td>6</td>
<td>670.7</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Light intensity x cultivar</td>
<td>12</td>
<td>499.5</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Error</td>
<td>84</td>
<td>134.1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: df, MS, and *** represent degrees of freedom, mean square, and significance at 0.1% probability levels respectively. Replicate effect non significant ($p < 0.05$).
Fig. 3.2: The effect of light intensity on haploid embryos recovered 14-20 d after pollination of wheat with maize. Graphed values are the means for 5 spikes, vertical lines represent standard error of the means.

Embryos from all of the environmental conditions were cultured successfully. No differences in the germination success rates of embryos taken from different environmental conditions were apparent.
3.5: Discussion

This study established that the ambient temperature and the light intensity at which pollinations were made and subsequent fertilisation and embryo development occurred, significantly influenced the frequency of haploid embryo formation. The optimal temperature for the recovery of haploid wheat embryos was day/night 22/17 °C (Fig. 3.1). However, the influence of temperature differed among wheat cultivars. Similar observations were noted with varying light intensities. The highest light intensity (1000 µmol m⁻²s⁻¹, day/night temp. 22°C/17 °C) produced the greatest frequency of haploid embryos (38% of florets pollinated), although the magnitude of this effect differed among wheat cultivars (Fig. 3.2). There was no relationship between those cultivars significantly affected by temperature and those significantly affected by light intensity. Belfield and Kotuku, for example, showed the greatest response to temperature but not to light intensity whereas, Rata was affected by light intensity and not affected by temperature. Otane, the only “true” spring cultivar in the study, was affected by both temperature and light intensity (Figs. 3.1 and 3.2).

In the study there was one environmental treatment (22/17 °C day/night and low light) which was similar in the temperature and light intensity experiments. Results from this treatment varied between the two experiments. The mean number of haploid embryos recovered was greater in the light intensity experiment than in the temperature experiment for all cultivars common to both experiments, except Belfield. A more modern growth cabinet was used in the light experiment to that previously used in the temperature experiment, to enable a more accurate control of light intensities. Variations in the overall growth cabinet environment between the experiments would account for the variation in plant response. This emphasises the subtle effects of the environment on the efficiencies of wheat x maize haploid production.

Fertilisation and embryo survival in wheat x maize crosses are independent events (Wedzony and van Lammeren, 1996). Thus, the influence of environmental factors seen in this study could be acting at one or two levels. Previous studies have improved haploid embryo recovery from 22 % (Laurie and Bennett, 1986) to 30 % of florets pollinated (Pienaar et al., 1996) through the manipulation of PGRs. PGRs probably decrease embryo abortion rates rather than improving fertilisation rates. This is because PGRs are applied at
least 24 hours after pollination and maize pollen has been shown to germinate on wheat stigmas within 30 minutes (Wedzony and van Lammeren, 1996). It would be of use to establish whether the effects of environmental factors seen in this study, are increasing fertilisation rates and/or increasing embryo survival rates. If the influence of the environmental factors were on fertilisation, which is reported to be relatively low (28% of florets pollinated; Laurie and Bennett 1988b), further investigation of environmental influences may lead to a significant improvement in the overall production of haploid embryos.

There are many possible physiological reasons for the influence of environmental factors on haploid embryo recovery. High light intensity could be influencing the accumulation of photosynthates as resources for haploid embryo development. However, all wheat plants were grown in the same environmental conditions until the booting stage. Therefore, any differences in photosynthate accumulation, affecting embryo recovery, would have had to occur in the time between transfer of the plants to the growth cabinets and embryo survival. Alterations in light intensity and temperature could be altering the fertilisation tract environment of the female wheat parent. This could affect the ability of the maize pollen to fertilise the wheat ovules successfully. Alternatively, different environmental conditions may influence endogenous PGR levels, or the sensitivity of cells to PGRs, leading to subsequent improvements in fertilisation rates and/or embryo survival rates.

In conclusion, the results of this chapter have clearly established that environmental factors can have a marked influence on wheat haploid recovery. This study indicates that seasons of the year with low light intensity or high temperatures are to be avoided in a wheat DH programme using the wheat x maize method. A greater understanding of the physiology underlying these results will enable manipulation of environmental conditions employed during wheat x maize crosses and other wide cereal crosses, which may lead to subsequent improvements in the overall efficiency of such systems.
4: The effect of light intensity on pollen tube growth and embryo survival in wheat x maize crosses

4.1: Introduction

The success of wide cereal crosses is controlled by four independent events: i) pollen tube growth, ii) entry of pollen tubes into ovules, iii) fertilisation and iv) embryonic growth (Ahmad and Comeau, 1990). Genetic and environmental factors can influence these events and may vary for different wide crosses and for each different event. As discussed in chapter one, temperature can influence the rate of pollen donor chromosome elimination in the production of barley haploid plants, which can determine whether the resulting embryo is haploid or a hybrid (Pickering, 1985, Pickering and Morgan, 1985). Poor seed set in some genotypes of *Hordeum vulgare* x *Hordeum bulbosum* crosses has been shown to be influenced by a single dominant allele located at a locus on chromosome seven of barley (Pickering, 1983), whilst poor seed set in other genotypes has been found to be strongly influenced by environmental factors rather than genetic effects (Bjørnstad, 1986). The action of *Kr1* and *Kr2* genes can affect the success of wheat x *H. bulbosum*, *H. bulbosum* x *Secale cereale* (rye), wheat x rye and wheat x sorghum crosses (Lange and Wojciechowska, 1976, Pickering and Morgan, 1985, Laurie and Bennett, 1989b, Inagaki and Mujeeb-Kazi, 1995). Dominant alleles of either locus will cause poor cross-fertility, with the *Kr1* allele having a stronger effect (Jalani and Moss, 1980). In genotypes homozygous for recessive alleles of these loci, environmental factors can influence the numbers of haploid embryos produced (Sitch and Snape, 1987b). Although wheat x maize, wheat x teosinte, wheat x pearl millet and wheat x *Tripsacum dactyloides* crosses are relatively insensitive to the actions of the *Kr* genes (Laurie and Bennett, 1987, Ohkawa *et al.*, 1992, Inagaki and Mujeeb-Kazi, 1995, Li *et al.*, 1996), other factors influence the success of the crosses. These include the type and concentration of PGRs applied, the timing of pollination and environmental factors (Laurie and Bennett, 1989b, Suenaga and Nakajima, 1989, O'Donoughue and Bennett, 1994, Pienaar and Lesch, 1994, Inagaki and Bohorova, 1995, Pienaar *et al.*, 1996, Inagaki and Mujeeb-Kazi, 1996, Morshedi *et al.*, 1996, Wedzony and van Lammeren, 1996).

The processes of pollen germination, pollen tube growth, fertilisation and embryonic
growth have been examined in wide cereal crosses. In most cases, these have been examined under one set of environmental and hormonal conditions (Ahmad and Comeau, 1990, O'Donoughue and Bennett, 1994, Li et al., 1996). However, Wedzony and van Lammeren (1996) investigated pollen tube growth and early embryogenic growth under two hormonal conditions and it was found the application of 2,4-D increased the success of both events. In another study examining fertilisation in wheat x H. bulbosum crosses at two temperatures (20 and 26°C) fertilisation was higher at the lower temperature (Sitch and Snape, 1987a). Sitch and Snape (1987a) suggested this may have been due to differences in the duration of pollen tube growth, differences in the receptivity of stigmas and/or differences in the female fertility of ovules.

Chapter three established that haploid embryo numbers in wheat x maize crosses were significantly influenced by differing temperatures and light intensities. Of the two environmental influences examined, light intensity had the greater effect on haploid embryo recovery. The influence of light intensity on the processes which control the success of this cross have not been examined. The aim of this chapter therefore was to examine the influence of light intensity on wheat x maize crosses at the levels of pollen tube growth and embryo survival. The electron transport rate (ETR) and photochemical quantum yield of wheat plants under differing light intensities was also examined to give an indication of photosynthetic rates. Two wheat cultivars, Karamu and Kotuku, were used as experimental material because of their differing response to light intensity in chapter three. The frequency of haploid embryo formation in Karamu was most affected by differing light intensities whereas in Kotuku no response to differing light intensities was observed.
4.2: Materials and methods

4.2.1: Plant material, growing conditions and pollination

New Zealand wheat cultivars, Karamu and Kotuku, were grown in glasshouse conditions (heating < 16 °C, ventilation > 25 °C, with a photoperiod of 16 hrs, supplemented with Phillips SON-T agn lights) until the booting stage. At the booting stage plants were transferred into a growth cabinet (25/20°C day/night with a photoperiod of 16 hrs, and relative humidity of 70%) at two different light intensities: 250 μmol m⁻²s⁻¹, P.A.R. (created with shading cloth) and 750 μmol m⁻²s⁻¹, P.A.R. (full light). Maize plants (Yates ‘Early Miracle’) were also transferred to the growth cabinet (at 750 μmol m⁻²s⁻¹, P.A.R.) prior to tassle production to provide environmental uniformity on pollen development. Wheat spikes were emasculated 1-2 days prior to anthesis and at the approximate time of anthesis florets were pollinated with fresh maize pollen using a small paintbrush.

4.2.2: Examination of maize pollen tube growth in whole mount preparations of wheat florets

Wheat pistils from a minimum of four spikes for each treatment were fixed 24 hours after pollination with maize (18-23 florets per spike). Pistils were fixed in ethanol: acetic acid (3:1) overnight and stored in 70% ethanol at 4 °C. The pistils were cleared overnight in 5M NaOH at room temperature. Following rinsing (3x) in distilled water, the pistils were placed in toluidine blue staining solution (0.01%) overnight to quench autofluorescence of cell walls and reduce non-specific fluorescence (Wędzony and van Lammeren, 1996). During this time, vacuum infiltration was applied for at least half an hour to remove air bubbles appearing after clearance. Pistils were rinsed in water (3x) and stained with 0.01% aniline blue in 15mM phosphate buffer, pH 10. Samples were examined with an Olympus IMT2 microscope fitted with an IMT2-RFC reflected light fluorescent attachment (excitation filter 365 nm, dichloric mirror 400 and barrier filter 420 nm). The presence of pollen tubes reaching the cavity between the ovarian wall and integuments and in the micropyle, was recorded for all samples examined.
4.2.3: Measurement of electron transport rate and quantum yield at two light intensities

The electron transport rate (ETR) and quantum yield were measured using a portable pulse amplitude modulation fluorimeter, PAM-2000, with DA-2000 data acquisition software (Heinz-Walz GmbH, Effeltrich, Germany). A total of 16 plants were measured (four plants for each cultivar under two light intensities). Three leaves were measured on each plant and three measurements were made on each leaf. Measurements were taken at one, two and three days after anthesis.

4.2.4: Examination of embryo numbers

A minimum of four spikes for each treatment (17-26 florets per spike) were pollinated with maize following wheat emasculation. Wheat spikes were sprayed with 100 mgL\(^{-1}\) 2,4-D 24 hours after pollination and florets were dissected 10-14 days after pollination to quantify embryo numbers.

4.2.5: Statistical analysis

All data in this chapter were analysed using Minitab© version ten. ANOVA was used to examine treatment means.
4.3: Results

4.3.1: Pollen germination and tube growth at two light intensities

Germination of pollen grains occurred in both cultivars at both light intensity treatments. The exact percentage of germination could not be calculated because ungerminated and poorly germinated pollen was usually washed away during the clearance and staining procedure. The presence of maize pollen tubes was observed at the micropyle of the wheat pistil 24 hrs after pollination and was found to be significantly affected by light intensity (Fig. 4.1). Individual analysis of each cultivar revealed that the number of Karamu pistils with pollen tubes present at the micropyle was significantly greater at the high light treatment ($750 \mu\text{mol m}^{-2}\text{s}^{-1}$) than at the low light treatment ($250 \mu\text{mol m}^{-2}\text{s}^{-1}$). The number of Kotuku pistils with pollen tubes reaching the micropyle was unaffected by differing light intensities (Fig. 4.1).
Fig. 4.1: The effect of different light intensities on maize pollen tube growth in wheat pistils. Graphed bars represent mean values for 4-5 spikes, vertical lines represent standard errors of the mean. Analysis of variance established highly significant \((p < 0.001)\) main effects of cultivar and light intensity and a highly significant \((p < 0.001)\) interaction effect of light intensity \(\times\) cultivar.

In both cultivars under both light intensities, some pollen tubes displayed aberrant growth. This mostly consisted of coiling and branching of pollen tubes. Many pollen tubes grew down the style (Fig. 4.2) and in some cases, pollen tubes bypassed the micropyle, coiling around the ovary. In most cases where pollen tubes had reached the wheat micropyle, there was at least one tube present. Although pollen tubes were observed entering the
micropyle this was not always apparent (Fig. 4.2). For this reason it was not known if fertilisation occurred.
Fig. 4.2: Maize (Yates ‘Early Miracle’)
pollen tubes germinating and growing down the style of wheat (Karamu). Magnification = x 116. PG, St and PT represent pollen grain, style and pollen tube respectively.
Fig. 4.3: Karamu wheat ovules 24 hours after pollination with maize (Yates ‘Early Miracle’). Magnification = 109 x. A=Karamu wheat grown at high light intensity (750 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) showing a pollen tube growing into the micropyle of the ovule. B=Karamu wheat grown at low light intensity (250 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) showing no pollen tube growth at the micropyle. OW, O, ES and PT represent ovary wall, ovule, embryo sac and pollen tube respectively.
4.3.2: ETR and quantum yield at two light intensities

The ETR of Karamu and Kotuku was significantly different at two light intensities (Fig. 4.4). In both cultivars the ETR was greater under the high light intensity (750 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)) than the low light intensity (250 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)). Quantum yield of Karamu plants was also significantly different under the two light intensities and was lower at the high light intensity (Fig. 4.5). In contrast, quantum yield of Kotuku plants was not significantly different under the two light intensities (Fig. 4.5).
Fig. 4.4: The effect of light intensity on electron transport rate (ETR) of Karamu and Kotuku wheat cultivars 1-3 days after anthesis. Graphed values are the means of 36 measurements, vertical lines represent standard error of the means. Analysis of variance of bulked data means established highly significant ($p < 0.001$) main effects of cultivar and light intensity and a highly significant ($p < 0.001$) interaction effect of light intensity x cultivar.
Fig. 4.5: The effect of light intensity on overall quantum yield of Karamu and Kotuku wheat cultivars 1-3 days after anthesis. Graphed values are the means of 36 measurements, vertical lines represent standard error of the means. Analysis of variance of bulked data means established significant \( (p < 0.01) \) main effects of cultivar and light intensity and a significant \( (p < 0.01) \) interaction effect of light intensity x cultivar.

4.3.3: Haploid embryo survival under two light intensities

In Karamu, haploid embryo survival was significantly affected by light intensity with 34% of pollinated florets producing haploid embryos at the high light intensity compared with only 4 % of pollinated florets producing haploid embryos at the low light intensity. Kotuku was unaffected by differing light intensities, with 33% of pollinated florets producing haploid embryos at the high intensity and 38 % of pollinated florets producing haploid embryos at the low light intensity.
4.4: Discussion

This study confirms results from chapter three that embryo survival in wheat × maize crosses is affected by differing light intensities. More importantly, it establishes that this resulted from the effect of light intensity on pollen tube growth. The highest percentage (65%) of haploid wheat pistils where pollen tube growth reached the cavity between the ovarian wall and integuments and in the micropyle, was observed in Karamu plants grown at high light intensity (750 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), whilst the lowest percentage (22%) was observed in Karamu plants grown at low light intensity (250 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). It is probable that increased pollen tube growth in Karamu plants grown at high light intensity has a direct influence on fertilisation frequency, or embryonic growth, as although each event may be controlled independently, the success of later events is dependent on the occurrence of preceding events. Previous studies on wide crosses have shown that the presence of a pollen tube in the embryo sac or at the micropyle does not guarantee fertilisation (Sitch and Snape, 1987a, Laurie and Bennett, 1987, Laurie and Bennett, 1988a). Therefore, as fertilisation was not examined in this study, it cannot be confirmed that light intensity affected fertilisation success.

The different responses in pollen tube growth at high and low light intensities shows that light intensity can influence wheat × maize crosses maternally and the effect of this can differ between genotypes. This is not unexpected since environmental factors such as temperature and photoperiod have been shown to influence wheat development at all stages in the life cycle and the precise influence can vary with the wheat genotype (Mohapatra et al., 1983, Rawson and Zajac, 1993, Slafer and Rawson, 1994). Light intensity can also have a marked influence on wheat development, in particular, on the time taken to reach to anthesis (Slafer, 1995). Slafer (1995) examined wheat development up to anthesis at two light intensities and found that at high light intensity (560 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) development was accelerated and the acceleration was greatest in the late anthesis stage. Slafer (1995) also found that the number of grains per spike was significantly affected by light intensity and that plants which were shaded (298 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) during the phase immediately preceding anthesis were those with the lowest number of grains per spike. In an examination of the effects of shading immediately after anthesis (1-8 days after), again it was found that shading reduced floret fertility (Grabau et al., 1990). This may be a consequence of reduced light intensity causing poor development of florets near the spike.
apex (Rawson and Ruwali, 1972), which may be related to sink capacity or source supply of photo-assimilates (Grabau et al., 1990). The number of grains per spike in other studies has been shown to be influenced by either the size of the spike and/or floret fertility (Slafer et al., 1990, Slafer and Andrade, 1993).

These studies examining the influence of light intensity on wheat development help in understanding the influence of light intensity on wheat x maize crosses. The reduced pollen tube growth and embryo survival of the Karamu cultivar at low light intensity could be related to poor development of florets near the spike apex and reduced maternal fertility and development. However, the magnitude of the result indicates that this may not be the only factor which influences pollen tube growth. In most pistils examined, maize pollen had germinated and grown down the style, therefore there may be another factor involved which stops the pollen tubes reaching the micropyle. This may be an alteration further down the fertilisation tract of the female wheat parent and/or differences in endogenous PGR levels, or the sensitivity of cells to PGRs, as proposed in chapter three.

Following pollination with maize, fertilisation of wheat has been shown to occur in 4-5 hours at 20 °C (Laurie and Bennett, 1989b). Thus, the ETR and quantum yield measurements were made during the time frame in which pollination, pollen tube growth and fertilisation would have occurred. Quantum yield and P.A.R. are used to calculate ETR which can be compared to the rate of CO₂ assimilation of O₂ evolution when divided by four. As expected the ETR was greater at the high light intensity than the low light intensity in both cultivars. Quantum yield was significantly different in Karamu plants, however there was no significant difference in quantum yield of Kotuku plants at the two light intensities. This supports the concept that photosynthesis may influence the success of wheat x maize crosses, because both pollen tube growth and embryo survival are also unaffected by light intensity in Kotuku plants. When Karamu and Kotuku are grown in the field for grain, in New Zealand conditions, Karamu is planted in spring whereas Kotuku is planted in autumn. This, along with the quantum yield results would indicate Kotuku is better adapted to winter conditions and responds more slowly to low light intensities than Karamu.

In conclusion, the results from this chapter show that pollen tube growth and subsequent embryo survival in wheat x maize crosses are significantly influenced by light intensity.
However, the effects of light intensity on pollen tube growth and embryo survival depend on the genotype of the female wheat parent. Pollen tube growth and embryo survival were significantly affected in Karamu plants but unaffected in Kotuku plants. Photochemical quantum yield measurements indicated that the two cultivars have different photosynthetic capacities under different light intensities, Karamu appearing to be more sensitive to light changes. This may account for, or contribute to, the differences in pollen tube growth and embryo survival observed in this chapter.
5: Near absence of aberrant genetic variation during the production of wheat x maize doubled haploid lines

5.1: Introduction

Doubled haploid (DH) technology offers considerable benefits to wheat breeding and genetical studies in New Zealand. However, there is still a lack of information about critical factors for applying DH technology predictably and routinely to a wheat breeding programme despite its use in breeding programmes. For effective use in wheat breeding programmes, genetic variation exhibited by the derived homozygous lines needs to reflect the heterozygous allelic variation present in the parental material. However, as discussed in chapter one, the genetic variation in the derived population may be influenced by the occurrence of mutations during DH production, particularly gametoclonal variation and by the influence of gametic and other forms of inadvertent selection (Laurie and Snape, 1990, Snape et al., 1992). Induction of gametoclonal variation during the culture of wheat anthers has been well documented (Baenziger et al., 1983, Skinnes and Bjørnstad, 1995, Baenziger et al., 1989b). This type of introduced genetic variation can be detrimental to the agronomic performance of wheat lines (Baenziger et al., 1983).

It is thought gametoclonal variation in populations derived from anther culture is induced during the callus stage of culture. No callus stage is required in the rescue of wheat x *H. bulbosum* or wheat x maize haploid embryos. However, Snape et al. (1988) found unexpected genetic variation in wheat DH populations, produced through the wheat x *H. bulbosum* method, compared with parent populations. They proposed that the use of colchicine for chromosome doubling was the cause of variation seen in their study. Recently, evidence for the heritable nature of colchicine induced variations in wheat has been reported (Hassan, 1996). The mechanisms underlying the changes caused by colchicine are not understood, but are believed to involve DNA modification. Another possible source of variation in the use of the wheat x maize method is in the application of 2,4-D to initiate embryo development. Plant growth regulators, such as 2,4-D, have been shown to induce chromosomal alterations and mutations in plant tissue cultures (Lee and Phillips, 1988, D'Amato, 1990).
The above factors highlight the need to examine genetic variation in New Zealand DH populations derived through the wheat x maize method. In a recent study, wheat x maize DH populations and anther culture populations of two hybrids were compared for the segregation of the 1BL-1RS chromosome (Lefebvre and Devaux, 1996). Expected inheritance ratios were found among wheat x maize derived haploids but not in anther culture derived haploids. This is promising for the use of the wheat x maize method, however, the study covered only a small proportion of the wheat genome and did not eliminate the possibility of induced genetic variation elsewhere in the genome.

The aim of this chapter was to determine if significant aberrant genetic variation is introduced into wheat DH populations produced by the wheat x maize method. To investigate this, inbred cultivars were used to eliminate any genetic variation arising from allele segregation. Consequently, any variation observed between the DHs and the parent cultivars can be attributed to aberrant genetic changes inherent to the method. To achieve this aim, variation in 13 quantitative traits, presumed to represent an adequate coverage of the wheat genome, was measured.
5.2: Materials and methods

5.2.1: Doubled haploid production

Spikes from five New Zealand wheat cultivars (Rata, Monad, Kotuku, Otane and Belfield) were emasculated one to two days before anthesis. At the approximate time of anthesis, spikes were pollinated with maize pollen (Yates “Early Miracle”) using a small paintbrush. Spikes were treated with synthetic auxins (40 mgL⁻¹ Dicamba and 20 mgL⁻¹ 2,4-D) one to two days after pollination. This plant growth regulator solution was injected at the first internode of the spike and Vaseline was used to prevent leakage. A drop of the solution was also placed into each of the floret cups. Seeds were dissected 14 to 16 days after pollination and developing embryos were rescued onto artificial nutrient medium containing ½ strength MS salts (Murashige and Skoog, 1962) supplemented with 20 gL⁻¹ sucrose and 7 gL⁻¹ agar. Embryos were incubated in darkness at 20 to 24 °C until germination and then transferred to a 16-hour photoperiod (65 μmol m⁻² sec⁻¹, P.A.R.). Haploid plants were transplanted to soil when 20 to 40 mm in height. To double chromosome numbers, haploid seedlings were placed in aerated colchicine solution (500 mgL⁻¹ colchicine, 2% DMSO) for five hours. The treated seedlings were then washed in tap water and transferred to pots containing fresh potting mix. Self-pollinated seeds were harvested at maturity.

Parent populations for each cultivar were grown in the glasshouse with DH populations to ensure both populations were subjected to the same glasshouse growing conditions prior to planting in the field.

5.2.2: Field trial

The field trial was planted (September, 1996-Lincoln, Canterbury, New Zealand) in a series of rows, 50 cm apart, with each parental cultivar and DH lines derived from them, in the same rows. In these rows the seed from the 40 DH lines (2-17 seeds/line) were sown 10 cm apart and seed from one of the five parent cultivars (10 seeds) were interspersed at least every second DH line to give 3-4 replicates of 10 plants for each parent cultivar. All plants were protected from weeds, birds and fungi and were moisture stress-free (irrigation scheduling) and buffered from wind with rows of Kotuku wheat.
Thirteen quantitative traits were measured, on a single-plant basis. Prior to harvest, flag leaf length, flag leaf width and days to ear emergence from planting were measured on all plants. Following harvest (March, 1997) plant height, spikes/plant, spike length, node number, awn length, number of grains/spike, weight/seed, grain weight/plant, grain protein percentage (NIR) and grain sedimentation values (Griffin, 1983) of all plants were measured.

5.2.3: Statistical analysis

All data in this chapter were analysed using Minitab© version ten. ANOVA was used to examine means among populations. Variation of means from all quantitative traits for the five cultivars was partitioned into the following comparisons: among parent lines, among DH lines and between DH lines and parent lines. Fisher's pairwise comparisons were used to analyse further any significant differences.
5.3: Results

The mean performances and standard errors of the parent and DH populations for the five cultivars and various characters are shown in Tables 5.1 and 5.2. There were no significant differences among any of the parent lines which were interspersed over the whole trial area, for all cultivars over all 13 traits (Tables 5.3 and 5.4). Therefore environmental influences were minimal and comparisons between single plants were justified in this small trial. Of the comparisons, few significant differences were observed for the measured traits in DH populations (Tables 5.3 and 5.4). Three of the five cultivars analysed (Kotuku, Belfield and Otane) showed no significant differences for all the 13 quantitative traits measured within DH lines. Within the Rata DH population a highly significant difference occurred in spike length. A dot plot of the data (Fig. 5.1) and Fisher’s pairwise comparisons showed DH line three had a longer spike length than the other DH lines.

Fig. 5.1: Spike length of Rata doubled haploid (DH) lines. Analysis of variance established a highly significant \( (p < 0.01) \) difference in Rata spike length within the DH population. Line three was significantly different \( (p < 0.01) \) to other lines in Fisher’s
Monad DH lines showed significant differences for three of the 13 quantitative traits measured. Significant differences were recorded for flag leaf length ($p < 0.05$), awn length ($p < 0.05$) and ear emergence ($p < 0.01$). Dot plots of data (Fig. 5.2, Fig. 5.3 and Fig. 5.4) and Fisher’s pairwise comparisons revealed DH line seven had a longer flag leaf length than other DH lines, DH line nine had a shorter awn length than other DH lines and DH line two matured faster to ear emergence than other DH lines.

**Fig. 5.2**: Flag leaf length of Monad doubled haploid (DH) lines. Analysis of variance established a significant ($p < 0.05$) difference in flag leaf length within the Monad DH population. Line seven was significantly different ($p < 0.05$) to other lines in Fisher’s pairwise comparisons.
Fig. 5.3: Awn length of Monad doubled haploid (DH) lines. Analysis of variance established a significant ($p < 0.05$) difference in awn length within the Monad DH population. Line nine was significantly different ($p < 0.05$) to other lines in Fisher's pairwise comparisons.
Fig. 5.4: Spike emergence time of Monad doubled haploid (DH) lines. Analysis of variance established a significant ($p < 0.05$) difference in spike emergence time within the DH population. Line two was significantly different ($p < 0.05$) to other lines in Fisher’s pairwise comparisons.

For all five cultivars there were no significant differences in comparisons between DH lines and their parent lines in any of the 13 quantitative traits measured (Tables 5.3 and 5.4).
Table 5.1: Mean performance and standard errors of doubled haploid lines of wheat compared with the parent lines (vegetative traits)

<table>
<thead>
<tr>
<th>Item</th>
<th>Flag leaf length (cm)</th>
<th>Flag leaf width (mm)</th>
<th>Ear emergence (days)</th>
<th>Height (cm)</th>
<th>Spikes/plant</th>
<th>Spike length (mm)</th>
<th>Node no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rata Std</td>
<td>163.5 ± 8.0</td>
<td>13.5 ± 0.5</td>
<td>98 ± 1</td>
<td>67.0 ± 1.0</td>
<td>24 ± 3</td>
<td>85.5 ± 3.0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Rata DH</td>
<td>163.0 ± 3.0</td>
<td>14.0 ± 0.5</td>
<td>96 ± 1</td>
<td>69.0 ± 1.0</td>
<td>25 ± 1</td>
<td>82.0 ± 2.0**</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Monad Std</td>
<td>180.5 ± 7.0</td>
<td>14.0 ± 0.5</td>
<td>97 ± 1</td>
<td>85.0 ± 2.0</td>
<td>14 ± 2</td>
<td>99.0 ± 3.5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Monad DH</td>
<td>185.0 ± 3.0*</td>
<td>14.0 ± 0.5</td>
<td>97 ± 1**</td>
<td>88.5 ± 2.5</td>
<td>18 ± 2</td>
<td>100.0 ± 2.0</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Kotuku Std</td>
<td>198.0 ± 9.0</td>
<td>15.5 ± 0.5</td>
<td>93 ± 1</td>
<td>73.0 ± 2.0</td>
<td>19 ± 2</td>
<td>98.0 ± 3.0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Kotuku DH</td>
<td>190.0 ± 9.5</td>
<td>14.0 ± 0.5</td>
<td>92 ± 1</td>
<td>70.5 ± 1.0</td>
<td>21 ± 2</td>
<td>97.0 ± 3.5</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Otane Std</td>
<td>160.0 ± 10.5</td>
<td>15.5 ± 1.0</td>
<td>85 ± 2</td>
<td>67.0 ± 2.0</td>
<td>11 ± 2</td>
<td>92.5 ± 2.5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Otane DH</td>
<td>156.5 ± 11.5</td>
<td>16.0 ± 0.5</td>
<td>83 ± 2</td>
<td>70.0 ± 1.5</td>
<td>12 ± 1</td>
<td>88.0 ± 3.0</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Belfield Std</td>
<td>154.5 ± 17.5</td>
<td>13.0 ± 2.0</td>
<td>92 ± 1</td>
<td>81.5 ± 14.5</td>
<td>27 ± 8</td>
<td>98 ± 0.5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Belfield DH</td>
<td>158.87 ± 8.5</td>
<td>13.5 ± 0.5</td>
<td>92 ± 1</td>
<td>79.0 ± 3.5</td>
<td>21 ± 2</td>
<td>101.5 ± 4.5</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Note: DH, std, * and ** represent doubled haploid, standard parent line and significance within group lines at 5% and 1% probability levels respectively.
Table 5.2: Mean performance and standard errors of doubled haploid lines of wheat with parent lines (grain traits)

<table>
<thead>
<tr>
<th>Item</th>
<th>Awn length (mm)</th>
<th>Grain/spike</th>
<th>1000 grain wgt</th>
<th>Grain wgt/plant</th>
<th>Grain protein</th>
<th>Sedimentation value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rata Std</td>
<td>72.5 ± 3.0</td>
<td>46 ± 3</td>
<td>5.4 ± 0.2</td>
<td>20.6 ± 3.2</td>
<td>12.3 ± 0.2</td>
<td>97.9 ± 0.7</td>
</tr>
<tr>
<td>Rata DH</td>
<td>71.0 ± 1.0</td>
<td>40 ± 2</td>
<td>5.4 ± 0.1</td>
<td>21.1 ± 1.6</td>
<td>12.4 ± 0.1</td>
<td>97.8 ± 0.3</td>
</tr>
<tr>
<td>Monad Std</td>
<td>59.5 ± 3.5</td>
<td>49 ± 4</td>
<td>6.7 ± 1.9</td>
<td>19.6 ± 2.7</td>
<td>12.7 ± 0.3</td>
<td>96.3 ± 0.7</td>
</tr>
<tr>
<td>Monad DH</td>
<td>61.0 ± 2.0*</td>
<td>53 ± 3</td>
<td>6.8 ± 1.3</td>
<td>25.4 ± 2.8</td>
<td>12.7 ± 0.1</td>
<td>95.9 ± 0.5</td>
</tr>
<tr>
<td>Kotuku Std</td>
<td>66.5 ± 3.0</td>
<td>55 ± 4</td>
<td>5.6 ± 0.2</td>
<td>24.7 ± 4.4</td>
<td>12.5 ± 0.4</td>
<td>93.9 ± 1.2</td>
</tr>
<tr>
<td>Kotuku DH</td>
<td>69.0 ± 3.0</td>
<td>46 ± 4</td>
<td>6.0 ± 0.2</td>
<td>21.5 ± 2.8</td>
<td>13.1 ± 0.3</td>
<td>95.0 ± 0.9</td>
</tr>
<tr>
<td>Otane Std</td>
<td>68.5 ± 5.5</td>
<td>54 ± 3</td>
<td>7.7 ± 0.5</td>
<td>26.0 ± 6.0</td>
<td>13.1 ± 0.3</td>
<td>93.0 ± 2.0</td>
</tr>
<tr>
<td>Otane DH</td>
<td>66.0 ± 3.5</td>
<td>51 ± 4</td>
<td>7.9 ± 0.3</td>
<td>19.9 ± 2.6</td>
<td>13.0 ± 0.3</td>
<td>90.3 ± 1.7</td>
</tr>
<tr>
<td>Belfield Std</td>
<td>Awnless</td>
<td>48 ± 1</td>
<td>5.9 ± 0.5</td>
<td>27.5 ± 9.7</td>
<td>12.4 ± 0.8</td>
<td>96.0 ± 1.0</td>
</tr>
<tr>
<td>Belfield DH</td>
<td>54 ± 3</td>
<td>6.3 ± 0.1</td>
<td>29.6 ± 4.5</td>
<td>12.1 ± 0.3</td>
<td>96.5 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

Note: DH, std, and * represent doubled haploid, standard parent line and significance within group lines at 5% probability level respectively.
Table 5.3: Analysis of variance for comparisons of doubled haploid lines of wheat with parent lines (vegetative traits)

<table>
<thead>
<tr>
<th>Item</th>
<th>Source of variation</th>
<th>Lin df</th>
<th>Mean squares</th>
<th>Flag leaf length (cm)</th>
<th>Flag leaf width (cm)</th>
<th>Ear emergence (days)</th>
<th>Height (cm)</th>
<th>Spikes/plant</th>
<th>Spike length (mm)</th>
<th>Node no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rata x maize</strong></td>
<td>Among Rata std lines</td>
<td>4 3</td>
<td>511</td>
<td>2.22</td>
<td>18</td>
<td>1.8</td>
<td>89.1</td>
<td>6</td>
<td></td>
<td>0.156</td>
</tr>
<tr>
<td></td>
<td>Among Rata DH lines</td>
<td>18 17</td>
<td>675</td>
<td>1.12</td>
<td>21.5</td>
<td>21.8</td>
<td>73.8</td>
<td>210**</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Between Rata DH pop. &amp; std pop</td>
<td>2 1</td>
<td>670</td>
<td>3.27</td>
<td>52</td>
<td>43.4</td>
<td>6.4</td>
<td>112</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Monad x maize</strong></td>
<td>Among Monad std lines</td>
<td>3 511</td>
<td>0.9</td>
<td>0.9</td>
<td>118.6</td>
<td>64.3</td>
<td>67</td>
<td>0.52</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Among Monad DH lines</td>
<td>10 984</td>
<td>287*</td>
<td>5.23</td>
<td>21.2**</td>
<td>154.8</td>
<td>104.6</td>
<td>139</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Between Monad DH pop. &amp; std pop</td>
<td>2 1</td>
<td>843</td>
<td>0.12</td>
<td>2.9</td>
<td>106</td>
<td>201.6</td>
<td>14</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Kotuku x maize</strong></td>
<td>Among Kotuku std lines</td>
<td>4 3</td>
<td>3256</td>
<td>3.2</td>
<td>20.9</td>
<td>66.7</td>
<td>49.1</td>
<td>219.3</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Among Kotuku DH lines</td>
<td>8 7</td>
<td>1854</td>
<td>2.9</td>
<td>28.7</td>
<td>20.7</td>
<td>98.4</td>
<td>281</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Between Kotuku DH pop. &amp; std pop</td>
<td>2 1</td>
<td>6750</td>
<td>14</td>
<td>8</td>
<td>55.7</td>
<td>60.1</td>
<td>16</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Otane x maize</strong></td>
<td>Among Otane std lines</td>
<td>4 3</td>
<td>481</td>
<td>3</td>
<td>18.7</td>
<td>14.1</td>
<td>30.1</td>
<td>2.1</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Among Otane DH lines</td>
<td>4 3</td>
<td>1293</td>
<td>1.37</td>
<td>5.6</td>
<td>27.3</td>
<td>19.3</td>
<td>92</td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Between Otane DH pop. &amp; std pop</td>
<td>2 1</td>
<td>39</td>
<td>0.34</td>
<td>10.8</td>
<td>40</td>
<td>1.7</td>
<td>91.3</td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Belfield x maize</strong></td>
<td>Among Belfield std lines</td>
<td>3 2</td>
<td>31</td>
<td>0.1</td>
<td>0.03</td>
<td>11</td>
<td>62.5</td>
<td>20</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Among Belfield DH lines</td>
<td>4 3</td>
<td>888</td>
<td>2.11</td>
<td>9.46</td>
<td>169.2</td>
<td>24.3</td>
<td>115</td>
<td></td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Between Belfield DH pop. &amp; std pop</td>
<td>2 1</td>
<td>633</td>
<td>2.19</td>
<td>6.58</td>
<td>135</td>
<td>48.2</td>
<td>129</td>
<td></td>
<td>0.29</td>
</tr>
</tbody>
</table>

Note: DH, std, * and ** represent doubled haploid, standard parent line and significance within group lines at 5% and 1% probability levels respectively.
Table 5.4: Analysis of variance for comparisons of doubled haploid lines of wheat with parent lines (grain traits)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Lines</th>
<th>df</th>
<th>Awn length (cm)</th>
<th>Grain/spike</th>
<th>1000 grain wgt</th>
<th>Grain wgt/plant</th>
<th>Grain protein</th>
<th>Sedimentation Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rata x maize</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Rata std lines</td>
<td>4</td>
<td>3</td>
<td>123</td>
<td>58</td>
<td>9.3</td>
<td>36</td>
<td>0.05</td>
<td>11.65</td>
</tr>
<tr>
<td>Among Rata DH lines</td>
<td>18</td>
<td>17</td>
<td>100.1</td>
<td>82</td>
<td>1.53</td>
<td>118</td>
<td>0.47</td>
<td>5.46</td>
</tr>
<tr>
<td>Between Rata DH pop. &amp; std pop</td>
<td>2</td>
<td>1</td>
<td>14.1</td>
<td>291</td>
<td>0.07</td>
<td>2</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Monad x maize</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Monad std lines</td>
<td>4</td>
<td>3</td>
<td>204</td>
<td>54</td>
<td>0.87</td>
<td>123.5</td>
<td>0.32</td>
<td>0.96</td>
</tr>
<tr>
<td>Among Monad DH lines</td>
<td>10</td>
<td>9</td>
<td>126*</td>
<td>488</td>
<td>5.46</td>
<td>207</td>
<td>1.19</td>
<td>8.08</td>
</tr>
<tr>
<td>Between Monad DH pop. &amp; std pop</td>
<td>2</td>
<td>1</td>
<td>60</td>
<td>176</td>
<td>0.09</td>
<td>997</td>
<td>0.02</td>
<td>10</td>
</tr>
<tr>
<td><strong>Kotuku x maize</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Kotuku std lines</td>
<td>4</td>
<td>3</td>
<td>154.5</td>
<td>344</td>
<td>0.13</td>
<td>279</td>
<td>0.57</td>
<td>1.1</td>
</tr>
<tr>
<td>Among Kotuku DH lines</td>
<td>8</td>
<td>7</td>
<td>193</td>
<td>197</td>
<td>0.69</td>
<td>211.5</td>
<td>1.28</td>
<td>16.8</td>
</tr>
<tr>
<td>Between Kotuku DH pop. &amp; std pop</td>
<td>2</td>
<td>1</td>
<td>47</td>
<td>620</td>
<td>1.2</td>
<td>82</td>
<td>3</td>
<td>6.9</td>
</tr>
<tr>
<td><strong>Otane x maize</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Among Otane lines</td>
<td>4</td>
<td>3</td>
<td>169</td>
<td>65.3</td>
<td>6.45</td>
<td>69</td>
<td>1.4</td>
<td>16.31</td>
</tr>
<tr>
<td>Among Otane DH lines</td>
<td>4</td>
<td>3</td>
<td>262.9</td>
<td>333.3</td>
<td>0.58</td>
<td>34.1</td>
<td>1.43</td>
<td>43.5</td>
</tr>
<tr>
<td>Between Otane DH pop. &amp; std pop</td>
<td>2</td>
<td>1</td>
<td>29</td>
<td>22.2</td>
<td>0.13</td>
<td>279</td>
<td>0.04</td>
<td>15.13</td>
</tr>
<tr>
<td><strong>Belfield x maize</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Belfield std lines</td>
<td>3</td>
<td>2</td>
<td>Awnless</td>
<td>422.5</td>
<td>0.22</td>
<td>8</td>
<td>0.01</td>
<td>1.41</td>
</tr>
<tr>
<td>Among Belfield DH lines</td>
<td>4</td>
<td>3</td>
<td>75.3</td>
<td>2.77</td>
<td>187</td>
<td>1.06</td>
<td>3.83</td>
<td></td>
</tr>
<tr>
<td>Between Belfield DH pop. &amp; std pop</td>
<td>2</td>
<td>1</td>
<td>128.6</td>
<td>1.44</td>
<td>86</td>
<td>0.53</td>
<td>2.82</td>
<td></td>
</tr>
</tbody>
</table>

Note: DH, std, and * represent doubled haploid, standard parent line and significance within group lines at 5% probability level respectively.
5.4: Discussion

To examine whether significant aberrant genetic variation is introduced into wheat DH populations produced by the wheat x maize method, this study was designed to minimise the opportunity for non-genetic factors to influence variation in the measured traits. Parent populations were subjected to the same glasshouse and field growing conditions as DH populations and parent seed was interspersed with DH seed in the same rows. This study established that of the five cultivars tested, no detectable (significant) genetic variation was introduced during the production of DHs for the cultivars Kotuku, Otane and Belfield over 13 quantitative traits. One quantitative trait was significantly different within Rata DH lines. Within Monad DH lines, genetic differences were detected in three of the quantitative traits. Rata and Monad were the cultivars where the most DH lines were examined. Therefore it is more likely that an off type line, which is presumably a rare event, would be detected within these cultivars. Laurie and Snape (1990) examined 29 DH lines, made from three wheat genotypes and showed a small amount of aberrant genetic variation. The present study extends these results. A further 40 DH lines produced from five commercial wheat varieties were examined for 13 quantitative traits and also showed minimal aberrant genetic variation.

Dot plots of data and Fisher’s pairwise comparisons revealed that of the four significantly different traits, it was usually only one DH line that was outlying from other lines. In Monad DH lines flag leaf length (line seven) and awn length (line nine) had lower mean lengths than other DH lines. It is possible that this was a result of a mutational event occurring during the production of these DH lines. Aberrant genetic variation has been shown to occur through chromosomal structural changes and/or behavioural deletions (Youssef et al., 1989). Baenziger et al., (1983) showed that this aberrant variation is generally deleterious to agronomic performance. Although an agronomic property was significantly lower in Monad DH lines seven and nine, the difference to other lines was minimal (few significant differences in Fisher’s pairwise comparisons). This would indicate that if a mutational event had occurred, the effects were small. Since such events are relatively uncommon and the DH lines were collectively not significantly different when compared with their parent lines (Tables 5.3 and 5.4), they would have minimal effect on a breeding programme.
The mean spike length of Rata DH lines was increased in the DH population and the mean ear emergence time was faster in the Monad DH population. In both cases one line (line three-Rata and line two-Monad) was an obvious outlier from all other lines. These changes can be considered non-detrimental with respect to a wheat breeding programme. Hassan (1996) reported that heritable changes in wheat induced by colchicine led to a significantly greater "improvement" in characters observed, including vegetative growth, flowering time, and grain yield. However, other studies of introduced genetic variation indicate it is unlikely that a random mutational event would cause such a non-deleterious change and these significant differences are probably a result of another sort of variation (Baenziger et al., 1989b).

In conclusion, the wheat x maize method for producing wheat DHs did generate some genetic variation above that present in the parent material. However, the magnitude of the variation was small and infrequent, with only four of 40 DH lines affected for over 13 quantitative traits and five cultivars. In addition, when the DH lines were compared with their parent lines, no significant differences were observed. Therefore the aberrant genetic variation seen in this study is unlikely to be detrimental in a wheat breeding programme.
6: Testing a model for the integration of marker assisted selection and doubled haploid technology into a wheat breeding programme

6.1: Introduction

The production of high frequencies of DH lines in cereal breeding allows the advancement of breeding lines to homozygosity in a single generation. This has the potential to considerably reduce the breeding time of new cereal cultivars. However, there is concern that if homozygosity is reached in early generations there is a loss of recombination events which could have created useful genetic combinations for breeders. Most barley haploids produced originate from an F1 cross (Snape and Simpson, 1981, Choo et al., 1985). In the presence of linkage it has been shown that the total genetic variance of DH populations derived from different generations differ, with the F1 generation having the least genetic variation (F1 < F2 < F3) (Snape and Simpson, 1981). Delaying DH production to the F2 or F3 generation allows for the breakup of repulsion linkages and creates increased opportunity to generate new allelic combinations at unlinked loci (Snape and Simpson, 1981, Choo et al., 1985, Patel et al., 1985).

In order to fully understand the impact early homozygosity has in a breeding programme, it is important to compare genetic variation of DH lines to those bred conventionally. This is difficult because residual heterozygosity, selection or dominance can affect the performance of conventionally bred lines (Bjornstad, 1987). Despite these difficulties, the use of DH technology in cereal breeding programmes needs to be fully evaluated if the technology is to reach maximum efficiency. Recently, proposed schemes for the use of DHs in breeding programmes have involved the integration of F2 derived DHs with marker assisted selection (MAS) (Howes et al., 1998).

Molecular markers are a potentially powerful tool in breeding programmes, enabling the direct selection for desirable traits. MAS relies on the existence of DNA polymorphisms between parents so that selection of progeny can be based on genotype rather than phenotype. The efficiency of MAS in breeding programmes depends on a number of factors, the most important being the number of markers involved and the closeness of the
markers to the desired genes (Lande and Thompson, 1990, Zhang and Smith, 1993).
When the numbers of markers are high and the linkages are close, then a high proportion
of the variation under selection can be accounted for by the markers.

In using MAS in breeding programmes, the breeding strategy used depends on the number
of genes under selection (Howes et al., 1998). At this stage, it is thought that combining
more than 12 marked gene loci is not feasible due to the large numbers of F2 plants which
would need to be screened (> 2000) (Howes et al., 1998). If heterozygote plants are
selected then they need to be retested in subsequent generations in case of marker
segregation, regardless of whether selections are made on early or late generations. This
would be time consuming and expensive when selecting for quantitative traits where many
genes may be involved (Edwards and Page, 1994). Selected genes can be fixed if a DH
step is used following marker selection and simulation studies have shown that DHs
increase the efficiency of MAS and offer faster strategies of combining large numbers of
genes with fewer marker tests (Howes et al., 1998).

MAS can be both expensive and labour intensive. For these reasons it is important to
balance the need to screen as few plants as possible with the need to retain genetic
variation in the MAS population. In conventional breeding, when a population has small
numbers, random genetic drift can cause significant fluctuations around the Hardy­
Weinberg equilibrium. For example, in a population where there are two alleles at one
locus, if the population size becomes too small, one of the alleles can easily be lost,
resulting in the other allele being “fixed” in the population. This process is compounded
by MAS as any genetic material which is closely linked to one of the markers will have a
high probability of being inherited and fixed along with the marker.

The aim of this chapter was to test a genetic model (appendix one) for the effects of
random genetic drift and MAS with DH technology on genetic variability. In doing this, it
should be possible to provide guidelines as to the minimum number of marker-selected
DH plants required by a breeder to maintain sufficient genetic variation for selection in
subsequent generations. An initial cross between two wheat cultivars, Rata and Monad,
was made and three polymorphic, independently segregating PCR-based (polymerase
chain reaction) HMW (high molecular weight) glutenin protein markers were used to test
the model. The model was tested by measuring the genetic variation in DH populations.
derived from the marker selected F₂ population and comparing this with the genetic variation in the unselected F₃ population.

6.1.1: The genetic model

The model outlined in appendix one estimates the proportion of genetic material that is fixed by the MAS and DH processes. For example, if ‘n’ progeny are produced, the proportion of genetic material which is identical in all progeny is estimated. The fixed genetic material includes the desirable genes selected for and may also include genes which co-segregate with the selected gene. Thus, with each selection a proportion of the chromosome is fixed (Fig. 6.1). In Fig. 6.1 the desirable allele “A” is selected to be present in all three progeny. As the progeny are DHs, each individual has two identical copies of the chromosome. The chiasmata produced during meiosis cause cross-over of genetic material, and the distribution of these across the gene pool of F₂ plants determines what fraction of the chromosome is fixed. The hatched areas in the composite chromosome are portions where all three individuals inherited DNA from only one of the parents. Fig. 6.1 also shows how genetic material can be fixed even when it is derived from chromosomes, or parts of chromosomes, which do not have selected markers. This corresponds to random genetic drift, ie, any small population in which genetic material can be permanently lost by chance.

In designing the genetic model the following assumptions were made:

i) That recombination events occur randomly on the chromosomes (ie there are no cross-over ‘hot-spots’).

ii) That all markers segregate independently (no linkage). The HMW-Glu markers used to test the model are present on the long arms of the group one chromosomes; chromosomes-1A, 1B and 1D. At each locus two genes are present which are tightly linked, they are known as the ‘x type’ and the ‘y type’ (Payne, 1987, Anderson and Greene, 1989). Only the HMW-Glu ‘x-type’ markers were used in this study.

iii) That the molecular markers used in selection are always linked to the gene of interest. In this study, the actual genes were used as markers, so linkage was not an issue.
In testing the genetic model, the positions of the three HMW *Glu-1* x-type markers were entered into the model to estimate the percentage of genetic variation which would be fixed in the selected population (Fig. 6.2). The positions of the three markers used were as follows: *Glu-1* A x-type gene is 8.3 cM from the centromere of the 1A chromosome which is 149.8 cM long, *Glu-1* B x-type gene is 7.1 cM from the centromere of the 1B chromosome which is 150.9 cM long, *Glu-1* D x-type gene is ~10.5 cM from the centromere of the 1D chromosome which is 182.8 cM long (Leroy *et al.*, 1997).

According to the model, less than 1% of the genome is fixed in a population of 100 DHs which are selected for being homozygous for three selected markers, while approximately...
10% of the genome is fixed in a population of five DHs which are selected for being homozygous for three selected markers (Fig. 6.2). To ensure 10% was the maximum genetic variation fixed in this study, we aimed to obtain a minimum of five plants with all selected markers from which to make DHs. Thus 364 Rata x Monad F2 plants were screened.

**Fig. 6.2:** The expected amount of genetic material which is fixed in doubled haploid populations produced following marker assisted selection
6.2: Materials and methods

6.2.2: Marker assisted selection

6.2.2.1: Plant material
Two wheat cultivars (Rata and Monad) were used to test the model, based on polymorphisms at the HMW Glu-1 locus of interest. HMW Glu-1 x/y locus configurations for Monad and Rata cultivars are as follows: Rata 1A: null, 1B: 7/9, 1D: 3/12, Monad 1A: 2*, 1B: 17/18, 1D: 5/10. Rata and Monad were crossed to produce F1 seed which was collected and re-sown to produce F2 plants for DNA extraction and selection (Fig. 6.3).

6.2.2.2: DNA extraction
Genomic DNA was isolated from leaf tissue (2 g) of 364 single plants in order to identify a minimum of five wheat plants homozygous for all three 'Rata' loci. Liquid nitrogen was added to the leaf tissue in a mortar. The leaf tissue was ground into a fine powder with a pestle. Four mL of extraction buffer (1% sarkosyl, 100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA: pH 8.5) was added to the thawed leaf tissue. Once homogenised, the extract was transferred to a 15 mL Falcon tube. Two mL of phenol solution (phenol/chloroform/iso-amyl-alcohol, 25:24:1) were added and tubes were placed on ice and mixed on a rotor for 15 min. Material was then centrifuged (400 rpm, 10 min, Eppendorf centrifuge 5810R) and the supernatant was poured into silica matrix tubes and re-extracted with 1 mL of phenol solution for 10 min and re-centrifuged (4000 rpm, 10 min). The supernatant was then poured into a fresh 15 mL Falcon tube. Four hundred μL 3M NaAc (pH 4.8) and 4 mL isopropanol were added to precipitate DNA. Following centrifugation (4000 rpm, 10 min), the supernatant was poured off and the resultant pellet was washed in 70% ethanol. The DNA pellet was then air-dried and resuspended overnight at 4 °C in 350 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA: pH 8.0).

The DNA quantity of each sample was measured using a Pharmacia GeneQuant RNA/DNA calculator and diluted to 50 ng μL⁻¹.

6.2.2.3: PCR amplification with HMW Glu-1 x type locus markers
PCR was performed in a 25 μL reaction volume containing one unit of Taq DNA polymerase (Boehringer), 1 x Taq PCR buffer (Boehringer), 200 μM of each
deoxyribonucleotide (Boehringer), 10 ng each of the two primers (Life Technologies) and 50 ng of genomic template. Primers were prepared as per D'Ovidio et al., (1995) and had the following sequences:

1A gene:

- 5' AGATGACTAAGCGGTGTGGTC 3'
- 5' CTGGCTGGCCCAACATGCGT 3'

1B gene:

- 5' ATGGCTAAGCGCTGGTCCT 3'
- 5' TGCCTGGTCGACAATGCGTGC 3'

1D gene:

- 5' ATGGCTAAGCGGTAGTCTCT 3'
- 5' CTGGCTGGCCCAACATGCGT 3'

The thermocycler used for all PCR reactions was an Eppendorf Mastercycler Gradient. Amplification of the entire coding and repetitive regions of the A and D HMW x-type glutenin genes included an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 62 °C for 2 min and 72 °C for 2 min 30 secs, followed by a final incubation step 72 °C for 7 min. Amplification of the entire coding and repetitive regions of the B HMW-Glu x-type gene included the same initial denaturation step followed by 35 cycles at 94 °C for 1 min, 58 °C for 2 min and 72 °C for 2 min 30 secs, followed by the same final incubation step.

Amplified products were analysed on a 1.5% agarose gel in TBE (0.085M Trizma base, 0.09 M boric acid and 0.05M EDTA) and stained with ethidium bromide (0.5 mg mL⁻¹) for half an hour before viewing under a UV light source.

6.2.3: Doubled haploid production

Spikes from the selected Rata x Monad F₂ plants were emasculated one to two days before anthesis. At the approximate time of anthesis, spikes were pollinated with maize pollen (Yates ‘Early Miracle”) using a small paintbrush. Spikes were mist sprayed with 100 mgL⁻¹ 2,4-D one to two days after pollination. Seeds were dissected 13 to 15 days after pollination and developing embryos were rescued onto artificial nutrient medium containing B5 salts (Gamborg et al., 1968) supplemented with 20 gL⁻¹ sucrose and 12 gL⁻¹
Embryos were incubated in darkness at 20 to 24 °C until germination and then transferred to a 16-hour photoperiod (65 μmol m⁻²s⁻¹, P.A.R.). Haploid plants were transplanted to soil when 20 to 40 mm in height. To double chromosome numbers, haploid seedlings were placed in aerated colchicine solution (500 mgL⁻¹ colchicine, 2 % DMSO) for five hours. The treated seedlings were then washed in tap water and transferred to pots containing fresh potting mix. Self-pollinated seeds were harvested at maturity.

6.2.4: Measurement of genetic variation

All plants were sown at 1.5 cm depth in 11.5 cm x 9 cm sized PB 2½ Planta bags (one plant per bag) which ensured equal spacing among plants on the glasshouse bench. Glasshouse conditions were as follows: heating below 15 °C and ventilation above 22 °C, daylength supplemented when needed with sodium and mercury lights and relative humidity maintained > 60%. Three populations were sown and randomly positioned in the glasshouse:

1) An unselected population of 200 F₃ seeds which were randomly harvested from 200 F₂ plants.
2) A DH population which consisted of 108 DH lines homozygous for markers Rata HMW Glu-x type, A, B and D.
3) Two parent (Monad and Rata) populations (15 plants per parent).

Six quantitative traits were measured in all three populations one week after anthesis: plant height, flag leaf length, flag leaf width, number of nodes, spike length and awn length.

6.2.5: Statistical analysis

Most of the data in this chapter were analysed using Minitab© version ten. Chi-squared tests were used to confirm that the observed segregation fitted the expected ratios. The means of the parent populations were compared using t-tests. The variances of the selected DH and unselected F₃ populations were compared using F-ratios and population means were compared using t-tests. Both populations were also tested for the shape of distribution (presence/absence of skewness and kurtosis).
DHs plants were grouped into separate lines according to which of the 15 selected plants they were derived from. One way ANOVA was then used to compare the different DH lines to test if the selected plant from which they were derived had any effect on the overall genetic variation in the selected population. If the plant from which they were derived had no influence on the DH progeny, then the means for a given characteristic, such as height, would be relatively homogenous across all lines. However, the distribution of one DH trait was not normal and as normality is a requirement for ANOVA to be statistically valid, a non-parametric bootstrapping method was also used to analyse the data (Efron and Tibshirani, 1996). Randomly re-assorting the DH progeny as if they had come from different plants would have had little effect on the means or the variance of the means. Bootstrapping was used to repeat the re-assortment of the DH progeny 10,000 times. The percentage of occasions where the re-assorted variance was greater than the observed experimental variance was measured. Statistical significance was reached if this percentage was less than 5%.

The distribution of height in the selected DH population was bimodal. The data were further analysed using a "mixture of two normals" model (Lynch and Walsh, 1998a). This model assumes that the data comes from either a normal distribution, with mean \( \mu_1 \) and variance \( \sigma_1^2 \) with probability \( p_1 \), or from an alternate normal distribution, with mean \( \mu_2 \) and variance \( \sigma_2^2 \) with probability \( 1-p_1 \). The location of the two peaks of such a bimodal distribution depend on \( \mu_1 \) and \( \mu_2 \); the width of the two peaks depend on \( \sigma_1^2 \) and \( \sigma_2^2 \); and the height of the two peaks relative to one another on \( p \) (as well as \( \sigma_1^2 \) and \( \sigma_2^2 \)).

The parameters \( \mu_1, \mu_2, \sigma_1^2, \sigma_2^2, \) and \( p \) were estimated using the expectation-maximisation (EM) algorithm for maximum likelihood estimators (Lynch and Walsh, 1998b). Likelihood ratio tests were used to test for significance. Both the bootstrapping and EM algorithm methods were performed using Matlab ©.

A summary of the methods used to test the model is represented in Fig. 6.3.
$F_1$ Harvested and resown in the glasshouse

$F_2$ DNA extracted from 364 plants and screened with three independent markers

$F_2$ (Selected) Homozygotes for one or two markers and heterozygotes for one or two markers selected

Selected plants crossed with maize to produce DHs. Haploid heterozygotes rescreened and reselected.

Genetic variation in DH population of selected plants compared to genetic variation in unselected $F_1$ plants.

**Fig. 6.3:** Diagram of experimental design to test the model for the integration of DH and MAS for accelerated wheat breeding
6.3: Results

6.3.1: Marker assisted selection

PCR products of Monad and Rata cultivars showed polymorphisms at all HMW-\textit{Glu} x-type loci.

6.3.1.1: HMW \textit{Glu}-D x-type locus

Progeny segregating as homozygous for the Rata type were selected (Fig. 6.4). Of the 364 plants assayed only 313 produced PCR products. The remaining 51 plants were not re-screened due to the time constraint of having to screen plants before anthesis. Of the 313 plants which produced PCR products, 67 were homozygous for the Rata HMW \textit{Glu}-D x-type marker. Although progeny which were homozygous for the Rata HMW \textit{Glu}-D x-type marker could be distinguished from heterozygotes, progeny which were homozygous for the Monad HMW \textit{Glu}-D x-type marker could not be distinguished from heterozygous progeny (Fig. 6.4). A goodness of fit Chi-square test confirmed that the segregation of heterozygotes + Monad homozygotes: Rata homozygotes (246: 67) fitted the expected 3: 1 ratio at the 5% probability level.

6.3.1.2: HMW \textit{Glu}-A x-type locus

The 67 plants which were homozygous for the Rata HMW \textit{Glu}-D x type allele were screened for the Rata HMW \textit{Glu}-A x type allele (Fig. 6.5). The segregation of Rata homozygotes: Rata/Monad heterozygotes: Monad homozygotes (14: 34: 19) was confirmed by Chi-square analysis to fit the expected 1: 2: 1 ratio at the 5% probability level. Due to the loss of 51 plants during screening with the HMW \textit{Glu}-D x type marker, both heterozygotes and homozygotes were selected (total 48 plants-14 + 34), as this increased the probability of obtaining five plants homozygous for all three Rata HMW \textit{Glu} x-type alleles. For the purpose of this study haploid plants had to be re-screened for the HMW \textit{Glu}-A x type allele.

6.3.1.3: HMW \textit{Glu}-B x-type locus

The 48 plants identified with the Rata \textit{Glu}-D and \textit{Glu}-A x type alleles were screened for the presence of the HMW \textit{Glu}-B x type allele (Fig. 6.6). Although progeny which were homozygous for the Rata HMW \textit{Glu}-B x-type marker could be distinguished from
heterozygous progeny, progeny which were homozygous for the Monad HMW *Glu-B* x-type marker could not be distinguished from the heterozygotes (Fig. 6.6). Chi-square analysis confirmed segregation of heterozygotes + Monad homozygotes: Rata homozygotes (33: 15) fitted the expected 3: 1 ratio at the 5% probability level.

### 6.3.1.4: Re-screening of haploid plants with HMW *Glu-A* x-type locus

All 15 selected plants were crossed with maize to produce haploid plants. The 56 haploid progeny from the six plants known to be homozygous for all three markers were not re-screened. The 106 haploid progeny from the remaining nine plants were re-screened with the *Glu-A* x type marker. Of these plants 52 had the *Glu-A* x type marker and along with the other 56 plants these were treated with colchicine to produce doubled haploids.
**Fig. 6.4:** Agarose gel illustrating segregation of the HMW *Glu-D* x-type marker. 

M=Monad parent, R=Rata parent, -ve=negative control, hz/m=heterozygous or Monad type progeny, **rat**=selected Rata progeny.
**Fig. 6.5:** Agarose gel illustrating segregation of the HMW *Glu-A* x-type marker. M = Monad parent, R = Rata parent, -ve = negative control, hz = heterozygous, mon = Monad type progeny, **rat** = selected Rata type progeny.

**Fig. 6.6:** Agarose gel illustrating segregation of the HMW *Glu-B* x-type marker. M = Monad parent, R = Rata parent, -ve = negative control, hz/m = heterozygous or Monad type progeny, **rat** = selected Rata progeny.
6.3.2: Comparison of genetic variation in selected and non-selected populations

The two parent populations, Rata and Monad were compared using t-tests. For the six traits measured, four traits were significantly different (Table 6.1).

### Table 6.1: Means and standard deviations of Rata and Monad parent populations

<table>
<thead>
<tr>
<th>Trait</th>
<th>Rata parent population</th>
<th>Monad parent population</th>
<th>t-test p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>56.5 ± 5.5</td>
<td>91.5 ± 10.5</td>
<td>&lt; 0.001 ***</td>
</tr>
<tr>
<td>Flag leaf length (cm)</td>
<td>25.0 ± 5.5</td>
<td>32.5 ± 6.0</td>
<td>0.002 ***</td>
</tr>
<tr>
<td>Flag leaf width (mm)</td>
<td>18.0 ± 2.0</td>
<td>19.5 ± 1.5</td>
<td>0.014 *</td>
</tr>
<tr>
<td>Node number</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>0.717 ns</td>
</tr>
<tr>
<td>Spike length</td>
<td>3.5 ± 0.5</td>
<td>4.0 ± 0.5</td>
<td>0.049 *</td>
</tr>
<tr>
<td>Awn length</td>
<td>93.0 ± 9.0</td>
<td>89.0 ± 5.0</td>
<td>0.163 ns</td>
</tr>
</tbody>
</table>

Note: ns, *, *** represent non significance and significance at 5% and 0.1% probability levels respectively.

Comparisons of the six measured traits between the unselected F₃ population and the selected DH populations showed that height was the only trait to significantly differ in both F-ratios and t-tests. The distribution of the height trait in the selected population was bimodal (Fig. 6.7). Distribution curves were relatively normal for both selected and unselected populations for each trait which had no significant difference in both F-ratios and t-tests (flag leaf length, flag leaf width, node number, spike length and awn length) (Figs. 6.7-6.12).
Fig. 6.7: The distribution of means for height in the unselected F₃ population (green) and the selected DH population (blue). Statistics for unselected population: mean = 70.5 cm, variance = 149.5 cm, skewness = -0.12, kurtosis = -0.82. Statistics for selected DH population: mean = 74.5 cm, variance = 230.5 cm, skewness = 0.311, kurtosis = -0.97. Population comparisons: F-test, populations significantly different, t-test, populations significantly (p < 0.01) different. Note: both F-ratios and t-tests require normality, therefore tests may not be valid because of the bimodal distribution of the selected population.

Fig. 6.8: The distribution of means for flag leaf length in the unselected F₃ population (green) and the selected DH population (blue). Statistics for unselected population: mean = 28.0 mm, variance = 29.0 mm, skewness = -0.11, kurtosis = 0.176. Statistics for selected DH population: mean = 29.0 mm, variance = 35.0, skewness = -0.11, kurtosis = -0.33. Population comparisons: F-test, non-significant, t-test, non-significant.
Fig. 6.9: The distribution of means for flag leaf width in the unselected F₃ population (green) and the selected DH population (blue). Statistics for unselected population: mean = 18.5 mm, variance = 4.5 mm, skewness = -0.06, kurtosis = 0.56. Statistics for selected DH population: mean = 18.5 mm, variance = 4.5, skewness = -0.45, kurtosis = 0.77. Population comparisons: F-test, non-significant, t-test, non-significant.

Fig. 6.10: The distribution of means for number of nodes in the unselected F₃ population (green) and the selected DH population (blue). Statistics for unselected population: mean = 4, variance = 1, skewness = 0.31, kurtosis = -1.12. Statistics for selected DH population: mean = 4, variance = 1, skewness = -0.02, kurtosis = -0.57. Population comparisons: F-test, non-significant, t-test, non-significant.
Fig. 6.11: The distribution of means for spike length in the unselected F₃ population (green) and the selected DH population (blue). Statistics for unselected population: mean = 89.0 mm, variance = 105.5 mm, skewness = 0.22, kurtosis = 0.61. Statistics for selected DH population: mean = 90.0 mm, variance = 86.5 mm, skewness = 0.31, kurtosis = 0.31. Population comparisons: F-test, non-significant, t-test, non-significant.

Fig. 6.12: The distribution of means for awn length in the unselected F₃ population (green) and the selected DH population (blue). Statistics for unselected population: mean = 88.5 mm, variance = 115.5 mm, skewness = -0.02, kurtosis = -0.22. Statistics for selected DH population: mean = 87.5 mm, variance = 121.5 mm, skewness = 0.19, kurtosis = -0.14. Population comparisons: F-test, non-significant, t-test, non-significant.
6.3.3: Analysis of genetic variation among selected DH populations

The groups of DH lines derived from each of the 15 selected plants were compared (Figs. 6.13-6.18). Height and awn length were significantly different among the 15 lines on ANOVA and were close to significantly different \((p = 0.06)\) on non-parametric bootstrapping.

**Fig. 6.13:** Comparison of height of selected DH lines. Analysis of variance established a significant \((p < 0.05)\) difference in height among the DH lines. Non-parametric bootstrapping established near significance \((p = 0.06)\).
Fig. 6.14: Comparison of flag leaf length of selected DH lines. Analysis of variance was non-significant ($p > 0.05$). Non-parametric bootstrapping was non-significant.

Fig. 6.15: Comparison of flag leaf width of selected DH lines. Analysis of variance was non-significant ($p > 0.05$). Non-parametric bootstrapping was non-significant.
Fig. 6.16: Comparison of node number of selected DH lines. Analysis of variance was non-significant ($p > 0.05$). Non-parametric bootstrapping was non-significant.

Fig. 6.17: Comparison of spike length of selected DH lines. Analysis of variance was non-significant ($p > 0.05$). Non-parametric bootstrapping was non-significant.
Fig. 6.18: Awn length of selected DH lines. Analysis of variance established a significant ($p < 0.05$) difference in awn length among the DH lines. Non-parametric bootstrapping established near significance ($p = 0.06$).

6.3.4: Further analysis of doubled haploid height data

The DH height data were analysed further due to their bimodal distribution using the "mixture of two normals" model described in section 6.2.5. The means of the two peaks of the DH population were similar to the means of the original homozygous parent population (Table 6.2). The estimated probability that a given individual DH came from distribution (or peak) one was 0.6354 whilst the probability a given DH individual came from distribution (or peak) two was 0.3646. The likelihood ratio showed that these $p$ values were significantly different to the expected probabilities of 0.5 and 0.5.

Table 6.2: A comparison of the height means and variances of the parent populations to the two peaks in the bimodal distribution of the selected DH population

<table>
<thead>
<tr>
<th></th>
<th>Rata parent</th>
<th>DH peak one</th>
<th>Monad parent</th>
<th>DH peak two</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height mean (cm)</td>
<td>56.5</td>
<td>64.5</td>
<td>91.5</td>
<td>92</td>
</tr>
<tr>
<td>Height variance (cm)</td>
<td>28</td>
<td>60</td>
<td>115</td>
<td>49.5</td>
</tr>
</tbody>
</table>
The probability density function curve produced from the "mixture of two normals" model was compared to the distribution of the raw DH data and shows an excellent fit (Fig. 6.16).

**Fig. 6.19:** A comparison of a probability density function curve produced from a "mixture of two normals model" to the raw selected doubled haploid data for height.
6.4: Discussion

The model tested in this study estimates that if three markers are used for MAS and a population of 100 DHs are produced, less than 1% of the genetic variation required by a breeder is lost (Fig. 6.2). This study established that in most traits this estimation is valid. Of the six traits measured four (height, flag leaf length, flag leaf width and spike length) differed significantly between the parents. Of these four traits, in the selected DH population three (flag leaf length, flag leaf width and spike length) were normally distributed and showed no significant differences in statistical comparisons to the unselected F3 populations. For the two other traits measured (node number and awn length) the selected DH distributions were normal and the variances equal to the unselected F3 population.

When the DH plants were grouped into separate lines according to which of the 15 selected plants they were derived from, ANOVA revealed that two traits (height and awn length) exhibited significant ($p < 0.05$) “line effects”. Because ANOVA works on the assumption that data are normally distributed, it may not have been an appropriate test for height. Therefore, a non-parametric test, bootstrapping, was also used to analyse the data. Of the six traits, no traits were significantly different, although both height and awn length were near significance ($p = 0.06$). These analyses, combined with the t-tests and F-ratios, indicate that in most traits there were sufficient numbers of DH lines to maintain genetic variation for a breeder. A breeder would still have enough DH lines to discard those derived from a “rogue” selected plant. However, if fewer than five plants were selected in the MAS process, it would be likely genetic drift would occur. For example, DH lines 9, 11 and 14 (Fig. 6.13) have a different distribution of height than the other DH lines and this difference is reflected in the statistical significance of the ANOVA. A breeder would have a moderate chance of obtaining such an unrepresentative sample if they only selected two, three or four plants from which to make DHs. This chance becomes much less when greater than five selected plants are produced.

While this problem of genetic drift confronts any breeder working with small numbers, it is exaggerated by MAS. As mentioned earlier, in the process of selecting for certain desirable genes a certain proportion of “passenger” genetic material in the vicinity of these genes is simultaneously selected. For example, from the model, if three markers were
used, a minimum of five selected plants would cause a maximum loss of 10% genetic variation. Presumably less genetic variation would be lost if more than one DH was produced from each selected plant. If only two plants were selected a maximum of 52% of the genome would be fixed and this may not be reduced significantly even with multiple DH progeny taken from each selected plant.

Height of the selected DH plants was distributed bimodally and was significantly different to the unselected F₃ population in both F-ratios and t-tests. The mean of the peaks in the bimodal distribution were similar to the parent means, indicating that the bimodal distribution is most likely to be a result of height being conveyed monogenically (the major gene involved was probably the \textit{Rht-2} dwarfing gene in Rata) as opposed to polygenically in the Rata x Monad cross. If a trait has a major determinant gene with two possible alleles, then a DH population will show a bimodal distribution with the two peaks representing the two homozygous states. A conventional F₃ population would have a more normally distributed population (as in Fig. 6.7 unselected population) reflecting the presence of heterozygosity. In future generations, when this F₃ population was selfed, its distribution would become bimodal as heterozygosity was reduced.

What is interesting about the height data is that the two peaks, or modes, of the DH population do not have the same weighting. If the major determinant gene in the Rata x Monad cross (eg. \textit{Rht-2}) was the only factor involved in the segregation, then a Mendelian segregation pattern would be expected. Each DH plant would have the same chance of inheriting its homozygosity from the Rata (semi-dwarf) parent as the Monad (tall) parent and \( p \) in the “mixture of two normals” model would be close to 0.5. This is not the case and more progeny were present in the first (semi-dwarf) peak. There are several possible explanations for this. Firstly, the major determinant gene could be weakly linked to one of the HMW-\textit{Glu} x type genes, causing the distorted segregation. Secondly, the expression of the major determinant gene could be affected by other genes, probably inherited from the other parent. This is an epistatic effect and could cause unexpected genetic drift in small populations. Thirdly, the dwarfing gene could produce a small survival advantage in the process of producing DHs (ie gametic selection in tissue culture), ensuring greater than 50% representation in the population.

If factors such as linkage and/or epistasis are affecting the distribution of height and
causing a difference in genetic variation for the breeder to select from, this has important ramifications for the use of greater numbers of markers than used in this study. Further analysis of the model using greater marker numbers and establishing the effects of factors such as linkage and epistasis would be of use.

In conclusion, the model in this chapter was designed to integrate DH technology and MAS into a wheat breeding programme and is the first of such models to be biologically tested. The results show that in five out of six traits the model estimation was valid and less than 1% of genetic variation was lost in the selected DH population. The sixth trait showed some unexpected results that illustrate some of the difficulties inherent in genetic manipulation. The results also show that the number of selected plants used to produce DHs is important and “DH line effects” may come into account if fewer numbers are used. This model should prove to be a valuable support tool for breeders in evaluating numbers of markers and sizes of DH populations required for successful integration into wheat breeding programmes.
7: General discussion

7.1: Major findings of thesis

The deregulation of the New Zealand wheat industry in 1987, combined with increased demand for improved and specialty wheat products, has meant that breeders have had to increase the range and quality of new wheat cultivars to meet market demands. Using DH technology allows a reduction in the time taken to produce a new wheat cultivar by up to four years. The goal of this thesis was to investigate the opportunity for increasing the efficiency of developing genetically improved New Zealand wheat cultivars by inducing direct homozygosity through wheat x maize crosses. In order to achieve this goal a number of objectives were formulated. This chapter discusses how each of the objectives contributed towards achieving this overall goal.

7.1.1: Development of the wheat x maize method for producing wheat doubled haploids

The first objective of this thesis was to establish a wheat x maize method suitable for New Zealand wheat cultivars and the New Zealand climate, which could be used routinely in a breeding or genetics programme. To achieve this objective, New Zealand wheat cultivars and hybrids were crossed with different maize lines during all seasons over two years (1995 and 1996). DHs were successfully obtained from all wheat genotypes. This verified the use of the wheat x maize method for producing DHs over anther culture which has been constrained by recalcitrant genotypes. Initially the major limitation in the crosses was poor embryo germination. This was apparent for all cultivars, but germination rates were particularly low in two cultivars (<20% in Belfield and Kotuku). A number of factors were identified which affected germination success. These included: timing of embryo excision, method of auxin application and media composition. Manipulation of these components led to improved embryo germination success for all cultivars (70-90%) and an overall improved method to produce DHs.

Although germination of embryos was improved, it was found that the numbers of haploid embryos per spike differed throughout the year. A significantly greater number of embryos were recovered from two hybrid crosses in summer months (18 and 28% of florets pollinated) compared with autumn (11 and 9% of florets pollinated) and winter
months (no haploid embryos). This could have been due to environmental factors affecting the wheat plants and/or the maize plants. Further investigation of these factors may lead to a more efficient system for producing wheat DHs.

7.1.2: The effects of environmental factors on wheat x maize haploid embryo recovery

To further improve the efficiency of the wheat x maize system developed in chapter two, it was important to identify environmental factors which influenced embryo recovery. These factors could include temperature, light intensity, spectral quality, photoperiod and nutrient status. Seasonal changes in these factors could be acting individually, or in combination, to affect the maize plant and/or the wheat plant. The objective of chapter three was to investigate the effect of factors most likely to be causing the seasonal differences observed. These were temperature and light intensity.

New Zealand wheat cultivars were grown in a glasshouse until booting, then transferred to growth cabinets at three temperatures (day/night; 17/12, 22/17 or 27/22 °C at an irradiance of 250 μmol m⁻² s⁻¹ P.A.R). In another experiment wheat lines were transferred to a growth cabinet at one of three light intensities (300, 500 or 1000 μmol m⁻² s⁻¹ P.A.R. at 22/17 °C day/night, with a photoperiod of 16 hrs).

The temperature and light intensity at which pollinations were made and subsequent fertilisation and embryo development occurred, significantly influenced the frequency of haploid embryo production. The optimal temperature for embryo recovery for three of the five cultivars was 22/17 °C. The greatest number of embryos was produced at a light intensity of 1000 μmol m⁻² s⁻¹.

It was not known why temperature and light intensity should have such effects on the success of wheat x maize crosses. Light intensity had a greater effect on haploid embryo numbers than temperature. Therefore, in chapter four the responses of two cultivars, Karamu and Kotuku, which responded differently to light intensity in chapter three, were examined in more detail. Pollen tube growth, embryo survival and indicators of rates of photosynthesis were measured at two light intensities (250 or 750 μmol m⁻² s⁻¹). It was found that pollen tube growth was significantly affected by light intensity in Karamu plants but not in Kotuku plants, despite both cultivars being pollinated from the same
maize source. The percentage of pollen tubes reaching the cavity between the ovarian wall and integuments and in the micropyle of Karamu plants at high light intensity (65%) was nearly three times greater than the percentage at low light intensity (22%). This showed that low light intensity, or shading, can affect the maternal wheat plant in a way which inhibits pollen tube growth and/or that high light intensity may be promoting pollen tube growth in Karamu plants.

Significant differences observed in ETR rates and overall quantum yields in plants grown at the differing light intensities indicated that the rate of photosynthesis may also have a direct effect on pollen tube growth, perhaps acting in conjunction with other factors such as PGR concentration.

7.1.3: A near absence of aberrant genetic variation in wheat x maize doubled haploid lines

Once the wheat x maize method of producing DH lines was established, it was critical to evaluate the DH lines to ensure the end products were actually homozygous and therefore true true breeding. Studies on the genetic variation in other DH populations show that genetic variation may be influenced by the occurrence of mutations during DH production and by the influence of gametic and other forms of inadvertent selection (Laurie and Snape, 1990; Snape et al., 1992). Thus, the objective of chapter five was to determine if aberrant genetic variation was introduced into wheat DH populations during their production by wheat x maize crosses.

Wheat x maize DH populations were made from homozygous inbred cultivars. Forty DH lines from five cultivars (Rata, Monad, Kotuku, Otane and Belfield) were compared with their parent lines under field conditions. DH lines from cultivars Kotuku, Otane and Belfield showed no significant differences for any of the 13 quantitative traits measured. The Rata DH lines differed significantly for spike length and the Monad DH lines differed significantly for flag leaf length, ear emergence time and awn length. The magnitude of the variation was small and infrequent and the variations could often be attributed to one outlying line. As well, the DH populations were not shown to be significantly different to the parent populations.
Overall, the results confirmed other studies (Laurie and Snape, 1990, Lefebvre and Devaux, 1996) and showed that most DH lines produced by the wheat x maize method resemble their wheat parent and that the introduction of low amounts of aberrant genetic variation is unlikely to be detrimental in a wheat breeding programme. Having established this, the next step in the thesis was to determine when DHs should be used in the New Zealand wheat breeding programme.

7.1.4: The testing of a model for the integration of doubled haploid and marker assisted selection technologies into a wheat breeding programme

The objective of chapter six was to determine where in a wheat breeding programme DHs should be used for maximum genetic gain. The successful use of DHs in cereal breeding programmes is dependent on a number of factors. These include at which filial generation DHs are made (Snape and Simpson, 1981) and the size of DH populations required to retain genetic variation sufficient for selection in future generations. Molecular markers are beginning to make a significant impact in wheat breeding worldwide. Recent simulations have shown that the use of DH technology in conjunction with MAS improves the efficiency of MAS (Howes et al., 1998). In chapter six a model, (appendix one) was partly tested for the integration of these two technologies into the New Zealand wheat breeding programme.

The genetic model showed that if a minimum of five plants homozygous for three markers were obtained, then approximately 10% of the genetic variation of the selected plants would be fixed. If 100 DHs were obtained then less than 1% of the genetic variation would be fixed. To test the model Rata and Monad cultivars were crossed, as these cultivars differ at three HMW *glu-l* loci. The F₂ generation was screened using three PCR based HMW *glu-l* x-type markers and 108 DHs were made from the 15 selected progeny. The genetic variation of the selected DH lines was then compared to the genetic variation of unselected F₃ plants.

The results showed that of the six traits measured five (flag leaf length, flag leaf width, node number, awn length and spike length) were normally distributed and had no significant difference in genetic variation to the unselected F₃ population. Height however, was bimodally distributed and analysis of the data using a "mixture of two
normals” model showed that although the inheritance of a major height gene/s probably caused the distribution, there may have been other factors involved such as linkage and/or epistasis. When the DHs were grouped and compared according to which of the 15 selected parents they were derived, analysis showed that “line effects” existed. This has implications for the selection of fewer plants than 15, in that there would be a greater chance of a high proportion of the DHs being derived from one or two “rogue” plants.

Overall, the model was shown to be a useful tool for wheat breeders allowing them to estimate the minimum DH population size, following MAS, required to retain sufficient genetic variation for selection in later generations.

7.2: Contribution of this thesis to cereal breeding and genetics

Current wheat breeding programmes used in New Zealand are based on pedigree and bulk methods for the production of new wheat cultivars. As previously discussed, it can take up to six years to reach homozygosity and up to twelve years before a new cultivar is released. The development of a reliable system for the production of wheat DHs over a range of genotypes is a valuable tool for use in current New Zealand wheat breeding programmes. The method developed enables the production of approximately 1-2 DH plants per spike pollinated. Therefore, if five spikes are pollinated per day, 10 to 20 days of crossing are required to produce a DH population of 100 plants. From the initial planting of wheat seeds, it takes eight to ten months for the final production of DH seed. Thus, populations of homozygous wheat lines can be produced within 12 months for evaluation in the overall wheat programme the following year. The breeding strategy used to implement DHs will depend on the breeding objectives for the various crosses.

The ability to produce homozygous wheat lines in a single generation is not only a valuable tool for New Zealand wheat breeding but it will also be an essential tool for progress in genetical understanding of New Zealand wheat. A considerable number of international wheat genome mapping projects are based on DH populations. Once the crosses of interest for mapping are made, DH populations are produced. These populations are particularly useful in mapping studies as they have fewer genotypic classes than F₂ populations, traits which are conveyed by recessive alleles are not masked by dominant alleles and any genetic markers found will not be lost during segregation in
following generations (Pauls, 1996). Projects are currently underway in New Zealand (Ahmad and Griffin, 1998) where wheat DH populations will be produced, using the method outlined during this thesis, to attain homozygous lines for genetic mapping for both quality traits and disease resistance traits. The use of DH populations will also be valuable in determining how genes interact in New Zealand wheats. For example, Choo and Reinbergs (1987) showed how DH populations can be used for detecting pleiotropy and linkage of genes. Analysis of traits such as this will be especially useful for examining genes which are unique to New Zealand wheats, for example, resistance to certain disease strains or some of the quality characteristics of interest in the specialty wheat programme.

Since wheat and maize were first crossed to produce DHs (Laurie and Bennett, 1986), most of the efforts to improve haploid embryo recovery has been in the manipulation of post-pollination PGR treatments, (Laurie and Bennett 1988b, Laurie 1989b, Guo et al.1994, O’Donoughue and Bennett 1994, Pienaar and Lesch 1994, Suenaga and Nakajima 1989). Although these efforts have been successful in improving the numbers of haploid embryos recovered, during this thesis and in other studies, differences in embryo numbers were found to occur at different times of the year. This study showed that both temperature and light intensity significantly affected the numbers of haploid embryos recovered from wheat x maize crosses. The effect of temperature has been noted in other wide crosses (Pickering and Morgan, 1985, Sitch and Snape, 1987a, Nkongolo et al., 1991, Molnár-Láng and Sutka, 1994) but the effect of light intensity has not been reported previously for any wide cereal crosses. In this study, the highest light intensity used (1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) produced significantly higher numbers of haploid embryos. This effect of light intensity was shown to act at the level of pollen tube growth which was dependent on the maternal parent’s response to differing light intensities. The effect of light intensity on pollen tube growth seen in this study may be relevant to many wide cereal crosses, as it shows that pollen tube growth can be altered by manipulation of the growing conditions of the maternal plant. If this is shown to be important in other wide crosses then an increase in pollen tubes reaching the micropyle of cereal pistils should see an improvement in fertilisation and overall success rates of wide crosses.

The effect of light intensity on pollen tube growth in wheat x maize crosses may also be of importance in wheat developmental studies which have shown that wheat fertility can be
affected by pre and post-anthesis shading (Grabau et al., 1990, Slafer, 1995). It is assumed
that the reduced fertility is a result of poor floret development near the spike apex
(Rawson and Ruwali, 1972), which may be related to sink capacity or source supply of
assimilates (Grabau et al., 1990). The results from this study indicate that photosynthetic
activity may be important in acting directly or in conjunction with other factors to alter
pollen tube growth in wheat x maize crosses. This may also be the case for wheat selfing
in the field.

Both anther culture and intergeneric crosses are used in cereal breeding for the production
of DHs. Wide crosses for producing cereal DHs are widely used, despite the cost
effectiveness of anther culture as up to 30% aberrant genetic variation has been shown to
be introduced into cereal DHs during the anther culture process (Baenziger et al., 1983, de
Buyser, 1985, Baenziger et al., 1989b, Skinnes and Bjornstad, 1995). This study
compared 13 quantitative traits, presumably covering a major proportion of the genome, of
DH lines and their parent lines and the results confirmed other preliminary reports, that the
production of wheat DHs through intergeneric crosses results in the introduction of only
very low amounts of aberrant genetic variation. This low level is not expected to have any
effect on a cereal breeding programme. Thus, the near absence of aberrant genetic
variation in wheat DH lines produced through the wheat x maize method allows
confidence in using the method to produce true-breeding wheat lines which are
particularly important for use in genetic studies and gene mapping work. It also allows
increased confidence in the use of intergeneric crosses to produce true breeding DH lines
in other cereal wide crosses.

There is still much debate as to how to integrate DH technology in cereal breeding
programmes for maximum results. Models have been designed to combine DH and MAS
technologies (Howes et al., 1998). Chapter six of this thesis is the first reported attempt to
validate such a model using biological data. The results showed that DH and MAS
technologies can be combined with minimal loss of genetic variation in a wheat breeding
programme. When 108 DHs were obtained from 15 plants selected with three markers,
less than 1% genetic variation was lost in most traits when compared with a conventional
F₃ population. Genetic variation of height did differ in the DH population indicating that
other genetic factors may be involved which may affect such models. The only way in
which genetic models can be designed with any degree of simplicity is by making various
genetic assumptions. Providing these assumptions are kept in mind by a breeder, the results show that the model tested in this study should be a valuable tool for estimating the numbers of markers and DH population sizes required to obtain maximum genetic variation for selection in subsequent generations.

7.3: Future directions for this work

The production of one to two wheat DHs per spike pollinated shown in this study compares favourably with other published wheat × maize methods (Inagaki and Tahir, 1990, Amrani et al., 1993, Lefebvre and Devaux, 1996, Zhang et al., 1996). However, Snape (1998) recently produced a review showing typical frequencies from work at the John Innes Centre and a UK plant breeding company. In these studies 1.4 DHs per spike were produced in four wheat genotypes and in another eight wheat genotypes, 4.1 DHs per spike were produced. The methods used to obtain the results in this review are unavailable due to commercial sensitivity, however they do suggest that there is potential for further improvement in the wheat × maize method developed in chapter two.

A direct comparison of DH production in optimal light and temperature conditions to 'non-optimal' conditions has not been made in this study due to time limitations. The improvements in embryo numbers at higher light intensities may lead to further improvements in the method in the next few years. At the optimal temperature (22/17 °C, day/night) and light intensity (1000 μmol m⁻² s⁻¹) found in this study, 38% of florets pollinated produced embryos. Assuming the average embryo germination rates (79%) and doubling rates (81%) found in chapter two would not change, then with 38% of florets pollinated producing haploid embryos the overall success rates could reach 6.1 DH plants per spike pollinated in some genotypes. In the temperature and light intensity experiments not all embryos were cultured to produce haploid plants and DRs. It may otherwise have been possible to show a direct result of improved environmental conditions on DH recovery as well as on haploid embryo recovery. In addition to this, a suitable growth cabinet to emulate the optimal conditions was not available at Crop & Food Research, Lincoln, to produce the DH populations that were used in following chapters of this thesis. These populations, therefore, could not be used to make this comparison.

As previously discussed, the influence of light intensity on pollen tube growth in Karamu
plants is relevant for other wide cereal crosses as well as wheat selfing in the field. However, the work done in this thesis is preliminary and future studies on the effect of light intensity on fertilisation in wheat x maize crosses and a more complex analysis of sucrose and other photosynthates would be needed to examine the effect further.

It would also be of importance to consider the possible influence environmental conditions could have on maize pollen. As mentioned in chapter two, viability of maize pollen grains are influenced by the environment in which they are produced (Barnabas and Rajki, 1976, Neuffer, 1982). Seasonal variation in the glasshouse, therefore, could have had an effect on pollen viability and subsequent wheat fertilisation. If optimal conditions for maize pollen viability, germination success and tube vigour were known for wheat x maize crosses, then this too may result in an improved system.

A number of studies have examined the distribution of RFLP markers in cereal DH populations to see if the DH method is introducing genetic variation (Bentolia et al., 1992, Murigneux et al., 1993, Lefebvre and Devaux, 1996). This overcomes the inherent disadvantages of field trials in dealing with environmental effects on quantitative traits as well as genetic variation. However, the advantage of using field trials is that agronomically important quantitative traits can be examined. Continuing research into QTL markers means that when they are available in the future they will be a better prospect for examining whether aberrant genetic variation is introduced into DH populations by any method.

Even with future improvements in the wheat x maize system, the method will always be limited to the number of florets which can be physically pollinated in a specific time. There is continued research into cereal microspore culture (Patel et al., 1994, Harwood et al., 1995, Kasha et al., 1998) which may provide the next breakthrough in DH technologies (Snape, 1998). This is because several thousand microspores can be obtained from a single spike and induced to undergo sporophytic development. Albinism and recalcitrant genotypes are still significant problems with the use of this technique (Raina, 1997), but if these limitations can be overcome, many more crosses could be handled than with the wheat x maize system. Until then, the wheat x maize method is superior for producing DH populations in wheat breeding programmes. This is largely because of the ‘true-breeding’ nature of the DH lines produced when compared to anther culture lines.
Aberrant genetic variation may also be introduced in microspore culture techniques and if these techniques come to the fore in the future, DH lines will need to be carefully re-evaluated.

If DHs are to be used to their full potential, the area requiring the most research is the integration of DHs into breeding programmes. This integration needs to be done without the loss of the genetic variation required by breeders and it needs to be performed in an efficient and economic manner. As shown in the general introduction section of this thesis, there have been a number of theoretical and practical studies in this area, particularly with barley breeding (Thomas et al., 1984, Bjørnstad, 1987, Bjørnstad and Aastveit, 1990, Kjær et al., 1990). Recent studies on integrating DHs into wheat breeding programmes have concentrated on their integration with MAS (Howes et al., 1998). Although this study tested a model integrating DH and MAS technologies into a New Zealand wheat breeding programme, this work is preliminary. To develop such a programme fully, a study would need to be conducted over a number of years. This may involve the evaluation of at least one cross in a number of DH and MAS schemes and comparing the breeding value of resulting plants to generations of the same cross in a conventionally bred programme. Although this would be a considerable task, the rapid improvements in gene mapping, plant screening and general understanding of the wheat genome means that the efficient integration of DHs into breeding programmes is essential if these advances are to have maximum effect.
8: Conclusions

Each objective of this PhD thesis contributed to the overall goal of this thesis: the increased efficiency of the development of genetically improved New Zealand wheat cultivars by inducing direct homozygosity through wheat x maize crosses.

- A method of wheat DH production through wheat x maize crosses, using New Zealand germplasm was developed.

- Optimal environmental conditions for the production of wheat DHs through wheat x maize crosses were defined.

- Wheat DHs produced through the wheat x maize method were found to be 'normal' and genetically stable for use in breeding programmes and genetical studies.

- A model was partly tested whereby DHs are integrated with MAS for maximum genetic gain in a wheat breeding programme.

Objectives achieved in this thesis combined with future work, particularly in the area of integrating DHs into wheat breeding programmes, should result in the more efficient development of improved New Zealand wheat cultivars.
9: Literature cited


**Inagaki, M. and Bohorova, N. (1995).** Factors affecting the frequencies of embryo


Suenaga, K. (1994). Doubled haploid system using the intergeneric crosses between


embryogenesis in wheat x maize crosses influenced by 2,4-D. *Annals of Botany, 77*: 639-647.


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“Your friend is the man who knows all about you and still likes you”.
Appendix one: Mathematical model

Note: This model was designed with considerable input from Dr Peter Campbell, c/o Flinders Medical Centre, Bedford Park 5042, South Australia.

To determine a statistical expression for the expected proportion of genetic material that becomes fixed by marker assisted selection (MAS) and doubled haploid (DH) technologies, we make the following assumption —

The chiasmata are distributed along the length of a chromosome, [0,L], according to a Poisson process with rate 1, where L is the length of the chromosome in Morgans.

Let n be the number of DH offspring produced by MAS assisted selection.

Consider a chromosome with a locus for one of the selected genes. Then, from the assumption above, the distribution of chiasmata along this chromosome is a Poisson point process with rate 1, for each individual.

We consider an imaginary ‘composite’ chromosome where the chiasmata for each of the n individuals are combined (Fig. 6.1). It is known from the theory of point processes that the ‘sum’ of n independent Poisson processes on [0,L] with rate 1 is a Poisson process with mean n.

Let Z be the length, in Morgans, of genetic material in the gene pool of the n individuals that is fixed. Let $X_i$, with $0 < X_1 < X_2 < \ldots \leq L$, $i = 1,2,\ldots$, be the order statistics for the distribution of chiasmata along the chromosome. Let $P_i$ be the indicator variable of fixed genetic material between chiasmata $I$ and $I-1$, such that —

$$P_i = \begin{cases} 1, & \text{if genetic material between chiasmata } i \text{ and } i-1 \text{ is identical for all } n, \\ 0, & \text{if genetic material between chiasmata } i \text{ and } i-1 \text{ has both parents' genes among } n. \end{cases}$$

It follows that —

$$Z = P_0(X_1 - 0) + P_1(X_2 - X_1) + P_2(X_3 - X_2) + \ldots$$
Because the $P_i$ and the $X_i$ are independent, we know that the expected amount of genetic material that is fixed in the population is given by —

$$E[Z] = E[P_0]E[X_1] + E[P_1]E[X_2 - X_1] + E[P_2]E[X_3 - X_2] + ...$$  \hfill (1)

**Calculating $P_i$**

The random variables $P_i$, for $i=1,2,\ldots$, can be viewed as a random walk on the chromosome.

Let $p_i(j)$, $i=1,2,\ldots; j=0,1,\ldots,n$, be defined as the probability that across all $n$ F1 offspring between chiasmata $I$ and $I-1$ there are precisely $j$ copies of the female’s chromosome (and hence $n-j$ copies of the male’s genetic material).

Consider the transition probability at a chiasma. Here, one (and only one) of the $n$ F1 offspring changes from one parent’s genetic material to the other parent’s. From the theory of Poisson point processes, each individual has an equal probability, $1/n$, of changing. Thus, the Markov chain transition probability matrix, $Q$, can be written as —

$$Q = \begin{bmatrix}
0 & 1 & 0 & 0 & \ldots & 0 & 0 & 0 \\
\frac{1}{n} & 0 & \frac{n-1}{n} & 0 & \ldots & 0 & 0 & 0 \\
0 & \frac{2}{n} & 0 & \frac{n-2}{n} & \ldots & 0 & 0 & 0 \\
\ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots \\
0 & 0 & 0 & 0 & \ldots & \frac{n-1}{n} & 0 & \frac{1}{n} \\
0 & 0 & 0 & 0 & \ldots & 0 & 1 & 0
\end{bmatrix}$$

From this, we know that the vector of probabilities after the $i$th chiasma is given by —

$$\begin{bmatrix}
p_i(0) \\
p_i(1) \\
\vdots \\
p_i(n)
\end{bmatrix} = \begin{bmatrix}
p_0(0) \\
p_0(1) \\
\vdots \\
p_0(n)
\end{bmatrix} Q^i,$$

where $\begin{bmatrix}
p_0(0) \\
p_0(1) \\
\vdots \\
p_0(n)
\end{bmatrix}$ is the starting distribution.

It follows that —
\[ \Pr(P_i = 1) = p_i(0) + p_i(n), \text{ and therefore,} \\
\mathbb{E}[P_i] = p_i(0) + p_i(n). \]

(2)

The starting distribution is easy to calculate. For chromosomes on which we select for a marker, we can consider the random walk starting at the marked gene locus. At this locus, the starting vector will be \([1 0 0 \ldots 0]\), because all offspring will have the marked gene from just one parent. Of course, the random walk will have to be performed twice, once for the 5' direction and once for the 3' direction.

For chromosomes on which we do not select for a marker, the number of copies from each parent will be distributed according to the binomial distribution with parameters \(n\) and 0.5, and the random walk can begin from either the 3' or 5' end.

**Calculating \(X_i\)**

With a point process, the distance from one chiasma to the next is called a renewal function. With a Poisson process on an infinite line, this renewal function is the simple exponential function. However, when the Poisson process occurs on a finite line such as a chromosome the renewal function is somewhat more complicated.

As above, we consider the end of the chromosome to be a chiasma. Then, with \(X_0 = 0\), the renewal function for \(X_i\) is

\[
f_{X_i}(x_i) = \begin{cases} 
  n e^{-na}, & 0 \leq x_i < L; \\
  \delta_L(x_i) e^{-na}, & x_i = L; \\
  0, & x_i < 0 \text{ or } x_i > L,
\end{cases}
\]

where \(\delta_L(x)\) is the Dirac delta function at \(L\).

Because the sum of any \(I\) exponential distributions with parameter \(n\) is a gamma distribution with parameters \(n\) and \(I\), we can generalise this to —
\[ f_{X_i}(x_i) = \begin{cases} \frac{n!}{(i-1)!} x_i^{i-1} e^{-\lambda x_i}, & 0 \leq x_i < L \\ \delta_i(x_i) \left[ 1 - \int_0^L \frac{n!}{(i-1)!} y^{i-1} e^{-\lambda y} dy \right], & x_i = L \end{cases} \]

It follows from this that —

\[ E[X_i] = \int_0^L \frac{n!}{(i-1)!} x_i^i e^{-\lambda x_i} dx_i + L \left[ 1 - \int_0^L \frac{n!}{(i-1)!} x_i^{i-1} e^{-\lambda x_i} dx_i \right]. \]

With a little manipulation, we can then find an expression for the difference \( E[X_i - X_{i-1}] \) —

\[ E[X_i - X_{i-1}] = \frac{1}{n} \left( 1 - e^{-nk} \sum_{j=0}^{i-1} \frac{(nL)^j}{j!} \right). \]  

**Completing the Model**

We can now calculate the expected amount of genetic material that is fixed by MAS, using equations (1), (2) and (3) above. Because the value of \( E[X_i - X_{i-1}] \to 0 \) as \( i \to \infty \), the sum quickly approaches its limit. In using the model we know that wheat has 42 chromosomes with known lengths in Morgans (Leroy et al. 1997).
Appendix two: List of publications and presentations


