PRODUCTION AND UTILIZATION OF DOUBLED HAPLOID LINES IN WHEAT BREEDING PROGRAMS

by

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Summary

For doubled haploids to become a valuable breeding technique, it is necessary that:

1. Success of doubled haploid production is not genotype dependent
2. The protocol used is efficient in time and cost
3. The doubled haploid population produced represents a random sample of gametes

For wheat, to date, intergeneric hybridization with maize has been shown to be more efficient than other methods used for other cereals such as anther culture or hybridization with *Hordeum bulbosum*.

The protocol which was established and later used in this project involved maize cultivar Illini Gold which was the most efficient pollen donor of the six maize and two sorghum lines tested. 2,4-D at 150 mgl^{-1} was applied after pollination to increase embryo formation rates. The best culture medium for embryo rescue and regeneration was found to be half strength MS.

This protocol was then used to produce 217 doubled haploids from the F_1 of a cross between Trident and Molineux wheats. This cross was polymorphic for a number of attributes. The genetic control of some of these characters was known and the segregation patterns of these characters were used to test for randomness of haploid production. The inheritance of the other characters which were of economic importance were unknown and the doubled haploids were used to investigate their inheritance.

Segregation patterns for high and low molecular weight glutenin subunits (*GluA1, GluA3, GluB3*), resistance to cereal cyst nematode (*Cre*), plant height, boron tolerance (*Bo1*) and stem rust resistance (*Sr38*) were investigated. In all cases these genes showed no distorted segregation ratios. The doubled haploids produced appeared to be from a random sample of wheat female gametes and so could confidently be used in gene mapping and to investigate the inheritance of other attributes.

The other characters segregating in the Trident/Molineux doubled haploid population included resistance to *Septoria tritici*, coleoptile length, leaf colour, heading date and grain yield. As many of these attributes appeared to be polygenic in nature, the four cumulants of their distributions viz. mean, variance, skewness and kurtosis were calculated. Also, the number of genes (or effective factors) controlling these attributes in this cross were estimated. The highest estimate was for coleoptile length of dwarf lines (10 genes) and semidwarf lines (8 genes), the height variation within the semidwarf group (8 or 9 genes) and leaf colour (9 genes). The results from two methods used for scoring *Septoria tritici* blotch reaction were in agreement with the results of other investigators and it was estimated that 2 to 3 genes determine resistance to this disease. Additive epistasis and gene interaction were observed in some biased distributions. Multivariate analyses and associations between some of these characters, for example, coleoptile length with plant height, leaf colour with yield and Septoria reaction with plant height and maturity are presented.

In a separate study a limited number of doubled haploids were produced for 24 different F_1 crosses. These doubled haploids were then compared with F_5 lines derived from the same crosses made in a conventional pedigree breeding program several years earlier. These populations were grown in hill plots in the field and measurements on grain yield, yield components, plant height and anthesis date were recorded and compared between the appropriate DH and F_5 populations. Very few significant differences were found. Thus, even small populations of doubled haploid lines could be used by wheat breeder to predict the likely success of individual crosses.

Unfortunately, the hill plot grain yields did not correlate with the grain yield of some of the lines under test (from which there was sufficient seed) when grown in conventional row-plots so the data produced could not be used to select for yield on farms.

The breeding value of the doubled haploids produced in these studies was evaluated and some of the lines are being multiplied for widescale evaluation as potential new varieties to replace Trident in South Australia. The Trident/Molineux population produced is a valuable resource for further genetic studies.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Chapter 1

General Introduction

Doubled haploids being completely homozygous are valuable germplasm for use in plant breeding, molecular biology and cytogenetics. The production of doubled haploids (DH) involves two steps: the production of haploid plants followed by doubling of their chromosome numbers. In cereals, there are two principal methods used for haploid production, namely, (i) anther culture and (ii) intergeneric hybridisation followed by embryo rescue.

Although anther culture has been developed as an efficient production system for haploids in species such as barley (Choo *et al.*, 1985; Lorz *et al.*, 1988; Foroughi-Wher and Wenzel, 1990; Pickering and Devaux, 1992; Frappell, 1993), this method needs to be developed further in wheat in order to efficiently produce sufficient plants for routine plant breeding purposes. So far, the intergeneric hybridisation system using maize, sorghum or pearl millet as pollen donor parents is considered to be more efficient than the other methods for providing sufficient number of doubled haploid populations. Using *Hordeum bulbosum* as the pollen donor parent for wheat haploid production showed less efficiency due to its problem with crossability genes (Laurie and Reymondie, 1991).

Plant breeding requires large numbers of doubled haploid plants containing sufficient genetic variability to enable improved varieties to be selected. The ultimate goal of plant breeding is to produce a superior variety with higher yield and quality and lower susceptibility to diseases, pests and other limiting abiotic factors (eg. nutrient deficiency, toxicity, water deficit). To achieve this aim, it is desirable to start with a large population

containing many combinations of these beneficial traits. In traditional methods of plant breeding, selection from a large number of gene combinations is time consuming and the period from the initial cross to the marketing of a variety often takes 15 years or longer (Lindsey and Jones, 1992). Finding a way to reduce this time period for the production of new varieties is of great interest to plant breeders. Time-saving and improving the efficiency of selection are the main advantages of using and doubled haploid breeding systems (Jensen, 1972; Chase, 1974). Each doubled haploid is a homozygous line, and therefore has the potential of being a new variety without reselection. The use of doubled haploids also has the potential to reveal more variation than some of the traditional methods (Choo *et al.*, 1985). Furthermore selection of the desirable lines among doubled haploids is easier than conventional methods as it requires less time and labour to achieve homozygosity.

There are still many problems associated with the doubled haploid procedure which need to be solved before it can be applied widely or routinely in plant breeding. Suenaga (1994) lists some of the essential characteristics of doubled haploid populations if they are going to be of real value in genetics and plant breeding, namely, (1) doubled haploid lines should be efficiently produced from all genotypes, (2) doubled haploids should be genetically normal and stable, and (3) doubled haploids must represent a random sample of the parental gametes (Snape *et al.*, 1980).

This thesis reports on studies undertaken to investigate some of these considerations particularly:

-To establish an efficient protocol for DH production using the wheat × maize system (see Chapter 3),

-To determine whether the DH production system used in this study causes distorted segregation of genes at loci polymorphic between parental cultivars (see Chapter 4),

-If DH lines are truly a random sample of gametes then to study the inheritance of some economic traits whose genetics is not fully understood (see Chapter 5),

-To investigate if populations of DH lines, which can be produced in two generations, can be used to predict the performance of F_5 lines obtained from conventional breeding programs after 5 generations (see Chapter 6), and

-To combine information arisen from this study to draw a recurrent selection plan to advance testing the DH lines for next steps of breeding program (see Chapter 7).

Part I

Production of doubled haploid lines in wheat

Chapter 2

Review of Literature

2.1. Doubled Haploids, Early History and Applications

Production of haploid plants in high frequency has recently received increased attention because of their potential use in plant breeding and genetic studies. There are many reports of haploid production in a wide range of higher plant species (Pierik, 1989). The utilization of haploidy in plant breeding, gene mapping and plant biotechnology has rapidly increased recently in many agricultural and horticultural crop plants. Cytologists have also used haploid plants as research tools to reveal the tendencies of non-homologous chromosomes to pair at meiosis (Bennett *et al.*, 1976).

Haploids, which are sporophytes containing the gametic number of chromosomes, can be classified into two main groups, monoploids and polyhaploids, depending on whether they are derived from diploid or polyploid parents, respectively (Choo *et al.,* 1985; Jensen, 1988; Thorn, 1992).

The first report on haploid plants was published by Johansen (1934) crossing with barley. However, the frequency of naturally occurring (spontaneous) haploids (Johansen, 1934; Kimber and Riley, 1963) or induced haploids (Muntzing, 1938; Tometorp, 1939; Clavier and Cauderon, 1951; Suzuki, 1959; Yoshida and Yamaguchi, 1973) initially was too low to permit their use in plant breeding programs (Jensen, 1982; Choo *et al.,* 1985). The most important points for the successful introduction of a haploid production system to

facilitate the breeding of crop plants are the efficiency of the system, the ability of producing a random sample from any given heterozygous genotype, and the ability of developing stable lines with high agronomic performance. By 1940, the sporadic occurrence of haploids either spontaneously or following chemical treatment, had been reported for *Hordeum*, *Oryza*, *Secale* and *Zea* (Kimber and Riley, 1963).

2.2. Applications of Haploids

2.2.1. Rapidly Achieving Homozygosity

Haploids can be used for the production of homozygous lines from heterozygotes (East, 1930). Alternatively, homozygosity in self-pollinated crops (eg. wheat) can usually be achieved after five to seven generations since heterozygosity is halved with each generation of selfing. Haploids resulting from heterozygotes are usually treated with colchicine to produce homozygous lines in one generation. These doubled haploid plants are completely homozygous except for any mutation induced by colchicine (Pickering, 1980). Doubled haploid breeding has received considerable interest in recent years because methods of inducing haploidy have become more efficient (Baenziger *et al.*, 1984) and recent reviews are shown in Table 2.1. Research on haploids has also resulted in the development of a number of cultivars such as Florin released in France in 1987 (for details see Kasha and Seguin-Swartz, 1983; Choo *et al.*, 1985; De buyser *et al.*, 1987; Morrison and Evans, 1988; Drew, 1993).

Table 2.1. Authors of recent reviews on doubled haploid production in plants

Author (s)	Year	Author (s)	Year
Reinert and Bajaj	1977	Baenziger and Schaeffer	1983
Nitzche and Wenzel	1977	Baenziger *et al.*,	1984
Kihara	1979	Choo *et al.*,	1985
Maheshwari *et al.*,	1980	Dunwell	1985
Hu and Shao	1981	Kasha *et al.*,	1989
Kasha and Reinbergs	1981	Pickering and Devaux	1992
Collins and Genovesi	1982		

2.2.2. Cytogenetic Research and Genome Mapping

Doubled haploids have also been widely used in cytogenetic studies. Research on wide crosses giving haploids (eg. *Solanum tuberosum* × S. *phureja; Hordeum vulgare* × *H. bulbosum*; *Triticum aestivum* × *H. bulbosum*; *T. aestivum* × *Zea mays; T. aestivum* × *Pennisetum americanum; T. aestivum* × *Sorghum bicolor*), and the genetic basis of haploidy (eg. chromosome elimination during embryo development) has been an important area of cytogenetic study in recent years (Kasha, 1974; Davies and Hopwood, 1980; Maluszynski, 1989).

Doubled haploids have also been widely used to produce mapping populations for the construction of genome maps with the help of random fragment length polymorphism (RFLP) markers (Laurie *et al.*, 1992; Suenaga and Nakajima, 1993a).

2.3. Haploid Production Systems for Wheat Modelled on Barley system

Choo *et al.* (1985) classified the systems for haploid production in barley into the four following categories: Gynogenesis (ovary culture), haploid initiation gene (pseudogamy), androgenesis (anther culture), and chromosome elimination (bulbosum method)(see also

Thorn, 1992; Pickering and Devaux, 1992). With wheat, haploid production has relied mainly on chromosome elimination and androgenesis, but modifications of the techniques have been necessary to increase the frequency of haploid progeny.

2.3.1. In vitro Androgenesis (Anther Culture)

Donor plant growing conditions, microspore stage, pretreatment to induce androgenesis and the culture medium are the most important factors affecting the success of wheat androgenesis systems (Foroughi-Wehr *et al.,* 1976; Zhou and Yang, 1980; Frappell, 1993). Although Xu and Sunderland (1982), Huang *et al.* (1984) and Xu and Huang (1984) have pointed out the importance of anther density in the culture vessels on the response of barley anthers to culturing, apparently this factor is not so critical for wheat. The size of the culture vessel has been implicated (Chuang *et al.,* 1978), as well as the effect of anther placement and orientation in the culture medium affect the response of barley, but not wheat (Hunter 1985; Shannon *et al.,* 1985; Powell *et al.,* 1988). Use of liquid media with Ficoll (Kao, 1981) or conditioned media (Xu *et al.,* 1981; Huang and Sunderland, 1982) had resulted in a high percentage of callus development (up to 90%) in anther culture.

One of the most serious problems with this androgenesis approach is the high frequency of albino plants. Albino production is mostly affected by genotype (Andersen *et al.,* 1987; Knudsen *et al.,* 1989; Simmonds, 1989; Zhou and Konzak, 1989), microspore stage (He and Ouyang, 1984), the physiological stage of the donor plants (Bullock *et al.,* 1982) and high culture temperatures (Bernard, 1980; Ouyang *et al.,* 1973; Chen, 1986). Consequently, the ratio of albino to green plants is highly important in determining the success of anther culture in cereal species (Wenzel *et al.,* 1977; Foroughi-Wehr *et al.,* 1982).

2.3.2. *Interspecific and Intergeneric Hybridizations*

The first important step in any breeding programs is the selection of suitable parental materials (Hadley and Openshaw, 1980). In most cases one of the parents is a current commercial cultivar which has widely been grown and usually plant breeders choose the other parent from the same biological species, because such crosses give fertile hybrids and there are few or no genetic barriers. However, in certain cases the other parent may be selected from another species to make interspecific crosses for the reasons discussed by Briggs and Knowles (1967):

i) to transfer one or a few desirable genes from an exotic species,

ii) to obtain new character expression not found in either parent,

iii) to produce a new species, and

iv) to determine the evolutionary relationship of one species to another.

For example, breeders have used interspecific crosses between *Hordeum vulgare* × *Hordeum spontaneum* to transfer desirable traits of wild species to cultivated barley and *Triticum aestivum* × *Secale cereale* with the aim of producing a new crop, Triticale (Muntzing, 1979). Wheat is crossable with many other species in the Triticeae and the potential of these inter- and intra-specific wide-crosses for wheat improvement has been reviewed by several investigators (Sharma and Gill, 1983; Zeller and Hsam, 1983; Riley and Law, 1984; Fedak, 1985; Goodman *et al.*, 1987; Islam and Shepherd, 1990).

2.3.2.1. Wheat × *Hordeum bulbosum* Hybrids

H. vulgare and *H. bulbosum* when hybridized at the diploid level usually give haploid *H. vulgare* progenies due to the preferential elimination of *H. bulbosum* chromosomes during embryo development (Kasha and Kao, 1970; Subrahmanyam and Kasha, 1973a). Barclay (1976) obtained a similar result when bread wheat was crossed with *H. bulbosum*. He used both diploid (*2n=2x=14*) and tetraploid (*2n=4x=28*) *H. bulbosum* as the male parent in

crosses with the wheat cultivar Chinese Spring and succeeded in producing haploid wheat plants in both crosses but the frequency was not high with 4× *H. bulbosum* (43.7%).

The difficulty with this system is that it depends on the wheat genotype used and no grains were set in crosses with cultivars such as Hope, Cappelle-Desprez and Vilmorin 27 with the tetraploid *H. bulbosum*, indicating that there is genetic variation for crossability between wheat cultivars (Barclay, 1975). Snape *et al.* (1979) extended Barclay's investigation using a wider range of wheat varieties to examine their crossability with *H. bulbosum* and also to elucidate the genetic control of crossability. They chose cultivars which were known to have high or low crossability with rye, *Secale cereale* (*2n=2x=14*) because the results of Barclay (1976) suggested that there is a relationship between the crossability of wheat with both rye and *H. bulbosum.* Chinese Spring has high crossability, and the other cultivars used by Barclay have low crossability with both species. They found a strong positive correlation between the crossabilities of wheat varieties with *H. bulbosum* and with rye. By using chromosome substitution lines of the non-crossable cultivar, Hope, into the crossable cultivar Chinese Spring, they identified that two chromosomes, namely 5A and 5B of Hope, markedly reduced the crossability of Chinese Spring. These chromosomes were already identified as being responsible for the crossability of Chinese Spring with rye by Riley and Chapman (1967), proving again that, at least in part, the same genetic system is influencing the crossability of wheat with both species. However, it has been shown that a given clone of *H. bulbosum* can behave differently when crossed with wheat (Sitch and Snape, 1987a). They found that the diploid clone, PB1, gave a low crossability with wheat even though it is highly crossable with barley (Simpson and Snape, 1981).

Snape *et al.* (1979) concluded that the crossability of wheat with *H. bulbosum* is restricted to a very few varieties and seems to be governed by genes *Kr1* and *Kr2* on chromosomes 5B and 5A respectively (Lein, 1943; Sitch *et al.,* 1985), which genes also determine crossability with rye. According to their study, wheat cultivars can be categorized into

crossable and non-crossable groups. The crossable group can be traced directly or indirectly to the variety Chinese Spring or to an Asian origin (Falk and Kasha, 1981). Most of the wheat cultivars highly crossable with rye, have their origins in China, Korea or Japan (Tozu, 1966; Riley and Chapman, 1967), or Australia (*al.,* 1979). In general, most wheat genotypes are non-crossable with *H. bulbosum* (Snape *et al.,* 1979; Falk and Kasha, 1981), and attempts to improve crossability with hormone treatments have not been successful (Falk and Kasha, 1982; Marshall *et al.,* 1983; Inagaki, 1986b).

2.3.2.2. Wheat × *Zea mays* Hybrids

Zenkteler and Nitzche (1984) were the first to report the production of globular haploid embryos using wheat × maize crosses. This finding encouraged Laurie and colleagues in 1986 to initiate extensive studies on wheat × maize crosses. They described the factors affecting crossability and frequencies of haploid embryo production and embryo rescue. The results of this work were reviewed by Laurie *et al.* (1990).

In their initial work Laurie and Bennett (1986) crossed Chinese Spring as the female parent with two maize genotypes Seneca 60, a single cross F_1 hybrid sweetcorn, and Zapalote Chico Oaxaca 57, a southern Mexican race. They found a relatively high frequency of embryo formation (22% overall).

They then studied the effect of the crossability loci *Kr1* and *Kr2* on fertilisation frequency in hexaploid wheat × maize crosses (Laurie and Bennett, 1987a). They selected three wheat cultivars based on the presence of homozygous recessive *kr1* and *kr2*, homozygous dominant *Kr1* recessive *kr2*, and homozygous dominant *Kr1* and *Kr2*, namely Chinese Spring, Chinese Spring (Hope 5B), a chromosome substitution line produced by E.R. Sears, University of Missouri, USA, Hope and Highbury, respectively. These female plants were pollinated by the Seneca 60 maize and the diploid rye cultivar, Petkus Spring. They concluded that the *Kr* loci were not important with respect to crossability with maize.

They also tested 25 out of 26 wheat × maize genotype combinations using spikelet culture, in which spikelets containing cross-pollinated florets were placed in culture medium two days after pollination (Laurie and Bennett, 1987b). They claimed that this technique was more successful than the conventional procedure and used it on non-crossable wheats with various combinations of *Kr* genes viz. Chinese Spring (*kr1, kr2*), Hope (*Kr1, Kr2*), Highbury (*Kr1, Kr2*), Chinese Spring (Hope 5A) (*kr1, Kr2*) and Chinese Spring (Hope 5B) (*Kr1, kr2*) (Laurie and Bennett, 1988c). The most significant finding of this study was that Hope (*Kr1, Kr2*), a variety that gave no seed set in cross with *H. bulbosum* (Snape *et al.,* 1979), gave haploid progeny in these crosses..

Subsequently, wheat × maize crosses have been used in many other studies (O'Donoughue and Bennett, 1988; Laurie and Bennett, 1988d, 1989; Suenaga and Nakajima, 1989; Laurie, 1989a,b, 1990; Laurie and Snape, 1990; Inagaki and Tahir, 1990; Laurie and Reymondie, 1991; Ushiyama *et al.,* 1991; Oury *et al.,* 1993; Kisana *et al.,* 1993; Suenaga and Nakajima, 1993a,b; Amrani *et al.,* 1993; O'Donoughue and Bennett, 1994a,b; Islam and Shepherd, 1994; Guo *et al.,* 1994). These investigations included using both hexaploid and tetraploid wheat cultivars in hybridization with maize and also compared the value of the wheat × maize method with anther culture as a means of producing wheat haploids.

2.3.2.3. Wheat × *Sorghum bicolor* Hybrids

Laurie and Bennett (1988b) and Ohkawa *et al.* (1992) studied fertilisation of wheat by sorghum and found that similar post-pollination events as found with maize resulted in the elimination of sorghum chromosomes. The largest sorghum chromosome is 7-fold smaller in length than the smallest wheat chromosome (Laurie and Bennett, 1988b). It was found that S9B sorghum is a superior pollinator for wheat haploid production since the observed frequency of fertilisation (69%) was twice that obtained with Chinese Spring wheat × Seneca 60 maize crosses (29.2%)(Laurie and Bennett, 1986, 1987b). The advantage of

using sorghum as a parent over maize is that it has the propensity to bloom throughout the year in a greenhouse without short-day treatment (Ono *et al.*, 1989) and thus provide a continuous supply of pollen. Ohkawa *et al.* (1992) tested five different sorghum genotypes and found that all the genotypes tested were able to produce haploid embryos with a frequency ranging from 8.9 to 21.1%. They found no significant difference among their sorghum stocks, but the differences between their finding and that of Laurie and Bennett (1988b) show that there is a great variation between sorghum and/or wheat genotypes to produce haploid plants or maybe the protocols used.

Despite these advantages sorghum has not been used as extensively as maize in the production of haploid wheat plants.

2.3.2.4. Wheat × *Pennisetum americanum* Hybrids

Laurie and O'Donoughue (1988) reported success in making wide crosses between tetraploid and hexaploid wheats and pearl millet. Laurie (1989) then used this cross to look for a more efficient pollinator than maize in wheat hybrid production. Zygotes were found to contain 21 wheat and 7 pearl millet chromosomes, but like with maize and sorghum, after a few cell division cycles all chromosomes of pearl millet were eliminated resulting in the production of haploid wheat embryos. Both cultivars Chinese Spring (*kr1*, *kr2*) and Highbury (*Kr1, Kr2*), gave similar frequencies of haploids in crosses with pearl millet indicating insensitivity to the action of the *Kr* loci (29 and 32%).

The embryos from the wheat × pearl millet crosses were more viable than those from wheat × maize, because the embryo developed along with an endosperm in most of florets in which fertilisation occurred. The ratio of fertilised egg cells to fertilised polar nuclei being 1.28:1 and 0.97:1 using pearl millet and 4.6:1 and 3.1:1 using maize as the pollen source for Chinese Spring and Highbury, respectively. Laurie concluded that there is no significant difference between Seneca 60 maize cultivar and Tit 23BE pearl millet variety

when they are used for production of haploid embryos in crosses with hexaploid wheat and so pearl millet could be used as a good alternative to the maize system (Laurie, 1989b).

Ahmad and Comeau (1990) tested eight different grain pearl millet accessions as pollen donor parents and a Japanese spring wheat cultivar Fukuho as female parent in the wheat × pearl millet system. They found no signs of incompatibility between pearl millet pollen and wheat stigmas.

Maize pollen needs a significantly longer time for pollen tube entry into the ovule than that observed in wheat × pearl millet crosses, since pistils fixed 8 hours after pollination did not demonstrate pollen tube entry whereas those fixed 24 hours after pollination often did. They also used two steps *in vitro* culture: 1) culture the ovules on Norstog II medium named U2.5 (Comeau *et al.,* 1990). 2) After ten days in culture, the ovules were dissected and scored for presence of embryos, which were then cultured until germination on another modified Norstog II, PNAC3 medium. Both steps were carried at room temperature in the dark.

In spite of the fact that wheat and pearl millet belong to different subfamilies of Poaceae (Gramineae), pearl millet pollen can normally germinate on wheat stigmas or grow into the wheat style and ovary. With regard to pollen germination and pollen tube entry into wheat ovules, Comeau *et al* (1990) reported that genotypic differences occurred among pearl millet accessions. There was not any definite relationship between percent pollen germination and percent pistil showing pollen tube entry into the ovule. For example, the pearl millet accession IP4021 showed the lowest pollen germination but highest pollen tube entry into ovules. Thus, it could be suggested that these two attributes may be independently determined.

Ahmad and Comeau (1990) found that in wheat Fukuho × pearl millet and wheat Fukuho × maize cv. Seneca 60 an average of 80% and 56% wheat pistils had one or more pollen tubes at the micropyle signifying potential fertilisation, while Laurie and Bennett (1987a)

reported that 61% of Chinese Spring wheat embryo sacs contained maize cv. Seneca 60 pollen tubes. So, Fukuho, like Chinese Spring was described as a good crossable cultivar with Poaceae subfamilies (Inagaki and Snape, 1982; Comeau *et al.*, 1985; Inagaki, 1986a). However, the presence of a pollen tube in the embryo sac or at the micropyle does not necessarily mean that fertilization has occurred, as has been clearly indicated in wheat × *H. bulbosum* (Sitch *et al.*, 1985), wheat × maize (Laurie and Bennett, 1987a) and wheat × sorghum (Laurie and Bennett, 1988a).

Four different events independently control the rate of viable embryo formation: Pollen tube growth, entry into ovules, fertilisation, and embryonic growth. For all of these events it is possible to define the genetic barriers which reduce the success rate. Ahmad and Comeau (1990) observed that compared to maize pollen, pearl millet pollen germinated better, but pollen tubes from the best germinating pearl millet lines did not necessarily enter the ovule more frequently than other lines. While the fertilisation rate in wheat × sorghum crosses is about 69% (Laurie and Bennett, 1988a), which is more than twice that found in wheat × maize crosses (Laurie and Bennett, 1987a), it was easier to obtain relatively large embryos from wheat × maize than from wheat × sorghum. A high frequency of fertilisation does not necessarily produce a high frequency of viable embryos. Producing a more effective male parent via recombination of the genes controlling the four mentioned events within *Zea*, *Sorghum* or *Pennisetum* may be an exciting step to further improve the frequency of haploid plants.

2.4. Chromosome Elimination

The phenomenon of chromosome elimination was first discovered in *H. vulgare* × *H. bulbosum* crosses (Kasha and Kao, 1970). Davies (1958) used three diploid plants from autotetraploid *H. bulbosum* × autotetraploid *H. vulgare* and believed that these plants had originated by male parthenogenesis. Later other workers (Kao and Kasha, 1969, Symko, 1969, Kasha and Kao, 1970, Lange, 1971a,b) also obtained haploids (or dihaploids from

tetraploids) from similar interspecific cross and postulated that preferential elimination of *H. bulbosum* chromosomes led to the formation of haploid embryos of barley.

Bennett *et al.* (1976) concluded that *H. bulbosum* chromosomes were eliminated preferentially, since no cells had less than seven chromosomes in any of the haploid embryos and all these chromosomes were from *H. vulgare*. In addition to the *H. vulgare* × *H. bulbosum* cross, chromosome elimination and haploid plants have been obtained from interspecific and intergeneric crosses between many other *Hordeum* species (Kasha, 1974, Subrahmanyam, 1983).

Also, haploid production in wheat resulting from selective elimination of the alien parent chromosomes has been achieved employing intergeneric crosses between hexaploid wheat (*Triticum aestivum*) and *H. bulbosum* (Barclay, 1975; Inagaki and Snape, 1982; Snape *et al.*, 1980; Sitch and Snape, 1986a,b), *Zea mays* (Laurie and Bennett, 1986; Inagaki and Tahir, 1990; Suenaga and Nakajima, 1989), *Sorghum bicolor* (Laurie and Bennet, 1988b; Ohkawa *et al.*, 1992), or *Pennisetum americanum* (Laurie, 1989b; Ahmad and Comeau, 1990). Therefore, the selective elimination of the alien parent chromosomes in wide crosses in the Triticeae appears to be a common phenomenon (Subrahmanyam, 1982; Bothmer *et al.*, 1983; Bothmer and Subrahmanyam, 1988; O'Dounoughue and Bennett, 1988; Amrani *et al* ., 1993).

In wheat × maize crosses, cytological studies have shown that fertilization occurs but hybrids are found to be karyotypically unstable, rapidly losing the maize chromosomes to give haploid wheat embryos (Laurie and Bennett, 1986, 1988a). Laurie and Bennett (1988a,d, 1987a) and Laurie (1989, 1990) observed that elimination of maize chromosomes occurred in young embryos and all embryos with four or more cells possessed micro nuclei. Elimination appeared to be complete after the third cell cycle, as no maize chromosomes were seen at metaphase in embryos with eight or more cells (Laurie and Bennett, 1988a).

2.4.1. *Time of Elimination of Chromosomes*

In wheat × maize crosses, Laurie and Bennett (1987a) found that in all crosses that they studied the cytological events were similar 48 hours after pollination. All but one embryo had undergone at least two rounds of cell division to produce four cells and most had eight or more cells. The cell numbers were similar to those of parental selfs of the same age. However, hybrid embryos invariably showed chromosome elimination, which was due to the failure of maize chromosomes to attach to the spindles. Laurie and Bennett (1987a) pointed out that according to Finch and Bennett (1983) rapid uni parental chromosome elimination is advantageous in these circumstances (Finch and Bennett, 1982) since the embryo reaches the balanced condition of a haploid wheat genome early in development.

Sitch (1984) pointed out that in crosses between cultivar Chinese Spring wheat and the *H. bulbosum* clone PB168 chromosome elimination began at the first embryo division and continued for several cell cycles. In the endosperm, elimination always began at the second division. The rate of chromosome elimination in both embryos and endosperms is greatest during the first days after fertilization. Laurie and Bennett (1989) found it interesting that crosses using different cultivars of barley as the female parent do not behave in precisely the same way. Karyotypically unstable barley × *H. bulbosum* crosses tend to show slower rates of elimination of *H. bulbosum* chromosomes at the zygotic mitosis. Elimination occurred at a maximum rate at 2 days after pollination, and at 5 days 94% of embryo nuclei, and all endosperm nuclei in Sultan barley, were haploid. With Vada barley the corresponding figures were 100 and 95.7%, respectively. Finch and Bennett (1983) obtained similar results using other genotype combinations. Subrahmanyam and Kasha (1973a) also reported a gradual loss of *H. bulbosum* chromosomes, but in their material about 6% of embryos still retained one or two *H. bulbosum* chromosomes 11 days after pollination.

2.4.2. *The Mechanism of Chromosome Elimination*

In wheat × maize crosses, maize chromosomes disappear after a few cycles of cell division (Laurie and Bennett, 1987). The maize chromosomes were observed to fail to move to the spindle poles during cell division, presumably due to a failure in attachment of their centromeres to the spindle microtubles at metaphase (Laurie *et al.*, 1990). The mechanism of maize chromosome elimination in wheat × maize crosses is thought to be the same as in karyotypically unstable interspecific *Hordeum* hybrids (Bennett *et al.*, 1976; Bennett, 1983; Finch and Bennett, 1983). Although the cytological and physiological mechanisms behind chromosome elimination in the hybrids from barley and *H. bulbosum* also are not completely understood, mitotic disturbances resulting from genetic imbalance between parental genomes (Lange, 1971a) and differences in cell cycle time (Subrahmanyam and Kasha, 1973b) have been suggested as possible explanations. Davies (1974) proposed that the *H. bulbosum* chromosomes are degraded by endonuclease products from the *H. vulgare* genome, but this was rejected later by Bennett *et al.* (1976) in their extensive investigations on chromosome elimination. While studying cell divisions during the first few days after fertilisation, they found that the *H. bulbosum* chromosomes were not subjected to any major degradation, although many abnormalities were found in the cells such as non-congressed chromosomes at metaphase, lagging chromosomes and bridges at anaphase and multipolar spindles. They observed that chromosome elimination from the embryo increased on the third day after fertilisation which coincided with increasing demand for protein synthesis and it was postulated that the *bulbosum* chromosomes might become less efficient in forming normal attachments to spindle proteins when protein becomes a limiting factor.

Orton and Browers (1985) also adopted the idea that spindle disturbances play an essential role in chromosome elimination. The cytogenetic disturbances they observed in a complex hybrid comprising three *Hordeum* species, *H. jubatum, H. compressum* and *H. vulgare*,

were very similar to what Bennett *et al.* (1976) had described earlier. They suggested that spindle disturbances were the general cause of chromosome elimination in the genus *Hordeum*.

Bennett *et al* (1981) also noted that chromosome size is correlated with centromere size and suggested that some male parent chromosomes might have sufficient microtubule binding capacity to reach the spindle poles in zygotes but, this ability is soon lost either because of modifications of the DNA of the centromeres from the male parent, as suggested for karyotypically unstable *Hordeum* hybrids (Bennett *et al.*, 1976; Finch and Bennett, 1983), or by dilution of components necessary for centromere function (Finch and Bennett, 1983). Finch and Bennett (1983) also noted that these male DNA cause a suppression of the nucleolar organizer (NOR) of the alien chromosomes, earlier represented by Lange and Jochemsen (1976b), but they suggested that this suppression was independent of the chromosome elimination process. Finch and Bennett (1983) compared the suppression of the centromere region of *H. bulbosum* chromosomes to a similar phenomenon in wheat lines which had been substituted with *Aegilops* chromosomes. In these lines the nucleolar organizers were suppressed and Flavell *et al.* (1983) postulated that this was caused by DNA methylation.

Finch and Bennett (1983) also suggested that methylation of centromeric DNA was the primary cause of chromosome elimination in *Hordeum*. Further strength to the assumption that centromere malfunction is the main cause of the elimination process was added by Noda and Shiraishi (1990). They induced lagging chromosomes in a hybrid between barley and *H. bulbosum* with cordycepin and cyclohexamide, chemicals which are known to inhibit protein synthesis. The result indicated that the inhibition of protein synthesis affects the spindle function hence the lagging chromosomes. The experimentally induced chromosomal aberrations were similar to those seen in cells undergoing chromosome elimination. The authors' conclusion was that RNA and protein synthesis related to the

spindle-centromere interaction during G2 and prophase may be partially suppressed in *H. vulgare* × *H. bulbosum* hybrids.

Laurie and Bennett (1989) suggested that under normal conditions (eg. self pollination) the parental chromosomes assemble complete functional centromeres from components resynthesised in the egg cell. Due to their small centromeres, sorghum, maize and pearl millet chromosomes might be unable to bind to wheat centromere so that they bind to spindle microtubules with low efficiency. Variation in the initial size of the centromere regions of the parental chromosomes, which is dependent on chromosome size (Bennett *et al.*, 1981), is the source of variation in stability. They concluded that following DNA replication in the two-celled embryo, centromere components from the male parent would be diluted and further chromosome loss would occur.

In crosses between Sultan barley and S60 maize, the maize chromosomes have well-differentiated centromeres in zygotes and are retained for more cell cycles than in crosses with hexaploid wheats (Laurie and Bennett, 1988a, 1989). Well-differentiated maize centromeres are not observed in hybrids with diploid or tetraploid *Triticum* or *Aegilops* species (cited by O'Donoughue, pers comm with Laurie and Bennett, 1989), suggesting that this difference is not a ploidy level effect *per se*. The maize nuclear organizer was expressed in Sultan × S60 zygotes, thus, the mechanism of maize chromosome elimination in wheat × maize crosses may be different from that of *H. bulbosum* in barley.

2.5. Efficiency of Haploid Production

This review of factors affecting haploid production has been concentrated on wheat × *H. bulbosum* crosses because this has been the first and most studied. Similar factors apart from the *Kr* genes affect other intergeneric haploid production including wheat × maize system.

The two main influences affecting efficiency of haploid production in wheat and barley using wide crosses are genotypic and environmental factors. The influence of both parental genotypes on the success of the haploid production techniques has been emphasised in numerous reports (Pickering and Hayes, 1976; Jensen, 1976; Novak *et al.*, 1977; Adamski, 1979; Pickering, 1979, 1980a,b, 1983, 1984; Simpson *et al.*, 1980; Snape *et al.*, 1980; Foroughi-Wehr *et al.*, 1981; Jensen, 1982; Inagaki and Snape, 1982; Inagaki, 1986a; Sitch and Snape, 1986a, 1987a,b,c; Laurie and Bennett, 1987).

In particular with wheat haploid production the frequency of haploids has been shown to be influenced by the genotype of the pollen donor parent whether it be *H. bulbosum* (Sitch and Snape, 1986a), maize (Laurie, 1989a), sorghum (Laurie and Bennett, 1988b) or pearl millet (Laurie, 1989b).

The ploidy level of the pollen donor species has affected the results of haploid production in wheat × *H. bulbosum* crosses, with higher seed sets being obtained with the tetraploid compared to diploid *H. bulbosum* (Barclay, 1975).

As an alternative scheme Lazar *et al.* (1984) have considered the use of anther culture for producing haploids of barley and wheat. The efficiency of the technique is limited by genotypic differences in the frequency of plant regeneration, and including problems arisen from a high frequency of albinos and variation in the ploidy level of the regenerated plants (De Buyser *et al.*, 1985).

2.5.1. Factors Affecting Seed Set

Seed set (caryopsis development) is influenced by an interaction between the parental genotypes. With wheat × *H. bulbosum* crosses, the crossability genes *Kr1* or *Kr2* have a large influence on seed set (Sitch and Snape, 1986a). Wheat parents also give varying numbers of seed depending on the *H. bulbosum* parent (Falk and Kasha, 1981; Li and Hu, 1983; Sitch and Snape, 1986a; Inagaki and Snape; 1982). Although the major effect of the wheat genotype on seed set is due to the presence of the crossability genes, with wheat

parents carrying recessive *kr1* and *kr2* crossability genes there is also an interaction with the *H. bulbosum* (Snape *et al.*, 1979). The cause of this interaction is not known, but it could be due to differences in the success of micropylar penetration and/or double fertilisation (Sitch and Snape, 1986a). Also there may be genotypic differences in the ability of the pollen tubes to penetrate the ovary wall (Sitch and Snape, 1987a).

Inagaki and Snape (1982) found that seed set in wheat by *H. bulbosum* crosses is greatly influenced by environmental conditions at pollination and hormone treatment of the pollination. High humidity and GA_3 treatment post-pollination give increased seed set. Based on such results, Inagaki and Snape (1982) suggested that the ovule of wheat needs to be mature to be at peak receptivity to the pollen of *H. bulbosum*.

2.5.2. *Factors Affecting Survival*

survival rates are influenced by three factors, namely wheat genotype, the post-pollination GA_3 application and the ambient temperature. In wheat × *H. bulbosum* crosses Sitch and Snape (1987c) showed that these factors all significantly affected seed survival, but the *H. bulbosum* had no effect.

survival was not related to crossability genes, since a high survival was obtained from pollination of both crossable and non-crossable varieties (Sitch and Snape, 1987c). The absence of an effect of the *Kr* loci on survival was expected since these loci are thought to operate only during pre-fertilisation. An effect of the wheat genotype on survival implies that because of the elimination of the *H. bulbosum* chromosomes early in development, the many developmental processes occurring during the early stages of hybrid development will be controlled by the wheat genotype. The effect of the *H. bulbosum* genotype on survival is probably due to its effect on the *bulbosum* chromosome elimination process early in hybrid development (Sitch and Snape, 1987c). They demonstrated a difference in survival rates between Chinese Spring and Chinese

Spring (Hope 5B) substitution and concluded that this difference implies an affect of a genetic factor on embryo survival located on the substitution Hope chromosome 5B.

With crossable wheat genotypes, the embryo survival is affected by ambient temperature. Optimum temperature of 20±2 allow more sustained embryo and endosperm development and thereby, improves embryo survival (Sitch and Snape, 1987c).

The control system of embryo survival is not clearly known, but embryo survival rate is expected to be improved by an increased rate of chromosome elimination. Rapid completion of the elimination of chromosome duration, will reduce the number of involved nuclei in elimination process because of the shorter time of completing the duration. Consequently, this may results in lower number of aberrant nuclei and an earlier production of a plantlet containing the haploid set of chromosomes.

2.5.3. Effects of Rate of Embryo Production, Embryo Size and Regeneration

The production of a high frequency of immature seed containing haploid embryos which will regenerate into plantlets is the main objective of a successful wheat haploid project.

Sitch and Snape (1986a) found that with winter wheat and triticale genotypes, 66.7% and 100% of the seeds contained embryos, respectively. However, they found that seed quality as normal by embryo size in wheat × *H. bulbosum* hybridizations was mainly influenced by the *H. bulbosum* genotype (Sitch and Snape, 1986a).

The *H. bulbosum* genotype was found to have an effect on barley embryo size (Pickering *et al.*, 1983b) and plantlet regeneration was found to be influenced by both the barley (Simpson *et al.*, 1980) and the *H. bulbosum* (Pickering and Morgan, 1981) genotypes. In wheat × *H. bulbosum* crosses, all three of these characteristics were influenced by the *H. bulbosum* genotype (Sitch and Snape, 1986a). These authors reported a highly significant relationship between embryo size and regeneration, indicating that haploid plantlets resulted only from the larger and presumably more differentiated embryos. The average

size of embryo produced varied according to the *H. bulbosum* genotype used, and this influenced the degree of embryo regeneration. Pickering (1980) defined the optimum length of barley haploid embryos for culturing to be 1.5 mm, following excision 11 to 20 days after pollination. Smaller embryos produced weaker plantlets. Sitch and Snape (1986a) found that, in wheat, excision of seeds 15 days after pollination tended to produce haploid embryos with a mean length of 1.19 mm which were smaller than the barley optimum.

2.5.4. Factors Affecting Frequency of Fertilization

Fertilisation frequencies are limited by the action of the crossability, *Kr*, genes in *H. bulbosum* crosses (Snape *et al.*, 1980). An incompatibility reaction at the base of the style and in the transmitting tissue of the ovary wall was responsible for controlling non-crossability in wheat × rye and wheat × *H. bulbosum* crosses. Lange and Wojciechowska (1979), Snape *et al.* (1980) and Sitch and Snape (1987a,b,c) observed that no rye or *H. bulbosum* pollen tubes penetrated the embryo sac and therefore fertilisation did not occur. However, Zenkteler and Straub (1979) suggested that a different mechanism is operating for wheat × *H. bulbosum* in comparison to wheat × rye crosses. Fertilisation occurred after pollinating non-crossable wheat varieties by diploid *H. bulbosum*, but the zygote and endosperm completely failed to develop.

This phenomenon was divided into two stages, pollen germination and growth, and fertilisation frequencies and post-fertilisation development, by Snape *et al.* (1980). They concluded that the variation observed in crossability is not the result of a differential effect on pollen germination or initial pollen tube growth. The pollen germinated on all genotypes and had penetrated and grown down the stigmatic hairs within 15 minutes. Then pollen tubes were observed near the base of the stigmas of all genotypes within 30 minutes. These events were similar to the pattern described by Zenkteler and Straub

(1979) and by Lange and Wojciechowska (1979) in pollination with diploid *H. bulbosum* and rye pollen, respectively, and were confirmed by Sitch and Snape (1987a) later.

Differences existing between crossable and non-crossable lines in the frequencies of fertilised ovules, in contrast to pollen germination and pollen tube growth rates were significant (Snape *et al.,* 1980). They found that fertilisation, when it occurred, took place within 8 hours of pollination and sperm nuclei were clearly visible appressed to the egg and polar nuclei. It was also confirmed by Zenkteler and Straub (1979).

Sitch and Snape (1986a) demonstrated that the frequency of fertilisation in wheat × *H. bulbosum* hybridizations is controlled by both the wheat and the *H. bulbosum* genotypes, through the action of the crossability genes.

2.5.5. Effects of Genotype

Sitch and Snape (1987a) realised a significant difference in the frequency of pollen tube penetration of the ovary observed between their two wheat cultivars, reflecting the genotypic differences at the *Kr* loci. They also observed pollen tube penetration as far as the top and the base of the ovule only in three ovaries of Highbury and the Chinese Spring. Therefore, not only the frequency of ovaries showing pollen tube penetration, but also because of the action of crossability genes, the extent of pollen tube penetration were obviously reduced (Snape *et al.,* 1980, Sitch and Snape, 1987a).

2.5.6. Effects of Gibberellic Acid (GA_3) Treatment, Temperature and Pre-Anthesis Pollination

Gibberrellic acid (GA_3) treatment (Sitch and Snape, 1987a), ambient temperature (Sitch and Snape, 1987a) and pre-anthesis pollination (Sitch and Snape, 1987b) significantly influenced fertilisation frequencies in wheat × *H. bulbosum* crosses.

Gibberellic Acid treatment significantly improves the percentage of successfully fertilised florets. An early application of the hormone, ie. immediately after pollination resulted in

the highest level of fertilisation.. Late GA_3 application 1-2 days after fertilisation did not improve the fertilisation frequency (Sitch and Snape, 1987a).

Pollination prior to normal anthesis increased fertilisation frequencies, whilst post-anthesis pollination resulted in a reduction (Sitch and Snape, 1987b). Comparable relationships between seed set and pollinating time have been reported in wheat × rye crosses (Kul'bii and Shestopalova, 1981; Thomas and Anderson, 1978; Tozu, 1966). Also, Sitch and Snape (1987b) found that even by very early (3 to 4 days) pre-anthesis pollination, fertilisation is still able to remain at a successful level, but much earlier pre-anthesis pollination may result in a reduction of fertilisation frequency due to ovary immaturity.

The effect of ambient temperature on the frequency of fertilisation is significant. Sitch and Snape (1987a) showed that overall fertilisation frequency is greater at 20°C than at 26°C. The influence of temperature, however, differed significantly between wheat genotypes. Inagaki (1986a) found that low-temperature condition after pollination proved to be disadvantageous to seed set. He indicated that seed sets on spikes at low-temperature (15°C) were significantly lower than those at intermediate (20°C) and high (25°C) temperature. One day in darkness had no effect on crossability (Inagaki, 1986a). Increased temperatures increased the rate of cell division (Brown, 1951) and stimulated the rate of chromosome elimination (Humphereys, 1978), thus with lowering seed set it may improve seed survival of those that do set (see section 2.5.2).

2.5.7. Effects of 2,4-D

Although GA_3 was used mainly for crosses between wheat and *H. bulbosum*, the results of its application were not as good as 2,4-D when maize, sorghum or pearl millet were used as pollen sources (Laurie and Bennett, 1986, 1988b; Laurie, 1989b).

Inagaki (1986b) found that application of 2,4-D significantly increased seed set on a Japanese wheat cultivar Fukuhokomugi although approximately half of those seeds did not contain any embryo. All the seeds became filled with liquid and those lacking embryos

could not be distinguished by external observation. When the culms were injected with 100 ppm 2,4-D solution, the highest efficiency of embryo production was 48.6% of florets pollinated, which was double that of the untreated ones (23.8%). He found that 2,4-D treatment also stimulated seed and embryo development in cv. Haruhikari but at very low frequencies. In addition using *H. bulbosum* clone S1-2 after 2,4-D injection in Fukuhokomugi, the frequency of seeds containing embryos increased to 58.2%, but there were no embryos obtained from Haruhikari using S1-2 as the pollen donor.

Droplet application of 2,4-D solution onto the emasculated florets in wheat × *H. bulbosum* induced parthenocarpic seed development and increased ovule size due to cell expansion without successfully overcoming cross-incompatibility (Marshal *et al.*, 1983). Inagaki (1986b) believed that seed development might be enhanced after fertilisation due to 2,4-D application rather than increasing fertilisation itself. Chung and Inagaki (1986) found that both *H. bulbosum* genotypes and 2,4-D application increased haploid production significantly. Inagaki (1986b) indicated that a selected clone of *H. bulbosum* and pre pollination application of 2,4-D enhanced the crossability of wheat cultivars Fukuhomugi, but the incompatibility of cv. Haruhikari was not sufficiently improved for practical haploid production.

Wheat varieties crossed with maize and treated with 2,4-D successfully developed embryos of sufficient size to regenerate haploid plants (Inagaki and Tahir 1990, 1992). Without the 2,4-D application, none of their wheat cultivars produced embryos. Although the process was not understood, they concluded that the application of 2,4-D enhanced the development of hybrid zygotes to haploid embryos, making laborious spikelet culture unnecessary (Laurie and Bennett, 1988d). They concluded that 2,4-D application had not positively influenced wheat haploid embryo production when it was crossed with *H. bulbosum*, but it enhanced the embryo production of both compatible and incompatible wheat cultivars.

Three main methods of using 2,4-D are: 1) spikelet culture in a culture medium containing 2,4-D solution, 2) dipping spikes into 2,4-D solution, and 3) injection of 2,4-D into the upper internode of the culm. Various concentrations of 2,4-D solution have been applied in each method by various investigators (Laurie and Bennet, 1987; Laurie and Reymondie, 1991;.Suenaga and Nakajima, 1989; Inagaki, 1986b).

Application of 2,4-D in culture media for spikelet culture resulted in producing better survival of haploid embryos (Laurie and Bennett, 1988d; Guo *et al.*, 1994), but none of these results were superior than the third method (Suenaga and Nakajima, 1989) and its slightly modified method (Laurie and Reymondie, 1991). The latter modified the former method using a drop of the same concentration of 2,4-D in the space between two remaining florets. Although in most cases investigators preferred to treat the spikes with lower than 100 ppm 2,4-D concentration, Islam and Shepherd (1994) reported better results using 150 ppm 2,4-D solution as the promoting substance. Guo *et al.* (1994) reported non significant differences in single embryo formation among the media with different promoting substances (1/2MS+2mg GA3/L, 1/2MS+2mg 2,4-D/L and 1/2MS+2mg KT/L) spikes having already been treated with 0.1 mg 2,4-D/L. The best result of their spikelet culture was achieved by using 2 mg KT/L (Guo *et al.*, 1994). In spite of its capability to keep the ovaries green, it was ineffective for ovary development especially for endosperm formation. They found that with combining 2,4-D treatment with spikelet culture, the ovary development was promoted while the frequencies of embryo development were also increased.

A post pollination spray of gibberellic acid and 2,4-D delays the onset of senescence and improves the number of high quality seeds up to more than 75% of the seed set. This improvement, in turn, results in increased survival of embryos for culture and subsequent plant regeneration (Pickering and Wallace, 1994). The mean numbers of seeds produced per spike significantly reduces if higher rates are used such as 100 mgl^{-1} 2,4-D mixed with

75% GA_3, however, it enhanced seed quality and the proportion of viable embryos increased.

2.6. Duplication of Chromosome Number

The final critical steps in producing doubled haploid lines from intergeneric crosses is doubling the chromosome numbers of the haploid plantlets recovered. Haploid plants are regenerated following embryo rescue on an artificial nutrient medium and their sexual fertility is obtained by doubling the chromosome number with a suitable method. Although the application of colchicine to plants is the most popular method, there are also a few other methods which have been tried with some success.

2.6.1. Spontaneous Chromosome Doubling of Haploids

Jensen (1974) and Subrahmanyam and Kasha (1973b) reported spontaneous chromosome doubling rates in haploid barley ranging from 1 to 3%. However, in wheat, the frequency is much lower (Laurie and Bennett, 1988a,e). Based on the number of doubled haploids needed, even in barley, the spontaneous rate is too low to be exploited in plant breeding and genetical studies and other methods must be used to increase this rate, or many more haploids must be produced.

2.6.2. Colchicine Treatment

As mentioned earlier, application of colchicine is the most efficient method of chromosome doubling for many crops including barley and wheat. Barley haploids can be treated with colchicine at various growth stages, however, the best result for wheat has been obtained by treating at the 2-4 tiller stage (Islam and Shepherd 1994). Different methods for the use of colchicine have been used.

Islam and Sparrow (1973) achieved 98-100% doubling success with haploid barley plants. When barley haploids have developed to the 4-5 tiller stage, a single tiller, preferably the main one, is treated with 0.1% colchicine by the inverted vial technique (Bell *et al.*, 1980).

They cut the main tiller off a few centimetre above the crown area and then inverted a vial of colchicine solution over it and repeated this treatment for a few days until the symptoms of colchicine toxicity (leaf necrosis) appeared on the other tillers' leaves. The application of colchicine prevents the formation of spindle fibres and cell division and homologous chromatids cannot separate from each other since there is no spindle. When the new nuclear membrane forms all chromatids are included at the same cell and then the number of chromosomes will be doubled. This happens only to dividing cells in the meristematic cells in the crown area. The treated plants are then grown to produce many tillers in the hope that one or more tillers or plants thereof will develop from a doubled cell, and produce a fertile spike or at least floret.

Jensen (1975) developed another method. He pruned the roots of barley haploids at the three- or four-tiller stage about 3 cm below their crowns and the plants were then placed into a glass vial containing 0.05% colchicine and 2% dimethyl sulfoxide (DMSO) plus a few drops of Tween 20 for 5 hours. Using this procedure he has obtained more than 90% doubled haploid plants.

In another technique Thiebaut *et al.* (1979) grew barley haploid seedlings to the three-leaf stage still in their culture vials and then immersed them to above the crowns in an aqueous solution of 0.1% colchicine, 2% DMSO, 0.3 ml/litre of Tween 20 and 10 mg/litre of GA3 for 5 hr at 25°-32°C. The seedling were then rinsed in water and transplanted to pots. Using this method they achieved over 70% success in producing doubled haploids.

In his method Pickering (1980) removed plants from a compost potting medium at the three- to four-tiller stage, washed the roots and trimmed them back to 1 cm. The plants, after placing a single incision at the stem base with a razor blade, were immersed to a depth of 5 cm in an aerated aqueous solution containing 0.05% colchicine and 2% DMSO at 25°C for 5 hr. The treated plants were washed with tap water for 2-3 min and then planted into John Innes compost No. 3 in shallow boxes. The success rate of this method was over 80%.

The methods described above were mostly used for doubling barley haploid chromosome number. For wheat slight modifications were employed by Islam and Shepherd (1994) in both physical treatments and the amount of chemical components that were needed. Haploid wheat seedlings were lifted from their pots with adhering soil and the soil carefully washed with water. Then the roots and crown region of plants were submerged into an aerated aqueous solution with 0.07% colchicine plus 3% DMSO for 5-6 hours, depending on the vigour of plants. After a brief wash in running water the seedlings were transplanted into new soil in 20 cm pots. Using this method the success rate of chromosome doubling was over 90%.

Thus colchicine is an efficient chemical for chromosome doubling of doubled haploids. However, it is expensive and worse, it is highly carcinogenic so must be handled with extreme care. Other methods which are safer to the operator but which are nearly as effective are highly desirable.

2.6.3. Application of Nitrous Oxide

Subrahmanyam and Kasha (1973b) found that nitrous oxide is also effective in doubling chromosome numbers of barley haploids. When the haploids were treated at the zygotic stage, the success rate of chromosome doubling was 0-5.4%, 5-60% and 23-100% at pressures of 21.1 x 10^3, 36.6 x 10^3 and 42.2 x 10^3 kg/m^2, respectively. However, at higher pressure it caused reduction in seed set and embryo formation. Nitrous oxide showed lower efficiency than colchicine solution for doubling the chromosome number of the haploid plants and has not been used extensively.

2.6.4. Application of Caffeine

Another less used method is the application of caffeine for chromosome duplication. However, the efficiency is very much lower than that obtained with colchicine treatment (Pickering, 1980).

2.7. Doubled Haploids as a tool in Barley Breeding. (see insert at back of thesis)

Chapter 3

Producing Doubled Haploids by a Wheat × Maize System

3.1. Introduction

In spite of successfully using many techniques in the production of barley (*Hordeum vulgare,* L.) haploid plantlets, there are some restrictions in the application of these methods in wheat. For example, anther (microspore) culture techniques are utilised extensively in barley haploid production but the efficiency of such techniques in wheat is highly genotype dependent and they are unsuitable for extensive use in wheat breeding programs. Also, haploid barley is frequently produced via intergeneric crosses using *Hordeum bulbosum,* but this technique in wheat is also genotype dependent. Most wheat varieties carry non-crossability genes (*Kr*) which prevent them hybridizing with some other species including rye and *H. bulbosum.*

To overcome these obstacles, maize (*Zea mays* L., 2n=20) was used as the pollen donor plant and compared with *H. bulbosum* using various wheat cultivars. Unlike *H. bulbosum,* maize appears to be insensitive to the action of the dominant *Kr* genes (*Kr1 or Kr2*) and produced similar rates of haploid embryo formation in wheat varieties which were characterised as non-crossable with rye and *H. bulbosum* as those regarded as crossable. Maize and sorghum (*Sorghum bicolor* L., 2n=20) have been used in the production of haploid wheat plants. Maize pollen will readily germinate soon after its placement on wheat stigmas and a few pollen tubes will reach the embryo sac. Laurie and Bennet (1989a) reported the success of maize pollen tubes reaching the embryo sac (in about 80% of florets) using the maize variety Seneca 60. In 28% of florets they found fertilization had

successfully occurred, a remarkably high frequency. However, it has been reported that there is variation for the effectiveness of haploid embryo production among pollen donors.

The critical post-pollination event in wheat haploid production following intergeneric hybridization is the elimination of the pollen parent genome (maize or sorghum) from the developing embryos in the initial zygotic division cycles. As explained in the literature review, following fertilization, most of the chromosomes of the pollen parent have usually disappeared after the first three-division cycles of the zygote. Laurie and Bennett (1986) found that zygotes with cells at metaphase had the expected F_1 combination of 21 large chromosomes from wheat and 10 much smaller chromosomes from maize when fixed one day after pollination. However, embryos fixed 3 to 6 days after pollination contained no maize chromosomes. Therefore, the maize (or sorghum) chromosomes are lost during the first few cell division cycles leaving cells that contain only a haploid complement of wheat chromosomes.

Wheat haploid embryos, which result from this chromosome elimination phenomenon, cannot develop on the plant due to the absence of endosperm and need to be rescued and cultured on a suitable culture medium.

Plantlet regeneration is another critical step in wheat haploid production. A key factor for successful plantlet regeneration is the culture medium used to induce regeneration of excised haploid embryos. Information on culture media for the induction of haploid embryos of wheat and their subsequent growth is very limited.

Comeau *et al.* (1992) used different media to grow 0-4 days old proembryos in a more heterotrophic manner, as much as possible without the help of other plant tissue. Amino acid mixtures have been shown to promote responses for anther culture (Zhu *et al.*, 1980), embryo culture (Raghavan, 1976) and in vitro pollination (Gegenbach, 1984), but in somatic embryogenesis trials, amino acids were not helpful (Tabaeizadeh *et al.*, 1990). Ghaemi *et al.* (1993) found that both genotype and culture media affected embryo formation in anther culture of tetraploid wheat. Media with a relatively high osmotic pressure, which is perhaps useful for the proembryos were found to be detrimental to larger embryos.

There appears to be no published reports on the effects of different culture media on plantlet regeneration in crosses between hexaploid wheat and maize.

This chapter reports on:

1) A preliminary experiment to develop a doubled haploid production protocol and assess some of the most widely-grown Australian wheat cultivars for suitability in DH production in crosses with maize genotypes.

2) The wheat F_1 hybrids derived from Trident × Molineux were pollinated with several maize genotypes to find an efficient pollen donor for future use and to produce a sufficient number of doubled haploid lines for the main experimental program. F_1 hybrids of wheat were used in the crosses with maize because: a random sample of recombinant DH lines is advantageous in both analyzing the linkage between marker genes and agronomic traits, and for selecting lines for breeding purposes. Therefore, an efficient method of haploid production in wheat is of great interest to wheat breeders. Trident and Molineux which were used as the parents for the main F_1 hybrid studied in the present program are polymorphic for several important agronomic and economic traits.

3) Using several different embryo culture media to select a medium which would give the best yield of haploid plantlets.

4) Production of DH populations from particular wheat crosses for which comparable lines were available from a conventional breeding program, so that both groups of lines could be compared in field tests. The production of these DH lines provided more information on the parameters involved in producing wheat doubled haploids.

3.2. Materials and Methods

3.2.1. Production of Doubled Haploids Using Different Wheat and Maize Genotypes

3.2.1.1. Plant Materials

Twelve Australian wheat cultivars (Table 3.1) and seven maize genotypes (Table 3.2) were periodically sown in pots in a glasshouse. Both the wheat and maize genotypes were grown in 25cm pots containing mixed potting soil enriched with complete nutrients. Wheat cultivar, Chinese Spring, was included because of its ease of crossability in many wide crosses. The other wheats included are cultivars widely grown in South Australia and thus of immediate interest as parents in varietal improvement programs in this region. Plate 3.2.1 shows the male and female parents which can result in haploid green plants following their intergeneric hybridization.

Table 3.1. Wheat cultivars hybridized with maize to produce haploid wheat

Cultivar	*Origin*	*Year of release*
Aroona	WARI, SA[†]	1981
Chinese Spring	-	-
Excalibur	RC, SA[‡]	1991
Janz	WRI, Toowoomba, QLD[§]	1988
Kite	NSW Exp. sta., Temora, NSW[¥]	1973
Machete	RC, SA	1985
Meering	Victorian Crops Res. Inst., Horsham, Victoria[£]	1984
Molineux	WARI, SA	1988
Oxley	WRI, Toowoomba, QLD	1974
Spear	RC, SA	1983
Tatiara	WARI, SA	1987
Yarralinka	WARI, SA	1991

[†] Waite Agricultural Research Institute, The University of Adelaide, South Australia
[‡] Roseworthy Campus, The University of Adelaide, South Australia
[§] Wheat Research Institute, Toowoomba, Queensland, [¥] NSW Experiment Station, Temora, New South Wales
[£] Victorian Crops Research Institute, Horsham, Victoria

3.2.1.2. Emasculation and Pollination

Pollinations were carried out during January to April 1993 under glasshouse conditions at the Waite Agricultural Research Institute. The methods used in this experiment were based

on that of Laurie and Reymondie (1991) with slight modifications based on a report by Ushiyama *et al.* (1991).

For a chosen wheat spike the upper and basal spikelets and all florets of the remaining spikelets were removed except for the primary and secondary florets in order to increase uniformity of floret development throughout the spike. The remaining florets were hand-emasculated one to two days before anthesis and then enclosed in a glassine bag together with the flag leaf. Awns were clipped, but not the glumes. Pollination involved the transfer of freshly collected maize pollen to the wheat stigmas, when most of the florets in the spike were at the developmental age equal to the day of anthesis (as described by Laurie, 1989a). The pollinated spikes were then re-covered with the same bags.

Table 3.2. Accessions of maize and sorghum used in the crossing program

Maize/Sorghum accession	Type	Plant Height	Source
Seneca 60	Sweetcorn	Short	Dr. P Banks, QWRI, Toowoomba, QLD
A619/A632	Dent	Medium	Mr. J M Colless, NSW Agriculture, Grafton, NSW
GH5009	Dent	Tall	"
K304/K305	Dent	Medium	"
SnoGold	Sweetcorn	short	"
GH5024	Dent	Tall	"
Pa405	Dent	Tall	"
Illini Gold	Sweetcorn	short	Dekalb Shand Seed Company Pty. Ltd.Tamworth, NSW
11775	Sweetcorn	short	Dr. A J Pryor, CSIRO Division of Plant Industry, Canberra
QLD36	Sorghum		Mr. B Boucher, Pioneer Overseas Corp. Toowoomba, QLD
TAM422	Sorghum		"

3.2.1.3. Injection of 2,4-D

On the day of pollination or the following day, the cavity of the upper-most internode of each pollinated wheat culm was injected with 100 mgl^{-1} solution of 2,4-

dichlorophenoxyacetic acid (2,4-D) using a syringe with a fine hypodermic needle (0.63 × 25mm). To help the 2,4-D solution pass easily into the cavity, a small hole was made at the bottom of the upper-most internode of each culm then the solution was injected from the top of the same internode until it flowed out through the bottom hole. To prevent further leakage of the 2,4-D, the bottom hole was then sealed with petroleum jelly. Plate 3.2.2 demonstrates 2,4-D injection into the upper most internode of female parent a few hours after pollination.

3.2.1.4. Embryo Rescue Procedure

The 2,4-D injected-spikes were collected 14-18 days after pollination. Caryopses obtained from the florets were surface sterilised with 70% ethanol for 30 seconds and then rinsed once in sterile de-ionised water. The sterilisation process was continued by using a chlorine bleach solution (200 mll^{-1}) for 4-5 min followed by rinsing twice in sterile de-ionised water. Caryopses were aseptically dissected and immature embryos were excised under a stereo microscope (2x) in a laminar air-flow hood wiped down with 70% ethanol. The immature embryos were placed in vials containing B5 culture medium (Gamborg *et al*, 1968). The normal selfed (large) and the haploid (small) caryopses are compared in Plate 3.2.3 and also the normal selfed (large) and the haploid (small) embryos are compared in Plate 3.2.4. Normal caryopses contain the normal triploid endosperms which allow the diploid embryos to develop rapidly after fertilization whereas the haploid embryos would die after a few days if left on the plant because of the abnormal liquid endosperm surrounding them. They need to be rescued and cultured on artificial medium to grow into haploid plantlets.

3.2.1.5. Incubation and Regeneration of Haploid Plantlets

Vials containing haploid embryos were incubated in the dark at 20±1°C for five to ten days. The plantlets were then subjected to 16/8 hour day/night photoperiods under constant temperature conditions in an incubator.

Depending on the size of embryos and their developmental stage when rescued, it took one to two weeks to regenerate and produce haploid green plantlets which were then transplanted into pots filled with an enriched steam sterilised potting mix in a greenhouse

with natural daylength conditions. The transplanted seedlings were covered for a few days with transparent plastic cups to assist acclimatization to the glasshouse conditions. Plate 3.2.5 shows a typical haploid seedling. It is noticeably thinner and weaker than a selfed regenerated plantlet grown under the same conditions.

Plate 3.2.1. Wheat haploid plants (centre) produced by pollinating wheat as the female parent (right) by maize as the male parent (left).

Plate 3.2.2. Injection of 2,4-D solution into upper internode of wheat after pollination with maize pollen using a syringe with a fine needle (0.63×25mm).

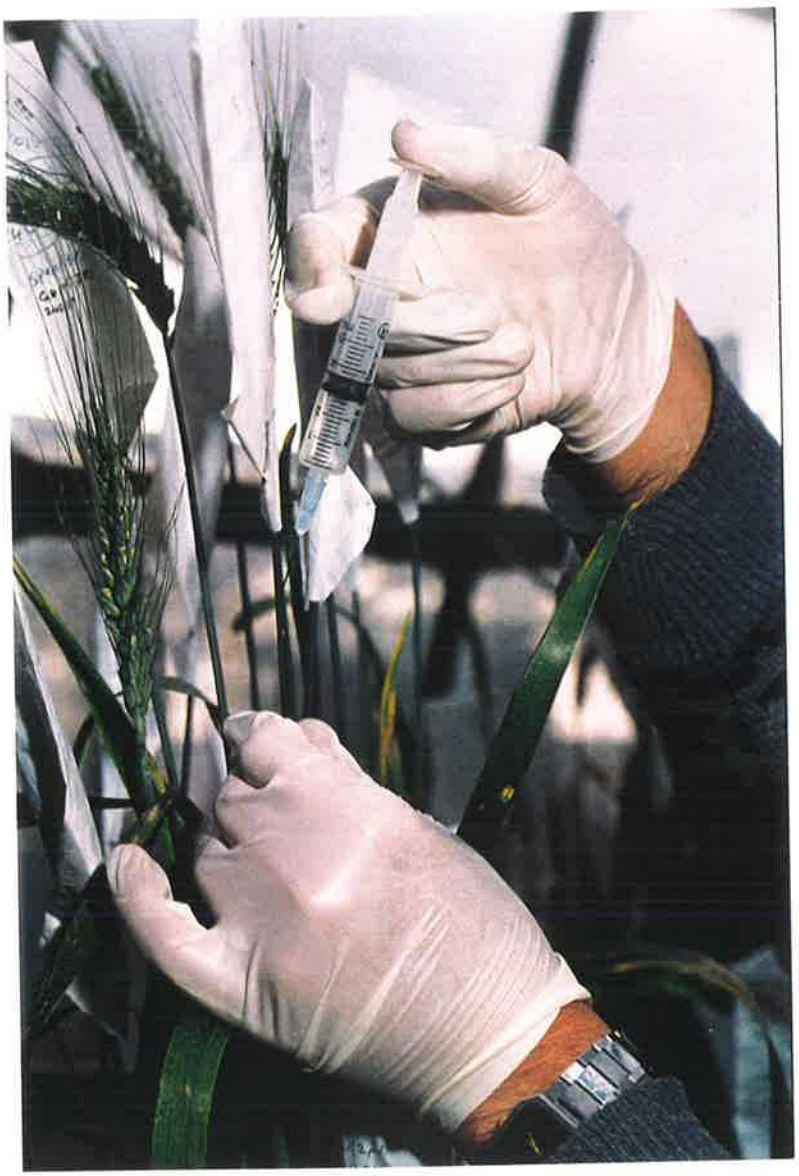
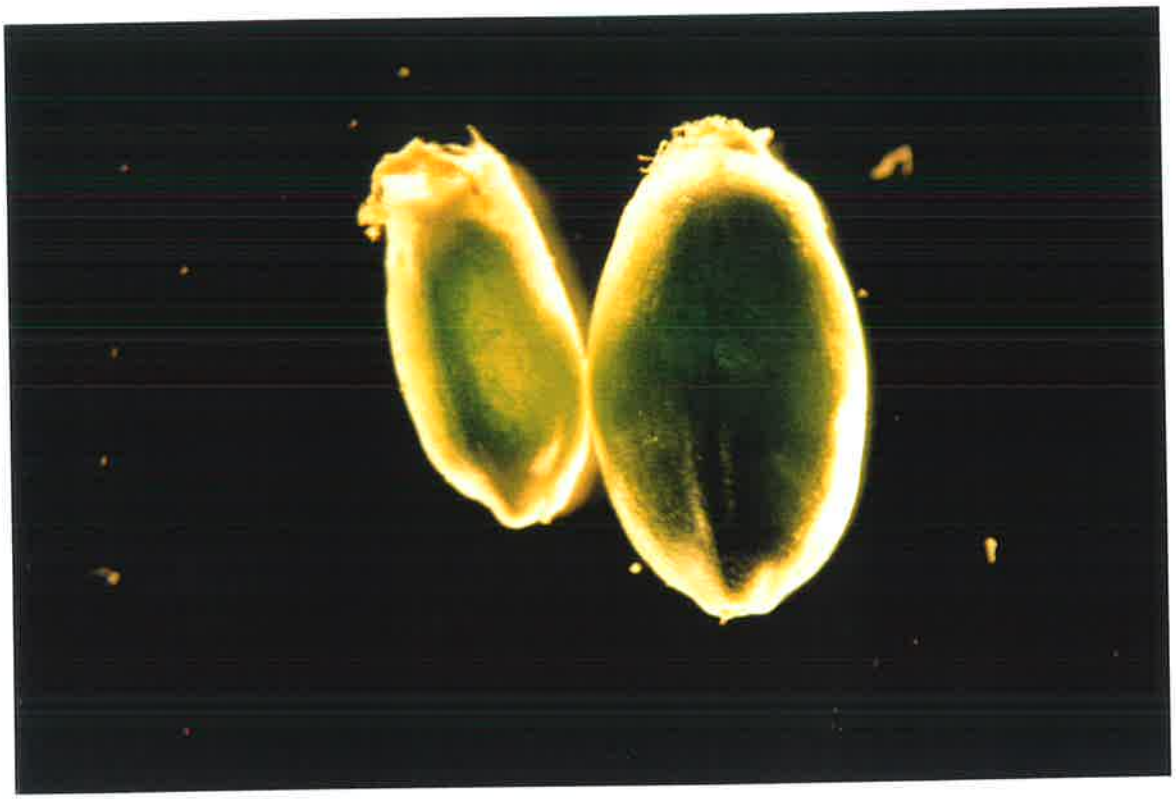


Plate 3.2.3. Comparison of haploid and diploid caryopses. The normal caryopsis (right) contain developing endosperm and embryo while the haploid caryopsis (left) is filled with fluid only and sometimes an embryo.

Plate 3.2.4. Rescued normal (below) and haploid embryos. Note the haploid embryos are much smaller than the diploid one and are twisted around two axes (20× magnification).



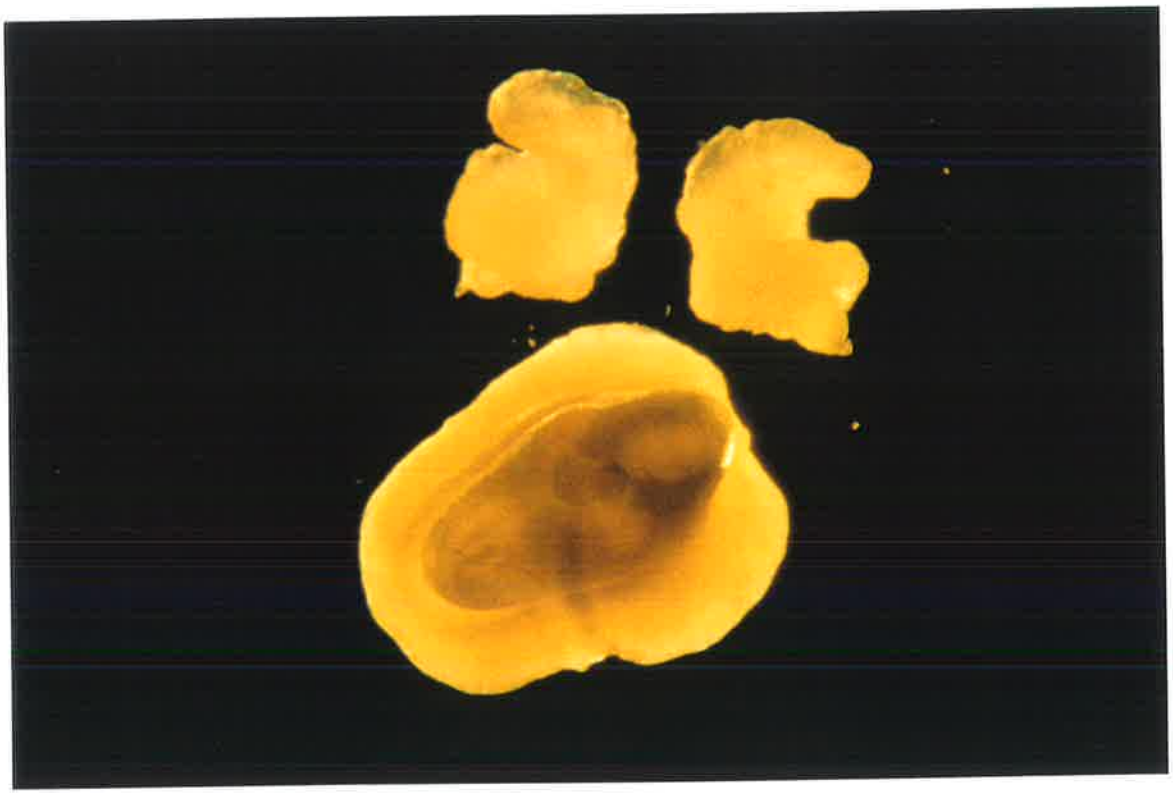


Plate 3.2.5. Comparison between diploid (top) and haploid (bottom) regenerated plantlets. The F_1 diploid plantlets are thicker and stronger and grow faster than haploid regenerants.

3.2.1.6. Chromosome Doubling

At the three to four tiller stage the plantlets were washed carefully to remove all soil from the roots, and then treated to induce chromosome doubling by immersing the roots in an aerated solution of 0.07% colchicine containing 3% DiMethyl SulphOxide (DMSO) for $5\frac{1}{2}$ hours. The seedlings were then repotted and grown in a glasshouse to maturity. Plate 3.2.6 shows the simple apparatus used for colchicine treatment. A few hours after treatment the leaves of old tillers begin to show the first symptoms of colchicine contact and leaves develop necrosis later (Plate 3.2.7). These tillers die and new shoots some with double the chromosome number appear at a later stage of growth and produce the doubled haploid seeds (plates 3.2.8).

3.2.2. Production of Doubled Haploids from F_1 Hybrid of Trident/Molineux

a) Using maize and sorghum pollen sources

To shorten the time needed for producing F_1 plants from a cross between two Australian wheat cultivars, Trident as female parent and Molineux as male parent, the immature seeds were harvested at 10-14 days after pollination and their immature embryos were rescued in sterile conditions, then cultured on B5 (Gamborg, 1968) culture medium and the regenerated plantlets were later transplanted and grown in soil in pots under glasshouse conditions. The F_1 plants were pollinated with seven maize (Seneca 60, A619/A632, GH5009, K304/K305, SnoGold, Illini Gold, 11775) and two sorghum (QLD36, TAM422) genotypes (Table 3.2). This F_1 hybrid was chosen for special attention and the production of large DH population because the two wheat parents are polymorphic for a number of economically important attributes. The resultant DH population could be used to investigate some of the criteria proposed by Snape *et al.* (1979) and also may lead directly to a new improved cultivar for South Australia.

The concentration of 2.4-D used in this experiment was 100 or 150 mg l^{-1}. A drop of the same 2,4-D solution was also placed in the space between each primary and secondary floret (the latter concentration was used for haploid production protocols described in

Plate 3.2.6. Apparatus used for colchicine treatment of haploid plantlets at the 3-4 tiller stage. The two cylinders contain colchicine solution into which the crown and rootlets of washed seedings were immersed for $5\frac{1}{2}$ hours. An aquarium pump aerates colchicine solution.

Plate 3.2.7. Appearance of leaves after colchicine treatment. They become necrotic from the tip within a few hours of treatment and several tillers may die.

Plate 3.2.8. A doubled chimera following colchicine treatment in a spike as shown by successful seed production. Close up in right hand side photograph.

section 3.2.3). Gamborg's B5 culture medium, which was used in the previous experiment, was replaced with half strength MS (Murashige and Skoog, 1962) supplemented with 8 g l^{-1} agar and 20 g l^{-1} sucrose in this experiment.

b) Using different culture media

The excised embryos were cultured in vials containing different media including MS medium(Murashige and Skoog, 1962), half strength MS and Gamborg's B5 basal medium (Gamborg, 1968) supplemented with 8 gl^{-1} agar and 20 gl^{-1} sucrose. Table 3.3 shows details of MS and Gamborg's B5 basal media components, respectively.

3.2.3. Production of Doubled Haploids from 24 Different F_1 Wheat Crosses

Fifteen wheat genotypes which had been used earlier to produce random F_5 selections in the Roseworthy Campus wheat breeding program were selected for DH production. Table 3.4 lists these 15 parental wheat cultivars together with their relevant pedigrees and origins. The parental cultivars were grown in the Roseworthy trial field in South Australia in 1993 and at anthesis heads were emasculated and crossed to produce the F_1s.

The parental genotypes were selected because of their high breeding value for South Australia. The F_1 combinations made were selected because: i) F_3 derived F_5 lines were available from the conventional wheat breeding program at Roseworthy for comparison with doubled haploid lines produced and ii) Some extra doubled haploids were produced from F_1s made to complete a diallel set. Thus this set of F_1 and the doubled haploids produced are not a random sample but a specially chosen set which would be useful to the wheat improvement work in South Australia. Trident is a high yielding, widely adapted variety recently released (Hollamby *et al.* 1993) and formed the main parent used in the conventional program at the begining of the current project.

Illini Gold was used for pollinating the resultant F_1 wheat hybrids grown in the glasshouse. Wheat × maize pollinations and embryo rescue occurred during the period November 1993 to February 1994. Chromosome doubling with colchicine occurred during April to May and viable DH seeds were harvested during late December 1994 to February 1995.

Table 3.3. Components of Murashige and Skoog and Gamborg's basal medium

	mgl^{-1}	
Components:	MS	B-5
Ammonium Nitrate	1650.000	-
Ammonium Sulfate	-	134.000
Boric Acid	6.200	3.000
Calcium Chloride Anhydrous	332.200	113.240
Cobalt Chloride Hexahydrate	0.025	0.025
Cupric sulphate Pentahydrate	0.025	0.025
Disodium EDTA Dihydrate	37.260	37.250
Ferrous Sulphate Heptahydrate	27.800	27.850
Glycine (Free Base)	2.000	-
Magnesium Sulphate Anhydrous	180.700	122.090
Manganese Sulphate Monohydrate	16.900	10.000
Myo-Inositol	100.000	100.000
Nicotinic Acid (Free Acid)	0.500	1.000
Potassium Iodide	0.830	0.750
Potassium Nitrate	1900.000	2500.000
Potassium Phosphate Monobasic	170.000	-
Pyridoxine Hydrochloride	0.500	-1.000
Sodium Molybdate Dihydrate	0.250	0.250
Sodium Phosphate Monobasic Anhydrous	-	130.500
Thiamine Hydrochloride	0.100	10.000
Zinc Sulfate Heptahydrate	8.600	2.000

The time required to carry out this DH program was underestimated in the initial planning of this experiment and thus the number of doubled haploids aimed for in each cross (25 each) was not realized in some cases. Also some lines because of their late maturity, could not be harvested in time to be included in the subsequent field experiments.

Table 3.4. Name, pedigree and origin of parental wheat cultivars

Cultivar	Abbreviation	Pedigree	Year of release	Origin	Reference
Trident	TRI	VPM1/5*Cook//4*Spear	1993	RC, SA	Hollamby *et al.* (1993)
Excalibur	EXC	RAC177/Uniculm492// Bayonet'S'	1991	RC, SA	Hollamby *et al.* (1991)
Frame	FRM	Molineux/3*Dagger	1994	WARI, SA	Rathjen *et al.* (1994)
Molineux	MNX	Pitic62/Festiguay//2*Warigal	1988	WARI, SA	Rathjen *et al.* (1989)
BT-Schomburgk	BTS	Halberd/Aroona//3*Schomburgk	1992	WARI, SA	Rathjen *et al.* (1994)
Machete	MCH	Son64//TZPP/Y54/3/2*Gabo// Madden	1989	RC, SA	Hollamby *et al.* (1989)
Reeves	RVS	Bodallin//Gamenya/Inia66	1987	Dept of Agric, WA	Whan (1991)
Sunfield	SNF	WW31/4/W3566/Topo/ WaiteLine/IRN70.511/2/Sun15B	-	Univ of Sydney, Narrabri, NSW	
RAC613-46	R613	WSF2-78-8-12/3/Lance/ Isr493//RAC177	-	Unreleased line, RC, SA	-
RAC702	R702	Pavon's'//Condor/Petit-Rojo/3/Pitic62/Festiguay	-	Unreleased line, RC, SA	-
RAC710	R710	Pitic62/Festiguay//Dagger	-	Unreleased line, RC, SA	-
RAC711	R711	Condor/Petit-Rojo//Condor /5*AUS10894	-	Unreleased line, RC, SA	-
RAC712	R712	RAC429//RAC177/Kite	-	Unreleased line, RC, SA	-
RAC719	R719	Excalibur'S'	-	Unreleased line, RC, SA	-
RAC745	R745		-	Unreleased line, RC, SA	-

The DH-lines from Trident/Molineux F_1 hybrids had been produced in a separate experiment described earlier (section 3.2.2). Therefore, in this experiment the results of these combinations are shown in a separate row at the bottom of Table 3.10.

3.3. Results

3.3.1. Production of Doubled Haploids Using Different Wheat and Maize Genotypes

3.3.1.1. Female Parent Effects

There are three necessary steps for production of DH lines namely efficient embryo formation, haploid plantlet regeneration and chromosome doubling of haploids (Inagaki and Tahir, 1990). Table 3.6 shows the results for embryo formation and regeneration of haploid plantlets. This work proved that the maize system can be used efficiently for wheat

haploid production for utilization in plant breeding programs. Many swollen caryopses did not contain an embryo. Meering, Oxley and Chinese Spring (88, 80 and 78%, respectively) showed a higher proportions of embryoless caryopses than the other cultivars. In terms of seed set containing an embryo, Excalibur exhibited the highest value (24%). The lowest percentage of embryo production was recorded for Oxley, Janz and Meering. Meering and Oxley produced lower rates of seed set as well. Spear (20%) and Yarralinka (19%) were the other varieties which showed greater than average embryo production.

3.3.1.2. Male Parent Effects

The male parent (maize) had strong effects on haploid and DH production in wheat by maize crosses (Table 3.6). The lowest proportion of seeds containing embryos (2%) was related to Pa405, a tall dent corn cultivar. GH5024, a tall dent genotype, produced 25% of seeds containing a haploid embryo, while it was only 16% for Pa405. The latter also had the lowest percentage of seeds containing no embryo. Due to fewer samples used for GH5024, its result may not be confidently compared with other genotypes. A619/A632, GH5009 and K304/K305 which had 22, 21 and 20% of embryo formation efficiencies, respectively, were the next most efficient genotypes (Table 3.6).

Table 3.6. Efficiencies of wheat and maize genotypes in haploid embryo, plantlet regeneration and doubled haploid production.

Cultivar		A619/A632	GH5009	GH5024	Illini Gold	K304/K305	Pa405	Sno Gold	Total
Aroona	FP		32		25	17	13	10	97
	EF		6 (18.8)†		3 (12.0)	2 (11.8)	1 (7.7)	1 (10.0)	13 (13.4)
	PR		2 (6.3)		2 (8.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.1)
Chinese Spring	FP		44	16			8		68
	EF		5 (11.4)	4 (25.0)			1 (12.5)		10 (14.7)
	PR		3 (6.8)	1 (6.3)			1 (12.5)		5 (7.4)
Excalibur	FP	118	82		102	132	54	230	718
	EF	32 (27.1)	23 (28.0)		39 (38.2)	46 (34.8)	1 (1.9)	30 (13.0)	171 (23.8)
	PR	24 (20.3)	10 (12.2)		10 (9.8)	38 (28.8)	0 (0.0)	4 (1.7)	86 (12.0)
Janz	FP	12				18	4	38	72
	EF	3 (25.0)				1 (5.6)	1 (25.0)	1 (2.6)	6 (8.3)
	PR	0 (0.0)				1 (5.6)	0 (0.0)	0 (0.0)	1 (1.4)
Kite	FP	86	14		18	28	10	68	224
	EF	16 (18.6)	1 (7.1)		1 (5.6)	10 (35.7)	2 (20.0)	2 (2.9)	32 (14.3)
	PR	13 (15.1)	1 (7.1)		0 (0.0)	9 (32.1)	0 (0.0)	0 (0.0)	23 (10.3)
Machete	FP		76	65		85		204	430
	EF		22 (28.9)	16 (24.6)		5 (5.9)		21 (10.3)	64 (14.9)
	PR		9 (11.8)	16 (24.6)		5 (5.9)		0 (0.0)	30 (7.0)
Mearing	FP					24			24
	EF					2 (8.3)			2 (8.3)
	PR					1 (4.2)			1 (4.2)
Molineux	FP		32		14				46
	EF		4 (12.5)		2 (14.3)				6 (13.0)
	PR		4 (12.5)		1 (7.1)				5 (10.9)
Oxley	FP				129			58	187
	EF				10 (7.8)			4 (6.9)	14 (7.5)
	PR				4 (3.1)			4 (6.9)	8 (4.3)
Spear	FP		122		84		14	112	332
	EF		26 (21.3)		9 (10.7)		1 (7.1)	29 (25.9)	65 (19.6)
	PR		20 (16.4)		1 (1.2)		1 (7.1)	18 (16.1)	40 (12.0)
Tatiara	FP		50		38	28			116
	EF		15 (30.0)		3 (7.9)	1 (3.6)			19 (16.4)
	PR		8 (16.0)		1 (2.6)	1 (3.6)			10 (8.6)
Yarralinka	FP	82	90		196		35	30	433
	EF	14 (17.1)	13 (14.4)		28 (14.3)		15 (42.9)	12 (40.0)	82 (18.9)
	PR	11 (13.4)	4 (4.4)		4 (2.0)		15 (42.9)	11 (36.7)	45 (10.4)
Total	FP	298	542	81	606	332	138	750	2747
	EF	65 (21.8)	115 (21.2)	20 (24.7)	95 (15.7)	67 (20.2)	22 (15.9)	100 (13.3)	484 (17.6)
	PR	48 (16.1)	61 (11.3)	17 (21.0)	23 (3.8)	55 (16.6)	17 (12.3)	37 (4.9)	258 (9.4)

FP=Florets pollinated, EF= Embryos formed, PR=Plantlets regenerated

† percentage in brackets

3.3.2. Production of Doubled Haploids from Trident/Molineux F_1 Hybrids

a) Using different maize and sorghum pollen sources

Tables 3.7, 3.8 and 3.9 show the number of florets pollinated, the number of seeds set, and the number of plantlets regenerated from crosses between wheat with maize or sorghum. Caryopses were classified according to the presence or absence of an embryo as per the method of Ushiyama *et al.* (1991). Embryos were cultured on half strength MS culture media.

Within maize genotypes, Illini Gold showed the highest production rate of embryos per florets pollinated (35%) and the highest efficiencies of plantlet regenerated per embryo cultured (77%) and per florets pollinated (27%)(Table 3.7).

Table 3.7. Effect of maize pollen source on seed set, haploid embryo formation, plant regeneration, and haploid green plants from Trident/Molineux F_1's

Maize genotypes	No. of florets pollinated	No. of Seeds set (% in brackets)		No. of plants regenerated	
		With embryo	Without embryo	(%)[1]	(%)[2]
11775	838	208 (24.8)	488 (58.2)	126 (60.6)	(15.0)
A619/A632	452	100 (22.2)	281 (62.2)	32 (32.0)	(7.1)
GH5009	272	64 (23.5)	147 (54.0)	28 (43.8)	(10.3)
Illini Gold	894	315 (35.2)	446 (50.0)	241 (76.5)	(27.0)
K304/K305	974	212 (21.7)	480 (49.3)	113 (53.3)	(11.6)
Seneca 60	923	256 (27.8)	433 (46.9)	199 (77.7)	(21.6)
Sno Gold	228	47 (20.6)	90 (39.5)	21 (44.7)	(9.2)
Total	4581	1202 (26.2)	2365 (51.6)	760 (63.2)	(16.6)

[1] Percentage of plants regenerated from the embryos obtained. [2] Percentage of plants regenerated from the florets pollinated.

The two sorghum genotypes showed very little difference in the percentage of embryos formed and number regenerated into plantlets. However, they had low efficiency compared to the maize genotypes tested.(Table 3.8).

Table 3.8. Effect of sorghum pollen sources on seed set, haploid embryo formation, plant regeneration, and haploid green plants from Trident/Molineux F_1's.

Sorghum genotypes	No. of florets pollinated	No. of Seeds set (% in brackets)		No. of plants regenerated	
		With embryo	Without embryo	(%)[1]	(%)[2]
QLD36	588	7(1.2)	484(82.3)	6(85.7)	(1.0)
TAM422	262	4(1.5)	201(76.7)	4(100.0)	(1.5)
Total	850	11 (1.3)	685 (80.6)	10 (90.9)	(1.2)

[1] Percentage of plants regenerated from the embryos obtained. [2] Percentage of plants regenerated from the florets pollinated.

The fertilization frequency (the percentage of haploid embryos cultured per florets pollinated) was 26% and 1% for maize and sorghum genotypes, respectively. However, sorghum genotypes showed greater efficiency in terms of the frequency of plant regeneration per embryo cultured than maize (91% vs 63%, respectively). In spite of this, maize was overall much more efficient than sorghum (17% versus 1%) in plant regeneration frequency when calculated as per florets pollinated (Tables 3.7 and 3.8).

Overall, 883 haploid embryos were formed out of 3243 florets pollinated. From these embryos 585 plantlets were regenerated, that is 18% of florets pollinated. Overall, 66% of the rescued embryos regenerated into green haploid plantlets (Table 3.9).

b) Effect of different culture media

Differences were observed between the different culture media used for haploid production. Pollen from Illini Gold was used most frequently with the Trident/Molineux wheat F1s and different media were used at different times (Table 3.9). The best plantlet regeneration was obtained when embryos were cultured on half strength Murashige and Skoog (1/2MS) basal culture medium, plantlets being raised from 88% of the haploid embryos rescued. Full strength MS and Gamborg's B-5 media were only 68% successful. The same was true for haploid embryos from other wheat F1s and other maize pollen sources (data not shown). The major effect on planlet regeneration is associated with the culture media and not the pollen source.

Table 3.9. Effects of different culture media on plant regeneration of wheat haploid embryos produced by pollination with maize cv Illini Gold pollen.

Culture media	Number of			
	Wheat florets pollinated	Seeds with embryo (%†)	Seeds without embryo (%)	Regenerants (%)
1/2MS	447	173 (38.7)	223 (49.9)	144 (32.2)
B5	269	88 (32.7)	125 (46.5)	60 (22.3)
MS	178	54 (30.3)	98 (55.1)	37 (20.8)
Total	894			

† Percentage of florets pollinated in brackets

3.3.3. *Production of Doubled Haploids from 24 Different F_1 Wheat hybrids*

The percentage of caryopses developed (CD), embryos formed (EF), plantlets regenerated (PR), doubled haploids obtained (DH) and the efficiency of embryo formation (EEF), plantlet regeneration (EPR) and doubled haploid production (EDH) for each of the 24 F_1 genotypes tested, using wheat × maize crosses are shown in Table 3.10.

All of the F_1 genotypes were crossable with maize cultivar Illini Gold and produced at least a few haploid embryos. Overall, 38% of florets pollinated contained haploid embryos. This result is an improvement over that initially with Trident/Molineux crosses (28%). The percentage of embryo formation (%EF) varied widely between genotypes, ranging from 25% for EXC/R719 to 60% for TRI/BTS. The efficiency of embryo formation (EEF) also showed considerable variation, ranging from 24% for FRM/R702 to 58% for TRI/BTS (Table 3.10).

Table 3.10. Haploid embryos, plantlet regeneration and doubled haploid production with 24 wheat F_1 hybrids crossed with maize.

F_1 hybrid (♀ / ♂)	NFP[1]	% CD[2]	% EF[3]	% PR[4]	% DH[5]	EEF[6]	EPR[7]	EDH[8]
TRI/BTS	189	97.4	59.8	69.1	64.5	58.2	40.2	25.9
TRI/FRM	164	96.3	32.9	57.7	53.3	31.7	18.3	9.8
TRI/EXC	518	97.3	43.7	51.8	47.4	42.5	22.0	10.4
TRI/MCH	288	94.1	35.8	66.0	53.1	33.7	22.2	11.8
TRI/R613	246	85.4	37.6	65.8	71.2	32.1	21.1	15.0
TRI/R702	214	94.9	34.0	60.9	28.6	32.2	19.6	5.6
TRI/R710	374	98.1	44.1	41.4	10.5	43.3	17.9	1.9
TRI/R711	246	95.1	37.2	44.8	10.3	35.4	15.9	1.6
TRI/R712	259	96.9	29.1	42.5	12.9	28.2	12.0	1.5
TRI/R719	307	93.5	48.8	43.6	23.0	45.6	19.9	4.6
TRI/R745	388	98.5	49.0	33.7	12.7	48.2	16.2	2.1
TRI/RVS	220	95.5	36.6	36.4	25.0	35.0	12.7	3.2
TRI/SNF	172	91.9	43.0	38.2	26.9	39.5	15.1	4.1
EXC/FRM	421	98.8	27.6	50.4	48.3	27.3	13.8	6.7
EXC/R702	414	99.0	32.7	31.3	35.7	32.4	10.1	3.6
EXC/R710	433	99.3	29.8	37.5	50.0	29.6	11.1	5.5
EXC/R719	319	98.8	25.4	41.3	24.2	25.1	10.3	2.5
FRM/R702	86	87.2	28.0	81.0	64.7	24.4	19.8	12.8
FRM/R710	374	98.4	37.5	33.3	21.7	36.9	12.3	2.7
FRM/R719	278	98.9	35.6	34.7	20.6	35.3	12.2	2.5
R710/R702	419	98.1	38.0	56.4	51.1	37.2	21.0	10.7
R710/R719	228	96.1	38.4	53.6	26.7	36.8	19.7	5.3
R719/R702	283	92.9	38.4	54.5	18.2	35.7	19.4	3.5
Total	6840	96.5	37.5	46.8	36.5	36.2	16.9	6.2
TRI/MNX[9]	6986	63.0	27.5	63.7	33.0	17.3	11.0	3.6

[1] NFP=No. of florets pollinated
[2] CD=Caryopses developed
[3] EF=Embryo formation
[4] PR=Plantlet regeneration
[5] DH=Doubled haploid production
[6] EEF=Efficiency of EF
[7] EPR= Efficiency of PR
[8] EDH= Efficiency of DH
[9] Main cross result discussed earlier

The percentage of plantlet regeneration showed considerable variation, ranging from 31% for EXC/R702 to 81% for FRM/R702. Only 10% of florets pollinated in EXC/R702 resulted in green plants much less than TRI/BTS with 40% (Table 3.10).

The number of doubled haploid lines produced for each cross combination is shown in Table 3.13. Outside of the main cross TRI/MNX most DH lines were obtained with the R710/R702 hybrids and the least with the FRM/R702 combination.

Table 3.13. Number of DH lines produced for each wheat F_1 hybrid combination

Cultivar	TRI	EXC	FRM	R710	R719
EXC	46				
FRM	16	27			
R710	4	21	9		
R719	12	4	7	11	
R702	9	13	3	41	9
BTS	38				
MCH	29				
MNX	217				
R613-46	34				
R711	4				
R712	5				
R745	7				
RVS	7				
SNF	7				

3.4. Discussion

These pilot trials with a series of wheat cultivars established a protocol for producing haploid embryos under glasshouse conditions in South Australia. Laurie and Bennett (1987) described the effects of the *Kr* cross incompatibility loci in wheat when using *Hordeum bulbosum* as the male parent. According to Snape *et. al.* (1979) some Australian wheat cultivars are cross compatible with *H. bulbosum* allowing them to be used in intergeneric haploid production. The maize system is less sensitive to the action of *Kr* genes so the apparent differences in crossability observed with different wheat cultivars used in the present experiment is probably caused by other factors such as the plant growth conditions, which were not strictly controlled. For example, the plants of Chinese Spring, Janz, Tatiara, Meering, and Aroona used in crosses were infected with powdery mildew and the plants were weakened by this disease. Another factor which may affect the efficiency of haploid production, is a difference between late and early tillers. The florets from main tillers may be more suitable for haploid production due to within plant competition for nutrients and the transpirational stream. Therefore, those cultivars which

have fewer tillers with stronger shoots and healthier heads could result in a higher frequency of haploid embryo.

There were differences in the haploid embryo size obtained on different wheat cultivars. Spear produced a high number of haploid embryos but the embryos were small and these showed a lower ability to germinate and give haploid plantlets. Kite produced moderate sized embryos, but the regenerated plantlets were not as strong as those from Excalibur and needed a lot of care after transplanting.

Some differences were obtained with the maize genotypes used as the pollen source but probably the main factor to consider is the ease of culture in the glasshouse of each maize genotype. Characteristics such as days to anthesis, length of anthesis period, size of tassel and height of plant in male parent should be taken into account. On this basis Illini Gold, K304/K305 and GH5009 are suitable pollinators.

Overall, the maize genotypes used in the current experiments showed a much higher rate of embryo formation in crosses with wheat (26%) than sorghum (1%). This is a small sample of sorghum genotypes. Research might find others that are more effective. Illini Gold, showed the highest rate of haploid wheat embryo production (35%) of all maize and sorghum genotypes tested. Seneca 60, which is used frequently by other investigators in wheat haploid production (Laurie, 1989; Laurie and Bennett, 1988a,b; Laurie and Reymondie, 1991) was the second most efficient pollinator with 28% (Table 3.7).

Culture media affect plantlet regeneration due to different chemical compositions. Theoretically, it should be possible to find a suitable culture medium for a special purpose such as growing wheat haploid embryos. This requires numerous culture media to be tested under properly randomized conditions. It is speculated that different maize genotypes will result in embryos differing in viability (Laurie and Reymondie, 1991; Suenaga and Nakajima, 1989; Islam and Shepherd, 1994). Illini Gold has previously been shown to be a good pollen source for wheat haploid production at all stages including seed set, embryo formation, and plantlet regeneration (section 3.3.1).

Using wheat × maize hybridization, haploid wheat plants were produced from 24 F_1 hybrids of wheat in glasshouse conditions. Unlike the *H. bulbosum* system, the maize

system seems to have no sensitivity for the action of *Kr* incompatible genes (Laurie and Bennett, 1987, 1988). However, there are still differences among wheat cultivars and their cross combinations using the maize system of haploid production but doubled haploids could be produced from all crosses attempted.

The whole procedure of doubled haploid production using a maize pollinator system is summarized in Table 3.14. The time needed for completing one round of DH production is about 13-14 months including the F_1 production period. Since the number of seeds produced directly from doubling of the chromosome number using colchicine treatment is not sufficient for further experiments, at least one additional generation of seed multiplication is a necessary part of each DH production program. An additional 7-9 months time is required for one round of seed multiplication and therefore, between 20-23 months should be spent on one round of DH production.

Excluding the production of wheat F_1 plants and seed multiplication periods, the DH production procedure itself can be divided into three separate steps, namely intergeneric crosses, embryo culture and chromosome doubling (Inagaki, 1989):

1) Production of haploid embryos

The first step is the production of haploid embryos through intergeneric crosses. Earlier in this chapter the maize cultivar Illini Gold was shown to be an efficient pollen donor parent for producing sufficient numbers of haploid embryos in all cross combinations (ranging from 25% to 59%, Table 3.10).

The wheat crosses used in this experiment all produced double haploid lines, though some more easily than others. This makes the wheat x maize system a viable method of doubled haploid production because wheat breeders will not be constrained by this system when choosing parents and making crosses in their programs, as they might if using anther culture or the 'bulbosum' method where certain genotypes are intractable.

Table 3.14. Procedure of DH production form F_1 hybrids in the maize system including one round of seed multiplication.

Process	Note	Duration
Planting wheat parents anthesis time and crossing for F_1 production	Emasculation of female and pollination with male pollen to produce F_1	50-70 days
planting F_1 seeds and maize cv to anthesis time	repeat the above procedure	50-70 days
Crossing	Anthesis time ± 2 day	10-11 days
Hormone application	150ppm 2,4-D solution immediately after pollination	
Embryo rescue	1/2 MS medium	5-6 weeks
Transplanting to soil	With acclimatization	4-5 weeks
Colchicine treatment	0.1% at glasshouse temperature (25±3°C) for 51/2 hours	depends on the varieties
Production of DH seeds		
One round of seed multiplication		7-9 months

One of the factors affecting the production of haploid embryos is the concentration of 2,4-D applied to promote the enlargement of caryopses. One of the observed effects of 2,4-D application is to prevent the natural shrivelling of seed produced by pollinating with maize pollen. In experiments with no 2,4-D, Suenaga and Nakajima (1994) found that the ovaries failed to grow and most of them were shrunken two weeks after pollination. The application of 100ppm 2,4-D before pollination resulted caryopsis development (73%), but no embryos were obtained. However, with the same concentration of 2,4-D applied after pollination they found that it markedly increased the efficiency of embryo formation (16%).

The application of both 100ppm and 150ppm concentration of 2,4-D resulted in an increased number of caryopses (96%) and it, in turn, resulted in a higher number of embryos formed (38%, Table 3.10). No differences were found in the formation of caryopses using 2,4-D prior or post pollination. All of the developed caryopses with or without embryo were filled with liquid, and a few of them contained a degenerated

endosperm. So far, no other hormones have been reported to be more effective than 2,4-D for maize pollination systems (Suenaga and Nakajima, 1994).

2) Plantlet regeneration

The second step in DH production is regeneration of haploid embryos. Regardless of the system used for haploid embryo production, the embryos have to rescued and grown on a supporting culture medium. At an early developmental stage of wheat seed after pollination, the endosperm ceases to develop and then it degenerates to form a residue (Inagaki, 1989). The immature embryos are not capable of completing their development in seeds filled with liquid lacking normal endosperm structure. After a while, the seed begins to shrink and rescue of the immature embryo is then needed.

Culture medium composition and the concentrations of its components is the most important factor affecting the rate of plantlet regeneration. As explained in section 3.3.2, three culture media were compared together and finally 1/2MS was chosen as an efficient medium for future cultures. A constant temperature of 20±2°C appears to be effective for growth of the embryos on the culture media. Also important is that the culture medium has little or no water left on the surface of the gel and that is has a sloping surface to increase the level of contact of embryos with the medium. Other important technical factors are (i) pouring the liquid off the gel before embryo planting, (ii) placement of only 2-3 embryos per tube, (iii) placement of each embryo on the gel with its scutellum in contact with the gel. (iv) using laminar flow cabinet for reducing contamination. Ambient temperature is also important in promoting plantlet growth. Optimum temperature has been proposed as 20±2°C.

Little is known about the nature of plantlet regeneration, but those crosses with higher embryo formation efficiency also had higher regeneration rates (Table 3.10). Total regeneration rate in the present experiment was 47%. FRM/R702 showed the highest percentage of plantlet regeneration (81%, No. of regenerants/No. of embryos × 100). It seems that most of its embryos had greater capacity to grow well on half strength MS culture medium in growth cabinet conditions when compared with the other genotypes (Table 3.10).

3) Chomosome doubling

The third step in DH production is doubling the chromosome numbers of these regenerated haploid plantlets. Improvement of the techniques for increasing the rate of chromosome doubling is critical for maximizing the number of doubled haploid plants. Using the technique described earlier in this chapter has resulted in better than 90% success when plants containing at least three tillers were used. In practice many regenerated haploids died due to their poor growth in agar or after transplanting into soil and were lost before colchicine treatment or in a few cases they were too weak to tolerate the treatment and died. This reduced the DH efficiency so that the best result was 26% from the TRI/BTS cross with a success of 65% from regenerated plantlets (Table 3.10).

The DH lines produced in the current study can now be used to investigate the value of doubled haploids in breeding programs, but some subsidiary questions need to be assumed first.

1) Does the doubled haploid procedure result in a random population of plants representing a random sample of female gametes or are some genotypes more disposed to doubling than others leading to distorted and unpredictable outcomes.

2) If doubled haploid production does not cause distorted ratios then: a) the genetics of other agronomic characters can be investigated and b) the time saving in getting homozygous lines compared to routine pedigree breeding methods might allow DH lines to be used for cross prediction.

Part II

Studies on Segregation Patterns in a Wheat Doubled Haploid Population

Chapter 4

Segregation Patterns of Known Major Genes in the Doubled Haploid Population Derived from (Trident/Molineux) Wheat F_1 × Maize Crosses

4.1. Introduction

If doubled haploid (DH) populations are to become an integral part of plant breeding or of general use for gene mapping, it is important to ascertain whether the genotype of each wheat F_1 gamete predisposes it to producing a haploid plant. If Mendelian ratios are not distorted by the doubled haploid protocol then minimum numbers of DH progeny plants required to recover certain gene combinations in a breeding program can be predicted. Furthermore, for gene mapping studies it is desirable that the DH population produced represents a random selection of F_1 gametes.

As indicated earlier, the F_1 hybrid Trident/Molineux is polymorphic for at least 8 different attributes controlled by known major genes (listed in Table 4.2.1 in Materials and Methods) and also some other traits whose genetics is less well defined. These will be discussed in Chapter 5.

One of these differences is in protein composition, which is important for bread making quality of wheat. Trident and Molineux are polymorphic at one locus controlling high molecular weight glutenin (HMW), namely *Glu*-B1, and two loci controlling low molecular weight glutenins (LMW), namely *Glu*-A3 and *Glu*-B3 (Cornish, 1994). Trident is heterogeneous for two alleles at the *Glu*-A1 locus so this could not be be used for experimentation (Cornish, pers.comm.).

Another difference is in their response to Cereal Cyst Nematode (CCN) infection. CCN is a serious root disease on many farms in Southern Australia (). Resistance, the inability of a cultivar to act as a host, and tolerance, the ability of a cultivar to perform well even when attacked, are highly desirable attributes in any new cultivar. Molineux is CCN resistant, the resistance, thought to be due to a single gene (Greene, pers. Comm.), is from one of its parents Festiguay.

High soil boron levels and boron toxicity is a major limitation to wheat production in parts of South Australia (Cartwright *et al.*, 1983, 1984). Boron tolerance in wheat has been identified as being under the control of several major additive dominant genes (Paul *et al.*, 1991). Therefore, screening for boron tolerant lines is a major selection criterion in seeking improved yields. Trident is known to carry *Bo1* from one of its parents, Spear.

Trident carries a translocation segment of VPM1 carrying the rust genes *Sr38, Lr37* and *Yr17* (Hollamby *et al*, 1993). In South Australian conditions these genes confer effective resistance to stem rust, leaf rust and stripe rust. This translocation from *Triticum ventricosa* is on chromosome 2A and is inherited as a linkage block because of lack of pairing of this segment with wheat chromosomal material (Bariana and McIntosh, 1993).

Screening doubled haploids to determine the segregation patterns for the loci controlling these characters and testing for distorted ratios will establish whether these loci, or chromosome segments linked with these loci, show Mendelian segregation in such populations. Because doubled haploids are a sample of female gametes, the expected ratio for a single locus will be 1:1.

4.2. Materials and Methods

Two hundred and fifty five doubled haploid lines were derived by pollinating F_1 florets from the cross Trident/Molineux with maize or sorghum. The quantities of seed produced from each doubled haploid varied considerably, and some lines produced extremely shrivelled seed, which could not be screened for all the attributes listed in Tables 4.2.1 and 5.2.1.

Table 4.2.1. Traits controlled by known major genes and showing polymorphism between wheat cultivars Trident and Molineux.

Trait	Alleles present in Trident	Molineux	Chromosome location	Reference
Stem rust reaction	*Sr38*		2AS	McIntosh (1996)
	Sr12		3BS	McIntosh *et al.*, (1995)
	-	*Sr5*	6DS	"
		Sr30	5DL	"
		Sr?	?	"
Stripe rust reaction	*Yr17*	-	2AS	"
	-	*Yr6*		"
Leaf rust reaction	*Lr37*	-	2AS	"
	-	*Lr1**	5DL	"
	-	*Lr2a*	2DS	"
Plant height	*Rht2*	-	4DL	Gale *et al*, (1975)
	-	*Rht1*	4Aα	Gale and Marshall (1975)
High soil boron	*Bo1*	*bo1*	7B	Paul *et al.*, 1991
HMW glutenin (*Glu-B1*)	7+8	7+9	1BL	Payne *et al*, (1980)
LMW glutenin (*Glu-A3*)	e	c	1AS	Cornish (1994)
(*Glu-B3*)	h	c	1BS	"
Cereal Cyst Nematode reaction	Susceptible	Resistant	Unknown	Greene, pers. comm.

4.2.1. Determination of Endosperm Protein Phenotypes

Payne and Corfield (1979) fractionated wheat proteins with cross-linked Sepharose gels. Glutenins of different molecular weights were then fractionated by polyacrylamide-gel electrophoresis in the presence of SDS-PAGE to characterise their polypeptide compositions. Payne *et al.* (1981) used this gel system to describe the genetic variation of HMW subunits in a large selection of hexaploid wheat varieties. The method developed by Laemmli (1970) has been modified by Payne *et al.* (1980). Wheat endosperm proteins have also fractionated with 10% one-dimensional polyacrylamide gel electrophoresis (Payne *et al.*, 1981). Singh *et al.* (1991) developed a simplified procedure for one-step one dimensional SDS-PAGE to fractionate both HMW and LMW glutenin subunits simultaneously. This method, slightly modified, was used in the present experiment. The Parents differed in HMW and LMW and glutenin bands.

4.2.1.1. Gliadin Analysis

The gliadin proteins of wheat are known to be partly determined by the loci *Gli-A1, Gli-B1* and *Gli-D1* which are tightly linked to the Low Molecular Weight glutenin subunit loci *Glu-A3, Gl-B3* and *Glu-D3* on chromosome arms 1AS, 1BS and 1DS, respectively, Therefore, determination of gliadin segregation patterns in the parents and DH lines can be used to help identify the more complex LMW bands (Singh *et al.* 1991).

The endosperm half of a single kernel of each DH lines was crushed into a fine powder using a hammer on a clean metal plate. This powder was placed into a 1.5 ml Eppendorf tube and the mixture was suspended in 300 µl of 70% ethanol by breaking it up with a fine spatula prior to vortexing. Then it was placed into a 60°C oven for 30 min with other vortexing at 10 min intervals. Following the final vortexing the mixture was centrifuged for 2 min at speed 10 using an Eppendorf tube centrifuge at 5000 rpm. The supernatant (100 µl) was transferred into a new Eppendorf tube and the ethanol evaporated at less than 60°C until 20 to 30 µl of supernatant was left then 100 µl 2x SDS sample buffer (80 mM Tris-HCl pH 8.0, 40% glycerol w/v and 0.02% bromophenol blue) was added. After the addition of a drop of glycine and remixing, the sample was centrifuged and then 14 µl of the supernatant were loaded into a sample well of a SDS-PAGE gel. The residue was retained for glutenin extraction.

4.2.1.2. Glutenin Analysis

The solid residue left after gliadin extraction was washed several times by resuspension in 1 ml of 50% v/v n-Propan-1-ol followed by centrifugation and discarding the supernatant using a fine tipped syringe attached to a tap-water vacuum pump.

Glutenin was extracted from the gliadin free residue in 100 µl of a compound solution of 50% n-Propanol and 80 mM Tris-HCl (pH 8.0) to which was added, just before use, 1% (v/v) dithiothreitol. After a brief initial vortexing the extract was then placed in an oven at 60°C for 30 min to reduce the glutenin aggregates. This was followed by a 3 min centrifugation. The extraction was completed using another 100 µl of the same compound solution plus (freshly mixed) 1.4% 4-vinylpyridine which was added to each tube and incubated in 60°C oven for 30 min for protein alkylation. The sample was

then centrifuged for 2 min and 50 µl of supernatant transferred to a new Eppendorf tube containing 100 µl of sample buffer, vortexed briefly and incubated for 15 min in the same oven for complexing SDS with the reduced and alkylated glutenin polypeptides. After centrifugation for 2 min, 14 µl of the supernatant were loaded into a sample well of the gel for SDS-PAGE separation of the glutenin subunits.

4.2.1.3. SDS-PAGE Preparation and Electrophoresis

The procedure for polyacrylamide gel electrophoresis (PAGE) involved a gel system for separating the protein molecules containing separating gel buffer (pH=8.8), acrylamide plus cross linker 1% (Acrylamide/Bis), Tetramethyl Ethylenediamine (TEMED) and catalyst Ammonium Persulphate (APS). The formulae of each part are given in Table 4.2.2.

The separating gel was prepared by pouring the separating gel solution between two glass plates which were separated by 1mm plastic spacers. After setting, the stacking gel which includes the sample loading wells was made according to the details in Table 4.2.2 and poured onto the top of the separating gel. After setting of this gel, the combs (28 wells) were removed and the wells were filled with electrode buffer. The samples were loaded into these wells and separated using 90 mA constant current and 400 V for 2-3 hours.

4.2.1.4. Staining and drying the gels

A stain solution with 0.25g Coomassie Brilliant Blue R (in 25ml H_2O), 57.8g Trichloroacetic acid, dissolved in 720ml H_2O plus 180ml Methanol and 63ml Glacial Acetic Acid was used for staining the bands with gentle shaking overnight on a platform rocker. To dry the stained gel a fixing solution with 40% Methanol, 10% Glacial Acetic Acid and 3% Glycerol by volume and made up to one litre with H_2O was used for one hour on the platform rocker then it was placed in between two layers of a cellophane membrane backing and left at room temperature for a few days to dry.

Table 4.2.2. Composition of SDS-PAGE gel used for separating the HMW and LMW subunits of glutenins in wheat DH lines.

Stock solution	pH	Amount used per gel	Chemical compounds	Concentration
Separating gel buffer (2×)	8.88	13ml	Tris	45.412g
			SDS	1.000g
			H_2O	in 460ml
Acrylamide solution for separating gel†		8.7ml	Acrylamide	75g
			BIS	0.75g
Distilled water		4.3ml	H_2O	up to 250ml
Final volume		26ml		
Temed		62µl		
APS		74µl		
Stacking gel buffer (2×)	6.8	5ml	Tris	6.06g
			SDS	0.4g
Stacking gel			H_2O	in 190ml
Acrylamide solution for stacking gel‡		1.3ml	Acrylamide	87.5g
			BIS	1.32g
Distilled water		3.7ml	H_2O	up to 250ml
Final volume		10ml		
Temed		20µl		
APS		50µl		

† Contains 30% acrylamide and 1% crosslinker, ‡ Contains 35% acrylamide and 1.5% crosslinker

4.2.1.5. Identifying and Scoring the bands

The bands were identified and scored according to Cornish *et al.* (1993). There were no problems in identifying the HMW bands. However, LMW patterns were difficult to identify, but made easier with the help of linked gliadin patterns.

4.2.2. Segregation of Genes Controlling Cereal Cyst Nematode Reaction (*Heterodera avenae*)

4.2.2.1. Setting up the Nursery

The protocol employed was developed by the South Australian Research and Development Institute (SARDI) for use by cereal breeders in South Australia. Mr John Lewis (SARDI) kindly supplied the materials and his group did much of the work of this bioassay. CCN inoculated media was prepared by mixing highly infested soil (known as Tailem Bend Sandy Loam, TBSL) with a washed builders sand to achieve approximately seven eggs per gram of soil. The infested TBSL was from paddocks in which highly susceptible varieties had been grown in previous years.

Black plastic P2 plant tubes (100mm deep with 50mm diameter at the top and 44mm diameter at the bottom) were filled with approximately 200-210g of CCN-infested moist soil and a single seed of the appropriate line was sown in each tube. Ten tubes of each test line (doubled haploid or parent) were planted. Tubes were kept in order in galvanized iron mesh crates (Plate 4.1a), 10 rows × 5 columns, ie 50 tubes per crate. The crates were then placed outside and exposed to normal environmental conditions as nematode infestation has been found to behave more consistently under these conditions (Plate 4.1b). The area was covered with netting to protect the nursery from birds. The nursery was watered by overhead sprinklers as required. A second replicate of 10 tubes per line was planted about one week later.

4.2.2.2. Scoring

In late October, 18 weeks after seeding, plants were carefully removed from their tubes (Plate 4.1c), and the number of developing white female nematode cysts on the outside of the rootball and, tube interface was counted under a large magnifier which is surrounded with a fluorescent tube (Maggy lamp, see Plate 4.1d). The number of cysts on each sample were recorded and later analysed using the software program POTS (a purpose made data base management program of SARDI).

4.2.3. Measurement of Plant Height and GA_3 Insensitivity

Both parental cultivars used to produce the doubled haploid population are semidwarf, with Trident (98.7cm tall) possessing the *Rht2* gene and Molineux (98.9cm tall) the *Rht1* gene. Therefore, the DH progeny are expected to segregate into four classes of homozygous genotypes (*Rht1Rht1Rht2Rht2*, *Rht1Rht1rht2rht2*, *rht1rht1Rht2Rht2* and *rht1rht1rht2rht2*) in equal proportions.

All DH lines were grown in pots in the glasshouse and in rows under field conditions. At maturity the height of the DH lines were measured from ground level to the base of the tallest spike. Since discrimination between the tall and semi-dwarf lines was often difficult a gibberellic acid (GA_3) insensitivity test was applied to plants from tall lines.

Plate 4.1. Cereal Cyst Nematode Screening Procedure.

Plate 4.1a. White CCN females on the roots of wheat at the soil-pot interface (1.5× magnification)

Plate 4.1b. Magnifier lens (Maggi lamp) used to count the white females and the computer for direct entry of results. The 50 tubes of a crate are depicted on the screen.

Plate 4.1. Cereal Cyst Nematode Screening Procedure.

Plate 4.1c. View of crate containing 50 polyvinyl tubes with 5 tubes in a row making up 1 replicate and each replicate is bar coded. (One seed was sown into CCN infested soil in each tube but in a few cases the plant did not grow).

Plate 4.1d. SARDI experimental field where the crates were placed for the growing season.

In this test two seeds of each of the suspect DH lines with uncertain genotypes were sown in a small plastic pot. A second pot was planted of each line as a control. Plants in one pot were sprayed with 100ppm gibberellic acid in water at the 3 and 4 leaf stage. Plant reaction was recorded as being sensitive or insensitive to the application of GA_3, when the response was compared to the unsprayed control plants. These data helped to classify semidwarf or tall lines into their relevant genotypic class. GA_3 sensitive plants showed rapid elongation of leaf sheaths, these were classified as carrying neither *Rht1* or *Rht2*, that is as talls.

4.2.4. Assay for High Soil Boron Tolerance

In boron intolerant wheat genotypes like Molineux, boron is absorbed passively by roots and swept along in the transpiration stream to accumulate at sites of transpiration especially leaf tips. High concentrations in the plant are toxic and reduce yields. Intolerant roots tend not to grow into soil high in boron, that is with concentrations greater than about 10ppm. Soils with toxic levels of boron are widespread in South Australia (Cartwright *et al.*, 1994). On the other hand genotypes tolerant to high soil boron, like Trident, actively exclude boron from being absorbed in the transpiration stream and so plant tips are not exposed to toxic concentrations. Roots on such plants tend to grow into high boron layers in soil.

Several methods have been developed in South Australian research programs to screen for boron tolerance. The method used with the Trident/Molineux doubled haploids was developed by Campbell *et al.* (1996). Seminal roots are challenged to grow into an aerated nutrient solution containing 100ppm boron added as boric acid as following:

Nylon mesh was glued to the base of plastic meat defrosting grids. This created a set of 18 × 6 cell (1×4cm) with a mesh 'floor' to hold 108 test lines where roots could grow through the 1.5 mm mesh (Plate 4.2). Five seeds of each test line were placed in each cell and the mesh was suspended to make contact with a reservoir of distilled water in such a way that all seeds were in contact with the water. After germination the

Plate 4.2. Screening for Boron tolerance. View of mesh grid (with 6 × 18=108 cells) with seedlings in place and the plastic box used as solution reservoir, at the end of Boron assay. Note: each cell contains two test seedlings.

two most vigorous seedlings (normal growth with roots growing downward through the mesh floor) were identified and the other 3 seeds were discarded. The distilled water was then replaced with a nutrient solution containing 100ppm boron, added as boric acid. A small aquarium aerator was used to aerate the solution. After ten days the length of the longest seminal root of each seedling was measured. Longer roots indicate better boron tolerance. Both parents and two control varieties Halberd (boron tolerant) and Oxley (boron intolerant) were included in the test.

The experiment was planted in two replications at two different planting dates (two week intervals) and the combined results were analysed by ANOVA.

4.2.5. Segregation of Genes Controlling Rust Reaction.

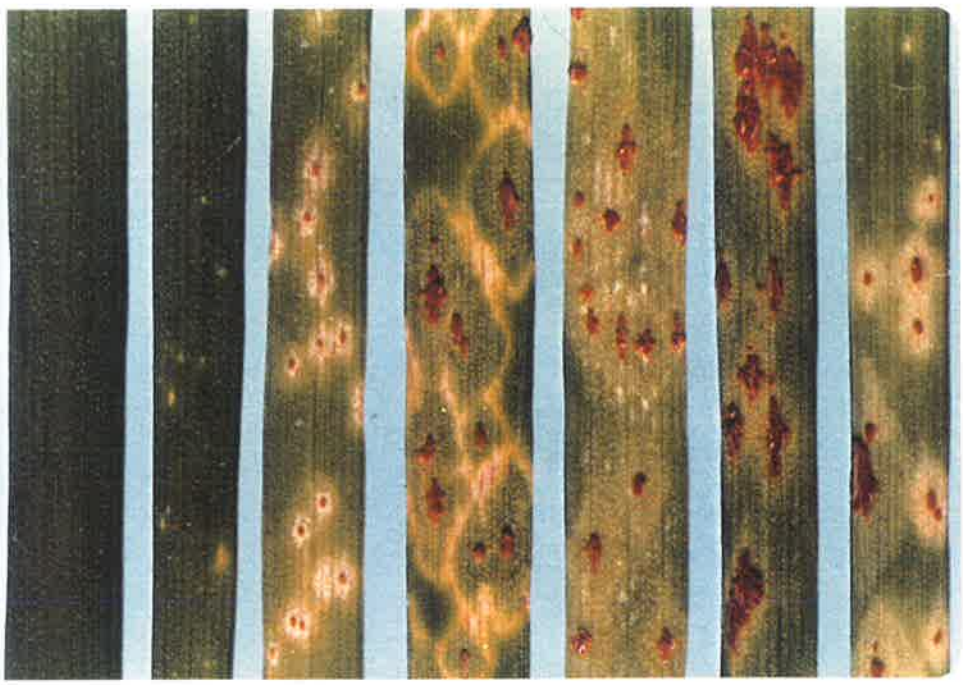
As shown in Table 4.2.1 several loci conferring resistance to stem rust were segregating in the DH progeny. The most important economically is the VPM1 segment located on chromosome arm of 2AL in Trident. This segment was originally derived from *Triticum ventricosa* (Bariana and McIntosh, 1993; Hollamby *et al.*, 1993) and in wheat it is inherited as a linkage block carrying *Sr38*, *Lr37* and *Yr17* (Bariana and McIntosh, 1993). As shown in Table 4.2.3 this cross contains several stem rust resistance genes which will be segregating and their individual detection will depend on using appropriate races of stem rust.

The stem rust analyses were kindly performed by Drs D. The and J. Bell, Plant Breeding Institute, University of Sydney, Cobbitty, New South Wales, using laboratory stem rust race 74L-1 which corresponds to race 34-1,2,3,4,5,6,7 in the Australian nomenclature system (McIntosh *et al.*, 1995).

Plate 4.3 Rust reaction types on seedling wheat leaves (reproduced with permission from McIntosh, pers. comm.)

From left to right:

0	immune
;	fleck hypersensitive reaction
1	small pustules, large hypersensitive halo
2	medium pustules, hypersensitive reaction not so marked
3	large pustules, very little hypersensitive reaction. Regarded as susceptible
4	very large pustules, no hypersensitive reaction. Fully susceptible
X	A mixture of pustule sizes with hypersensitive reactions. Effectively resistant



Ten seeds of each of 252 doubled haploid lines along with their parents were sown in a glasshouse for inoculation at the two leaf seedling stage. Stem rust reaction was scored approximately two weeks after inoculation. Plate 4.3 illustrates the reaction types of stem rust.

DH lines were classified for the presence or absence of *Sr38* (Table 4.2.3). Reaction types X, X- and X= contain *Sr38* alone or in combination with other resistance genes. Those DH lines not containing *Sr38* had reaction types ranging from 2= to 4 depending on other genes carried. It is not possible to unequivocally identify presence or absence of genes other than *Sr*38 because with the stem rust races available the phenotypes overlap.

Table 4.2.3. Stem rust genes segregating in Trident/Molineux cross and their reaction type (phenotype) in response to stem rust race *74L-4*.

Genotype	*Sr5* locus	*Sr12* locus	*Sr30* locus	*Sr38* locus	Unknown *Sr?*	Type of reaction (phenotype)
Trident	-	*Sr12*	-	*Sr38*	-	X
Molineux	*Sr5*	-	*Sr30*	-	*Sr?*	3-→3
Sr5	*Sr5*	-	-	-	-	3+→4
Sr12	-	*Sr12*	-	-	-	3+→4
Sr30	-	-	*Sr30*	-	-	3→4
Sr?	-	-	-	-	*Sr?*	3+
Sr38	-	-	-	*Sr38*	-	X

In a field test experiment held at Roseworthy, the DH-lines were growing under irrigated conditions. Each DH-line, with 20 seeds available, were planted in a single row in two replicates. Observations on reactions of each DH row to a natural stripe rust infection were recorded as a score between 1 to 5, with 1 representing little or no infection to 5 being highly infected.

4.3. Results

The data collected and the classification of each doubled haploid are detailed in Appendix 1.

4.3.1. Determination of Endosperm Protein Phenotypes

Plates 4.4a and b show electrophoresis gels for HMW and LMW glutenin subunits and gliadins, respectively, illustrating the different protein banding patterns. Tables 4.3.1. and 4.3.2 show the DH progenies classified into each of the eight possible genotypes

from the simultaneous segregation at the 3 loci *Glu-B1*, *Glu-A3* and *Glu-B3*. No variation was observed at the other HMW and LMW loci, as expected, because both parents carried the same alleles at these loci.

At the *Glu-B1* locus 96 DH progeny carried the allele controlling the 7+9 HMW glutenin subunits and 84 had the allele for 7+8. This did not vary significantly from the expected 1:1 ratio ($\chi^2_{[1]} = 0.8, P = 0.75$). Segregation of the other loci were similar and

Table 4.3.1. Segregation of HMW and LMW glutenin subunits controlled by genes of three loci.

Genotypes			Observed no.
Glu-B1	*Glu-A3*	*Glu-B3*	of DH Lines
7+8	c	b	28
7+8	c	h	13
7+8	e	b	24
7+8	e	h	19
7+9	c	b	20
7+9	c	h	30
7+9	e	b	21
7+9	e	h	25

Table 4.3.2. Segregation of seed protein phenotypes in DH lines from Trident/Molineux

Locus	Frequencies of phenotypes in DH lines		Expected ratio	$\chi^2_{[1]}$	P
	Trident type	Molineux type			
HMW (*Glu B1*)	(7+8)	(7+9)			
	84	96	1:1	0.80 ns	0.40
LWM					
(*Glu A3*)	(e)	(c)			
	91	89	1:1	0.02 ns	0.89
(*Glu B3*)	(h)	(b)			
	93	87	1:1	0.20 ns	0.67

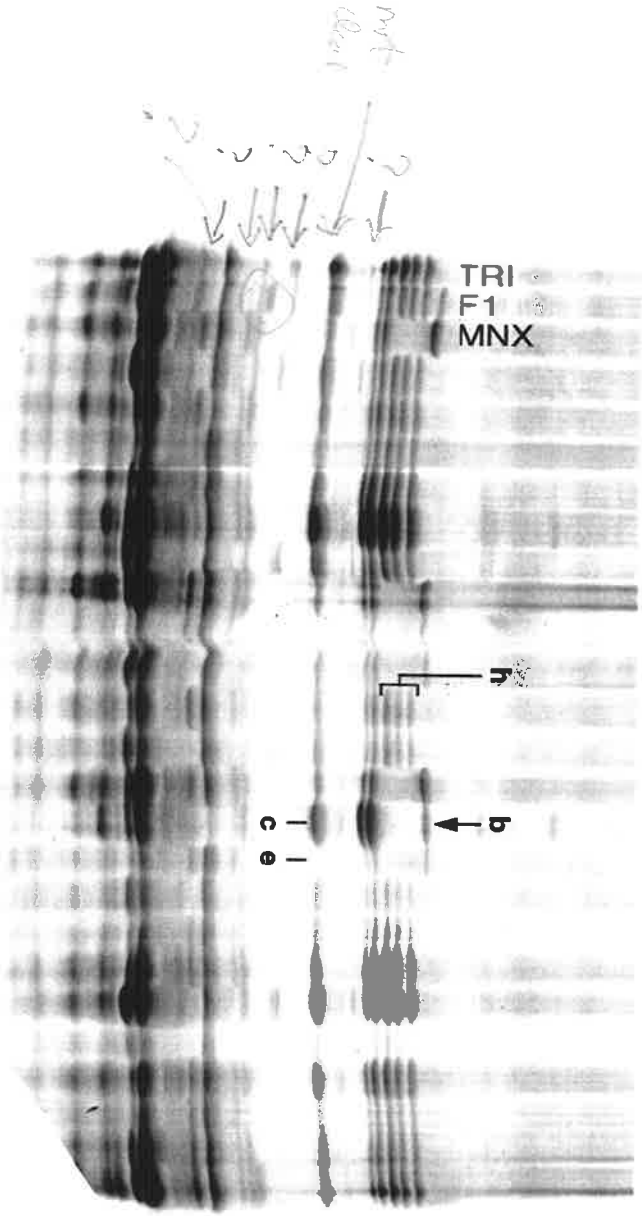
Plate 4.4a. Electrophoretic banding patterns of Trident, Molineux, their F_1 progeny and 23 doubled haploid lines derived from intergeneric crosses of their F_1 with maize:

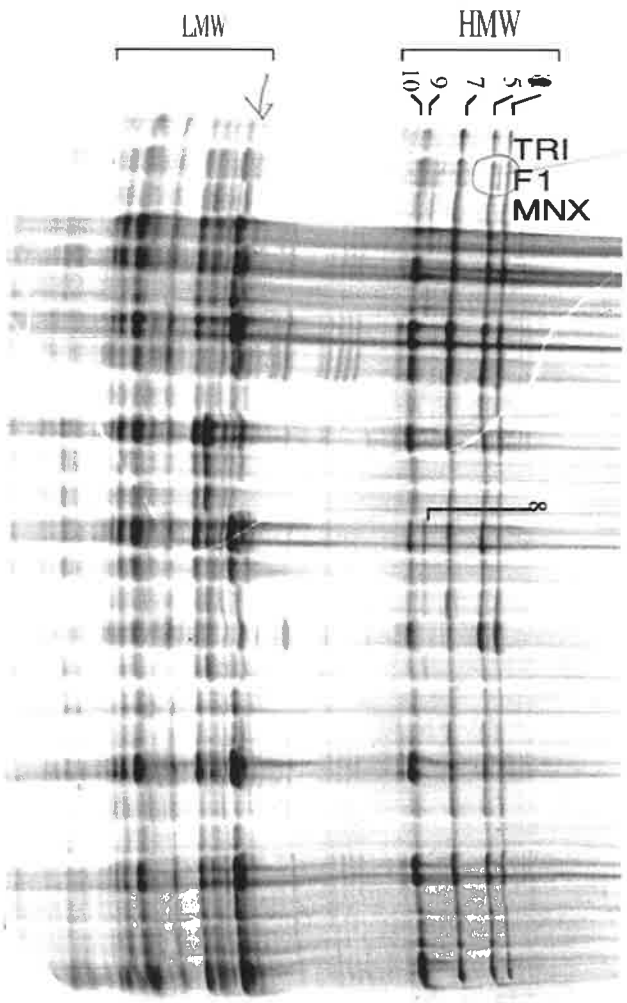
High Molecular Weight glutenin subunits show variation for *Glu-B1* locus for Trident and Molineux and doubled haploid progenies and Low Molecular Weight glutenin subunits show variation for *Glu-A3* and *Glu-B3* loci.

The Trident parent of the F_1 shown here would have carried the allele 2* rather than 1 at *Glu*-A1 hence the extra band in the HMW glutenins.

Plate 4.4b. Gliadin banding patterns of Trident, Molineux and their F_1 progeny and 23 doubled haploid lines derived from intergeneric crosses of their F_1 with maize:

Gliadin band patterns were used to decipher the LMW glutenin subunits present. The *Gli*-1 group of loci show complete linkage with the *Glu*-3 loci and the band pattern produced is much less cluttered than that of the LMW. The gliadin bands marked are linked to the LMW glutenins according to their labels. Thus alleles b and h at *Glu*-B3 can be identified, and alleles c (band present) and e (band absent) at *Glu*-A3 can be identified.


TRI
F1
MNX
f
c
e
d


LMW
HMW
10
9
7
5
TRI
F1
MNX
8

when the three loci were considered jointly no significant departure was found from all 8 genotypes occurring in equal proportions ($\chi^2_{[7]} = 9.16, P = 0.25$). Therefore, no evidence of any distorted segregation for protein phenotypes was found using these DH progeny.

4.3.2. Segregation of Genes Controlling Cereal Cyst Nematode Reaction

Table 4.3.3 shows the scoring criteria related to CCN white female number counted on the soil-pot interface. Lines showing white females numbers equal to or less than the resistant parent, Molineux, were regarded as resistant lines, those with the same number or more white females than the susceptible parent, Trident, were regarded as susceptible lines and those in between are doubtful and ideally should be retested. Time constraints prevented these assays being repeated.

Table 4.3.3. Scoring criteria based on the number of white females counted on the pot-soil interface

Number of females counted	Phenotype	Symbol
<4.5	Resistant	R
4.5-7.5	Uncertain	R-S
>7.5	Susceptible	S

The analysis of variance of DH-lines for the number of nematodes counted in root interface is presented in Table 4.3.4. The number of nematodes counted on different DH lines exhibited highly significant differences (LSD=4.6, P=0.01).

Table 4.3.4. Analysis of variance between DH-lines for number of white females counted.

Source of Variation	*df*	*SS*	*MS*	*F*
DH-lines	217	20332.69	93.70	21.55**
Replication	1	0.26	0.26	0.06
Error	217	943.51	4.35	

Figure 4.1 shows the frequency distribution of DH-lines for the number of white females counted on the roots at the pot-soil interface in the bioassay. The data graphed represented the means of up to 20 plants. Molineux had an average of less than 4.5 white females on the root interface, whereas Trident, the susceptible parent had more than 7.5.

The distribution is clearly bimodal, suggesting that the CCN resistance segregating in this Trident/Molineux population is due to segregation of alleles at a single locus. Because the two sub-populations, resistant and susceptible overlap in the range 4.5 to 7.5 white females/plant it is not possible to determine an exact discrimination between the two. Thus in Table 4.3.5 different cutoffs have been used and the hypothesis of a single gene segregation tested for five different segregation patterns. Except for using a cutoff of 4.5 cysts/plant to distinguish between resistant (<4.5) and susceptible (>4.5) which gave a significant departure from a 1:1 expectation there was no significant distortion.

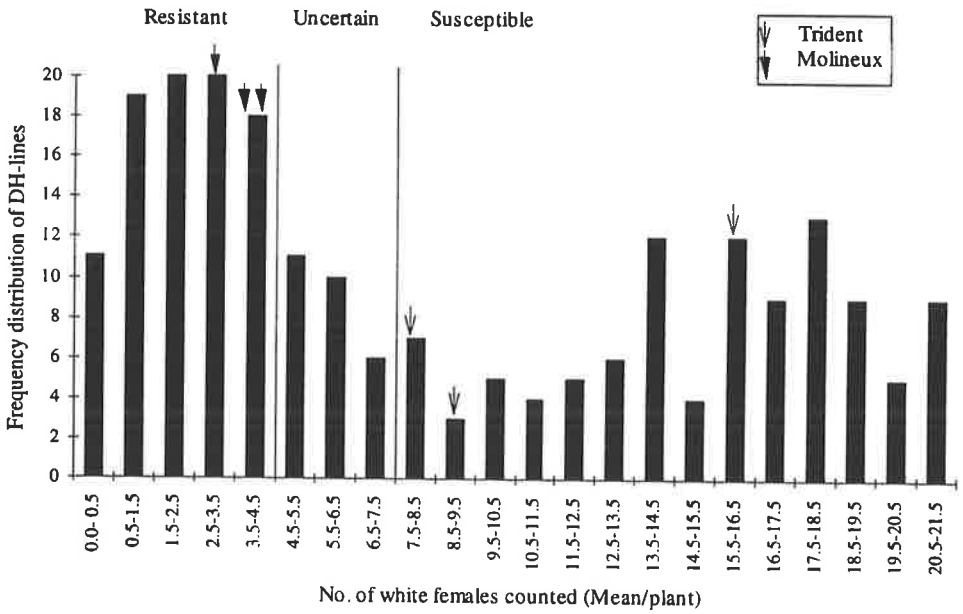


Fig 4.1. Frequency distribution of DH-lines for number of white females counted in bioassay.

Table 4.3.5. Ratios of resistant to susceptible progeny based on different cutoff point for classifying resistant progenies.

Cutoff point	Resistant	Susceptible	$\chi^2_{(1)}$	P%
4.5†	88	130	8.09	<1%
5.5	99	119	1.83	18%
6.5	109	109	0.00	100%
7.5	115	103	0.66	43%
8.5	122	96	3.10	8%

† White females per plant

4.3.3. Segregation of Genes Controlling Plant Height and GA_3 Insensitivity

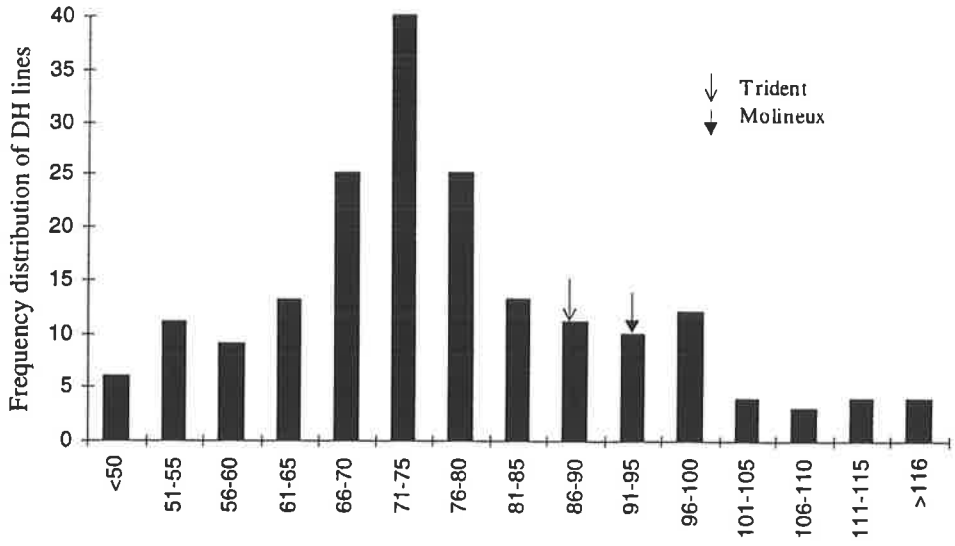
The frequency distribution of lines for plant height measured on DH plants in the greenhouse is depicted in figure 4.2.

It has been shown that there is a close linkage between the major genes *Rht1* and *Rht2* controlling height in wheat and those controlling gibberellic acid insensitivity (Gale *et al.*, 1975), namely *Gai1*, *Gai2*, etc. For example *Gai1* and *Rht1* are very closely linked on chromosome 4BS while *Gai2* and *Rht2* are very closely linked on chromosome 4DS (Gale *et al.*, 1975). Therefore, GA_3 insensitivity test was used to identify 'tall' semidwarfs (ie *Rht1*, *rht2* and *rht1Rht2*) from 'short' talls (*Rht1Rht2*). Test crosses would be necessary to unequivocally classify short lines into double dwarfs and semidwarfs. Double dwarfs do however have a different general appearance to short semidwarfs and this allowed some precision to classifying the short lines into putative genotypes. The frequency distribution of the putative genotypes is tabulated in Table 4.3.6.

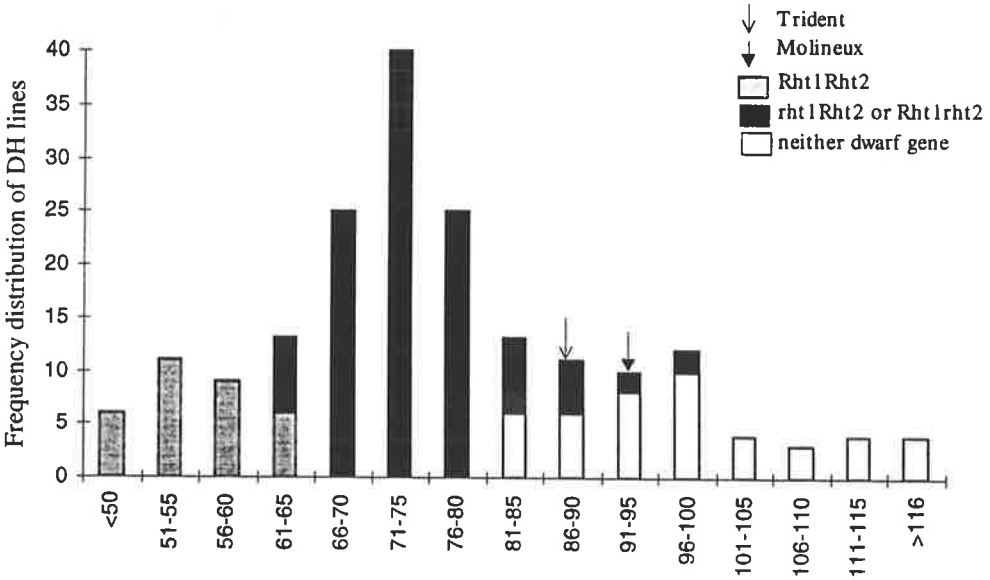
Table 4.3.6. Approximate number of lines carrying *Rht1* or *Rht2* genes based on their phenotypes.

Phenotype	Genotype	No. of DH line	Range of height
Double dwarf	*Rht1Rht2* †	52	44-67
Semi-dwarf	*Rht1rht2* *rht1Rht2*	119	66-100
Tall	*rht1rht2*	45	82-129
Total		216	χ^2=2.69^{ns}

† *Rht* is assigned to show the presence of dwarfing gene, even though it is recessive
ns Not significant at 5% level of probability



(a) Plant height (cm)



(b) Plant height (cm) with GA_3 response superimposed

Fig. 4.2. Frequency distribution of DH lines from Trident/Molineux cross for plant height (a) without GA_3 response and (b) with GA_3 response superimposed.

The expected Mendelian ratio for doubled haploid lines segregating for two complementary height genes is 1 double dwarf: 2 semidwarf: 1 tall. The frequencies observed were in close agreement with this expectation, $\chi^2_{[2]} = 2.7, P = 0.27$. Thus, for these two loci the doubled haploid protocol has not affected the randomness of the lines produced.

4.3.4. Assay for High Soil Boron Tolerance

Plate 4.5 shows variation in root length between DH lines and control cultivars when grown in a 100ppm concentration of boron solution. Halberd is moderately tolerant due to the dominant gene *Bo1*(Paul *et al.*, 1990, Chantachume *et al.*, 1995). It is the same source of the boron tolerance exhibited by Trident, as the pedigree tree in figure 4.3 illustrates.

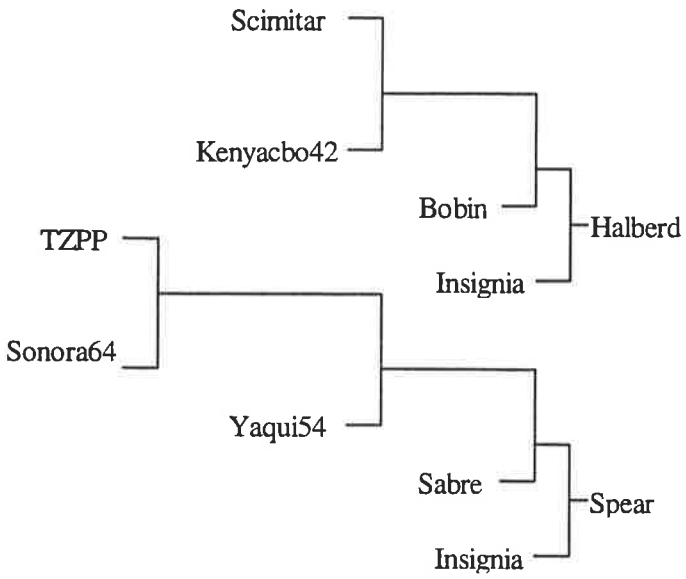


Fig. 4.3. Family tree of Halberd and Spear (a parent of Trident). Insignia which is a common parent of these cultivars is the source of boron tolerance in both Halberd and Trident

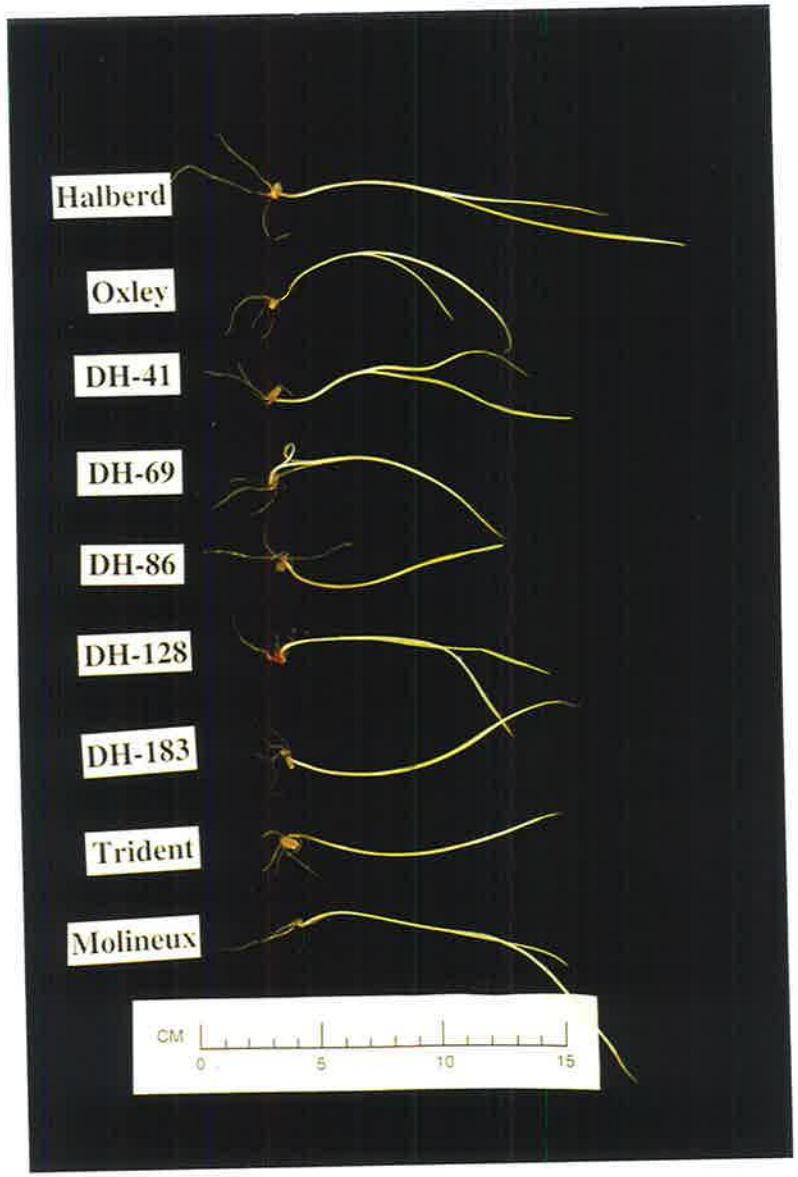
Relative seedling root growth in solution high in boron (100ppm) is used as a measure of boron tolerance (Campbell *et al.*, 1996). DH lines showed significant differences in tolerance to boron concentration (Table 4.3.7). Analysis of variance of the root length of doubled haploids when grown in 100ppm boron solution showed highly significant differences between them ($Lsd_{(p=0.05)}$=2.18). The mean ranged from 2.15cm to 10.18cm, see appendix 1.

Table 4.3.7. Analysis of variance between DH-lines for tolerance to high soil boron .

Source of Variation	*df*	*SS*	*MS*	*F*
DH line	159	123035.7	773.80	4.4**
Replication	1	7960.05	7960.05	45.5**
Error	159	27843.2	175.12	
Total	319	158838.9		

** Significant difference at 1% level

Plate 4.5. Root lengths of doubled haploid lines and their parents two weeks after growing in a 100ppm concentration of Boron solution. Halberd and Oxley were used as controls.


Halberd
Oxley
DH-41
DH-69
DH-86
DH-128
DH-183
Trident
Molineux
CM
0
5
10
15

No doubled haploids however were significantly more tolerant than Trident or less tolerant than Molineux, and the frequency distribution for root length was distinctly bimodal as displayed in figure 4.4. These data are consistent with the segregation of 2 alleles of a single locus. The distribution of the tolerant lines overlaps that of the

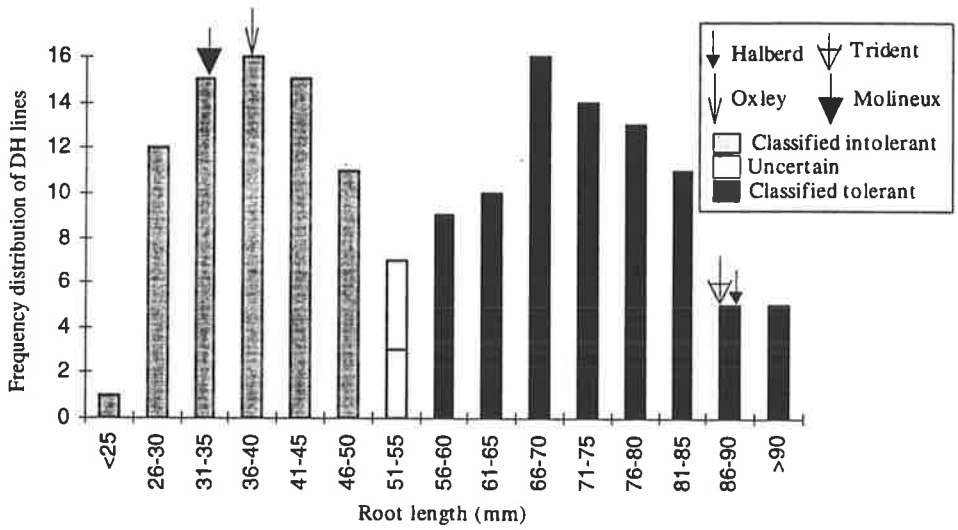


Fig. 4.4. Frequency of DH lines with indicated root length when grown in nutrient solution with high boron concentration

intolerant lines. In order to test for a Mendelian segregation of 1:1 the total number of lines in the overlapping root length class 51-55mm was split. This gave an observed segregation of 87 tolerant and 73 intolerant lines. This pattern is not significantly different from the 1:1 expectation ($\chi^2_{[1]} = 1.2, P = 0.27$). Thus, with respect to the *Bo1* locus, and genes closely linked to it, results are consistent with having a random sample of F_1 gametes in the DH population.

4.3.5. Segregation of Genes Controlling Rust Reaction

The observed frequency of reaction types to stem rust race 74L-1 was:

98 doubled haploid lines with resistance reaction types X, X- or X= and

119 doubled haploid lines with reaction types different to X.

This segregation was not significantly different from that expected of 1:1 ($\chi^2_{[1]} = 2.03, P = 0.14$).

Field stripe rust reactions recorded in the Roseworthy field trial produced a preponderance of lines scored as resistant. The susceptible lines were not distributed randomly over the area but were concentrated in one section indicating that there was probably an initial focus of infection at that location and the infection had not spread completely across the nursery. Consequently, there would have been many escapes. The stripe rust data were therefore discarded as being inaccurate. Thus it was not possible to identify with the stem rust race available and because of an ineffective stripe rust nursery those doubled haploids carrying the VPM1 chromosome segment with *Sr38, Yr17* and *Lr37*. A DNA marker might be more successful.

4.4. Discussion

For use in genetic studies, especially gene mapping, it is crucial that doubled haploids produced from a cross represent a random sample of gametes. For plant breeding it is also desirable that a random sample is obtained because then it is possible to calculate minimum number of doubled haploids that must be produced to have a particular expectation of recovering the most desirable gene combination. If some gametes because of their genotype are more or less prone to becoming a doubled haploid than others then the outcomes from a doubled haploid breeding procedure will be unpredictable and distorted.

The presence of distorted segregation have been reported by Thompson *et al.* (1991) for microspore-derived barley plants in which four of ten investigated loci showed segregation distortion in microspore-derived barley plants. Moreover, distorted segregation were reported in anther culture derived DH lines in rice (Guiderdoni *et al.*,

1989) and in barley DH lines obtained through the *H. bulbosum* technique, but less severe than that of microspore-derived DH lines (Pickering, 1983). Overall, it was concluded that DH lines produced by the *H. bulbosum* technique in comparison with those of microspore culture and anther culture techniques are more representative of a random sample of parental gametes. In the case of wheat less work have been done to test for distorted frequencies of segregating economically important genes. Laurie and Reymondie (1991) compared three systems of haploid production in wheat and found that maize is less genotype-dependent than *H. bulbosum* or anther culture technique. Suenaga and Nakajima (1991) on the other hand reported a considerable varietal difference in the efficiency of embryo formation in wheat × maize crosses. Therefore, they planned a trial to test a number of genetic markers in a separate study but found no distorted segregation ratios (Suenaga and Nakajima, 1993).

The current study of major genes on different chromosome arms gave no case of distorted segregation patterns showing departure from expected Mendelian ratios. Some gamete genotypes could be unequivocally determined, eg. the protein alleles. Other characters which were classified on the basis of quantitative measurements such as boron tolerance and CCN resistance gave some room for misclassification because of the overlap of the distributions in the progeny. Retesting would have helped classify such plants more accurately. This retesting is essential if this population of doubled haploids is to be used for gene mapping.

Amongst the loci investigated one, the CCN resistance, has not yet been fully located. The bimodal frequency distribution and the non-significant departure from a 1:1 ratio among DH-lines supports the previous claims about the monogenic nature of resistance to CCN (Cook, 1974; Nilson, 1966; O'Brien *et al.*, 1980). Although Slootmaker *et al.* (1974) and O'Brien *et al.* (1980) have characterized a dominant allele at a locus (*Cre1* or *Cen1*) on chromosome 2B in the line AUS10894 (Loros) which has been extensively used as a source of resistance in breeding programmes at Horsham, Victoria, Greene and Michael (1993) found that the Festiguay gene in Molineux is different from that of AUS10894. Therefore, based on their results the Festiguay gene involves another locus. Since doubled haploids are the best materials for molecular work, this population presents an opportunity to map this gene.

Some genotypic combinations had similar phenotypic classifications (eg. stem rust) so individual loci could not be investigated. In such cases further screening using different disease races or test crosses are needed to investigate the individual loci. But because the combined phenotypes did not show distortion it is unlikely that distortions individual loci. An RFLP marker detecting *Sr38* can be used to test the lines for only this gene without interaction effects of the other genes polymorphic in these parents. Recently an RFLP marker (BCD 175) has been discovered which is linked to this gene (Langridge, pers. Comm.) and the use of this marker would help confirm the current findings.

Chapter 5

Inheritance of Some Agronomic Attributes in the Doubled Haploid Population Derived from Trident/Molineux F_1 × Maize Crosses

5.1. Introduction

Investigation of the segregation ratios of known major genes segregating in the doubled haploids from the Trident/Molineux cross showed no obvious distortions so it was assumed that the DH individuals represent a random sample of gametes from the F_1 plants. Given this, this same population was used to investigate the inheritance of other agronomic attributes segregating in these progenies.

This chapter describes a study of these characters. Many of these characters are under polygenic control and so follow quantitative inheritance. Information about the number of genes involved and the types of gene action involved is useful for breeders in the determination of selection strategies.

5.2. Materials and Methods

The attributes considered and the phenotypes of the parents are detailed in Table 5.1. The yields of the doubled haploid lines under field conditions were also assessed.

Table 5.1. Attributes showing polymorphism between Trident and Molineux but inheritance pattern not established

Attribute	Trident	Molineux
Septoria tritici blotch	Very susceptible	Moderately resistance
Coleoptile length	Moderately Long (90mm)	Medium (80-85mm)
Leaf colour	Dark green	Pale green
Time to heading	Early	3 days after Trident

Statistical considerations

The characters studied in this chapter were appraised for the occurrence of major gene effects, which would be expressed as distinctly bimodal distribution patterns. If these effects were absent then quantitative inheritance was assumed and the four cumulants of the population namely, mean, variance, skewness and kurtosis were calculated. If skewness and/or kurtosis are significant, that is the distribution is not normal, then gene interactions are likely. Choo and Reinbergs (1982a,b) described the use of doubled haploids for estimating the number of genes which control quantitative traits and their interactions. The estimation method they proposed is:

$$\frac{|D|^2}{\sigma^2_{DH}} = \frac{(kd)^2}{kd^2} = k$$

where $|D|$ is the absolute deviation of the most extreme doubled haploid line (either the best or the poorest) from the population mean,

σ^2_{DH} is the genotypic variance of the population,

$\pm kd$ is the greatest deviation from the finite population mean,

kd^2 is the genotypic variance of the finite population, and

k is the number of segregating loci or effective factors

Note that $D=\pm kd$ and $\sigma^2_{DH}=kd^2$ only under the assumptions of absence of additive epistasis, lack of linkage and equal effects of genes. The above formula was derived for DH lines based on Mather and Jinks (1971) and here the notations are changed according to these authors.

5.2.1. Segregation of Genes Controlling Septoria tritici Blotch Reaction

5.2.1.1. Screening 1995

Doubled haploid lines were planted into a field disease nursery at the Roseworthy Campus, University of Adelaide, in 1995 in single rows alternating with a spreader row, the very susceptible cultivar, Madden. Rows were 1.5m long and spaced 15cm apart with a 30cm

gap between every 3rd and 4th row and planted in two replications. Inoculum was prepared from an aggressive isolate of *Septoria tritici* kindly supplied by Dr H. Wallwork, SARDI, by spreading a dense spore suspension onto potato dextrose agar (PDA) plates and incubating for 7 days at 15°C under near ultraviolet light (16h/day). Pycnidiospores were washed off plates and the spore concentration adjusted to 1×10^6 per ml. Tween 20 was added (0.1%) to act as a wetting agent. The first inoculation was sprayed onto the plants from all sides at tillering stage with an atomizer (20 psi) until runoff occurred. After inoculation the plants were kept moist as much as possible using a sprinkler irrigation system. The inoculation was repeated 4 times during the plants early growth stages until the visible symptoms of Septoria tritici blotch appeared on the lower leaves of the spreader cultivar or the susceptible DH lines.

Disease reaction of each line was scored from 0 to 5 using the scoring system developed by Rosielle (1972):

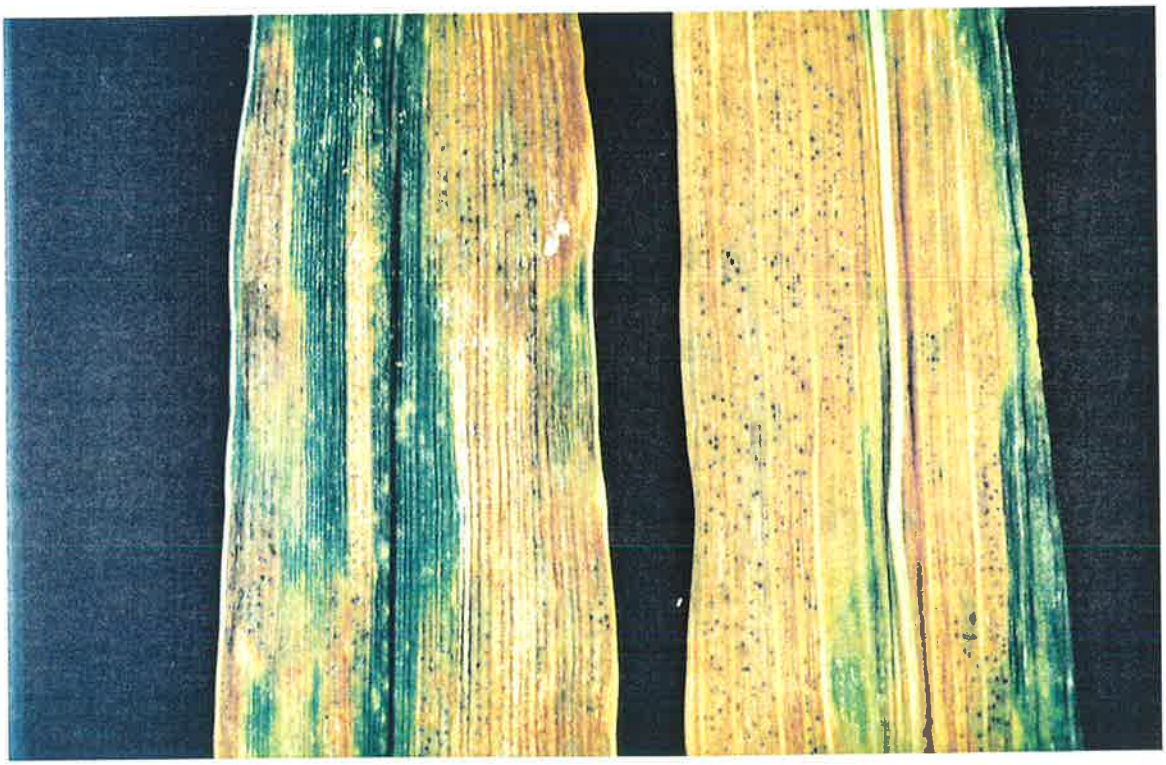
0 - Immune (Imm) - No pycnidial formation, no symptoms or occasional hypersensitive fleck.
1 - Highly resistant (HR) - No pycnidia or only occasional isolated pycnidia formed, particularly on the older leaf tissue, hypersensitive flecking in younger leaf tissue.
2 - Resistant (R) - Very light pycnidial formation. Some coalescing of lesions mainly toward the leaf tip and in older leaf tissue.
3 - Intermediate (I) - Light pycnidial formation. Coalescing of lesions normally noticeable towards the leaf tip and elsewhere on the leaf.
4 - Susceptible (S) - Moderate pycnidial formation, with many lesions coalesced.
5 - Very Susceptible (VS) - Large, abundant pycnidia, lesions coalescing extensively.

The progression of the disease was followed by scoring three times at 10, 17 and 25 days post-inoculation. The Septoria tritici blotch and its pycnidia are shown in Plate 5.1.

Plate 5.1. Screening for Septoria tritici blotch at Roseworthy 1995.

5.1a. The Septoria nursery.

5.1b. Progressive symptoms of Septoria tritici blotch on the leaves of susceptible wheat lines following artificial inoculation with overhead watering.



5.2.1.2. Screening 1996

A natural infection of Septoria tritici blotch occured in a field trial involving many of the doubled haploid entries at Roseworthy in 1996. Septoria spreads by rain splash from residues on the soil to lower leaves and then progressively up the plant and the Saari-Prescott 0-9 scale (Saari and Prescott, 1971) was considered to be more appropriate for rating the natural infection than the previous method used for rating artificially inoculated plants. Only a single digit scale was used where,

0 = No lesions
1 = Lesions on lower leaves only
3 = lesions on lower third of leaves
5 = lesions on all but top two leaves
7 = lesions onto flag leaves
9 = lesion on spike

Plant height could affect this score as shorter plants with the same degree of genetic resistance as taller plants will be more prone to infection by rain splash.

5.2.2. Segregation of Genes Controlling Coleoptile Length

Coleoptile lengths were determined for doubled haploids derived from the Trident/Molineux cross. Halberd (long coleoptile) and Oxley (short coleoptile) were used as standard control varieties and Trident and Molineux as the parents in each test.

Ten seeds of each DH line were first surface sterilized using 0.5% sodium hypochlorite then placed embryo downwards at a spacing of 2cm across the base of a 45 × 32cm filter paper (Ekwip 32*46 cm grade R6) which had been soaked with distilled water and drained for 1 minute. The filter paper was then rolled up and sealed in aluminium foil to keep the seeds in dark conditions and stop them drying out. These rolled up papers were placed upright and incubated at 15°C.

After 12 to 14 days, when all the coleoptiles had been broken by the seedling leaves, coleoptile lengths were measured to the nearest mm. Results from the number of germinated seeds out of ten initially planted were averaged to give one mean coleoptile length. The test was repeated a second time.

5.2.3. Segregation of Genes Controlling Leaf Colour

The wheat cultivar Trident, like its recurrent parent Spear, has much darker green leaves than most other cultivars, including Molineux, grown in South Australia. In 1996 a three replicate yield trial was grown at Buckleboo on Eyre Peninsula, South Australia, involving 116 randomly selected doubled haploids from the Trident/Molineux cross planted in plots 4 rows wide and 4m long. At anthesis five flag leaves were taken at random from each plot and leaf colour was measured using a MINOLTA Chroma Meter. The mean of five readings taken on the upper surface of the leaves was used as the measure of colour.

The MINOLTA apparatus measures three parameters, a* the red-green axis, b* the yellow-blue axis and L* a measure of greyness (brightness)(Minolta manual, 1991, see Plate 5.2). The colour data were analysed using TwoD spatial analysis (Gilmour, 1992) to take account of any spatial trends in the trial area due to nutritional factors, such as varying nitrogen nutrition, which could affect leaf colour.

5.2.4. Segregation of Genes Controlling Heading Date

The date on which 50% of the ears of a plot were fully emerged from the boot was recorded as heading date. In South Australian field conditions the actual date of maturity, usually described as 'yellow under the ear', is very difficult to determine because increasing temperatures during the latter stages of grain filling cause sudden desiccation rather than allowing a gradual maturity. Heading date is a good guide to the length of the grain filling period.

Heading dates were recorded in the Septoria nursery described in Section 5.2.1, and in all field experiments.

5.3. Results

All data for the individual doubled haploid lines are detailed in Appendix 1. Table 5.2 summarises the 4 population statistics of the characters measured using the method described in Sokal and Rohlf (1969). Frequency distributions for the attributes measured are shown in Figure 5.1 and 5.2 for Septoria tritici blotch reaction, Figure 5.5 for coleoptile length, Figure 5.7 for Minolta b* of leaf colour and Figure 5.9 for heading date.

For leaf colour, the measures for a*, b* and L* were closely correlated, see Table 5.6, so statistical and genetical analysis are presented only for the parameter, b*.

Table 5.2. Mean, variance, coefficient of skewness (g_1) and coefficient of kurtosis (g_2) for five characters in the Trident/Molineux wheat DH population

Character	Sample size	Mean	Variance	g_1	g_2
Septoria Score, 1995	216	3.6	1.1	0.05^{ns}	-1.30^{***}
Septoria Score, 1996	112	4.5	3.2	-0.25^{ns}	-0.79^{ns}
Coleoptile length					
Double dwarf	34	61.5	89.3	0.89^{*}	1.83^{*}
Semidwarf	82	71.9	119.4	0.40^{ns}	0.56^{ns}
Tall	39	91.6	196.1	0.24^{ns}	-0.70^{ns}
Plant height I[‡]					
Double dwarf	34	59.8	47.1	-0.82^{*}	-0.46^{ns}
Semidwarf	82	78.2	52.0	0.84^{***}	0.41^{ns}
Tall	39	96.9	103.1	0.68^{ns}	-0.16^{ns}
Plant height II[‡]					
Double dwarf	52	59.0	41.6	-0.47^{ns}	-0.89^{ns}
Semidwarf	119	78.7	55.3	0.76^{***}	-0.16^{ns}
Tall	45	99.0	141.5	0.79^{*}	-0.07^{ns}
Leaf colour	115	15.4	2.0	0.56^{*}	0.40^{ns}
Heading date	114	23.5	9.6	0.23^{ns}	0.45^{ns}
Yield	114	1106.9	10464.9	-0.34^{ns}	-0.05^{ns}

[‡]Plant height I is the same population as that used for coleoptile length, [‡]Plant height II includes all doubled haploids.
^{ns,*,**,***}, nonsignificant and significant at 5, 1 and 0.1% level of probability, respectively

Plate 5.2. Diagram for attributing the numeric measurement of a*, b* and L* of Minolta Chroma to their colour (Minolta manual, 1991).

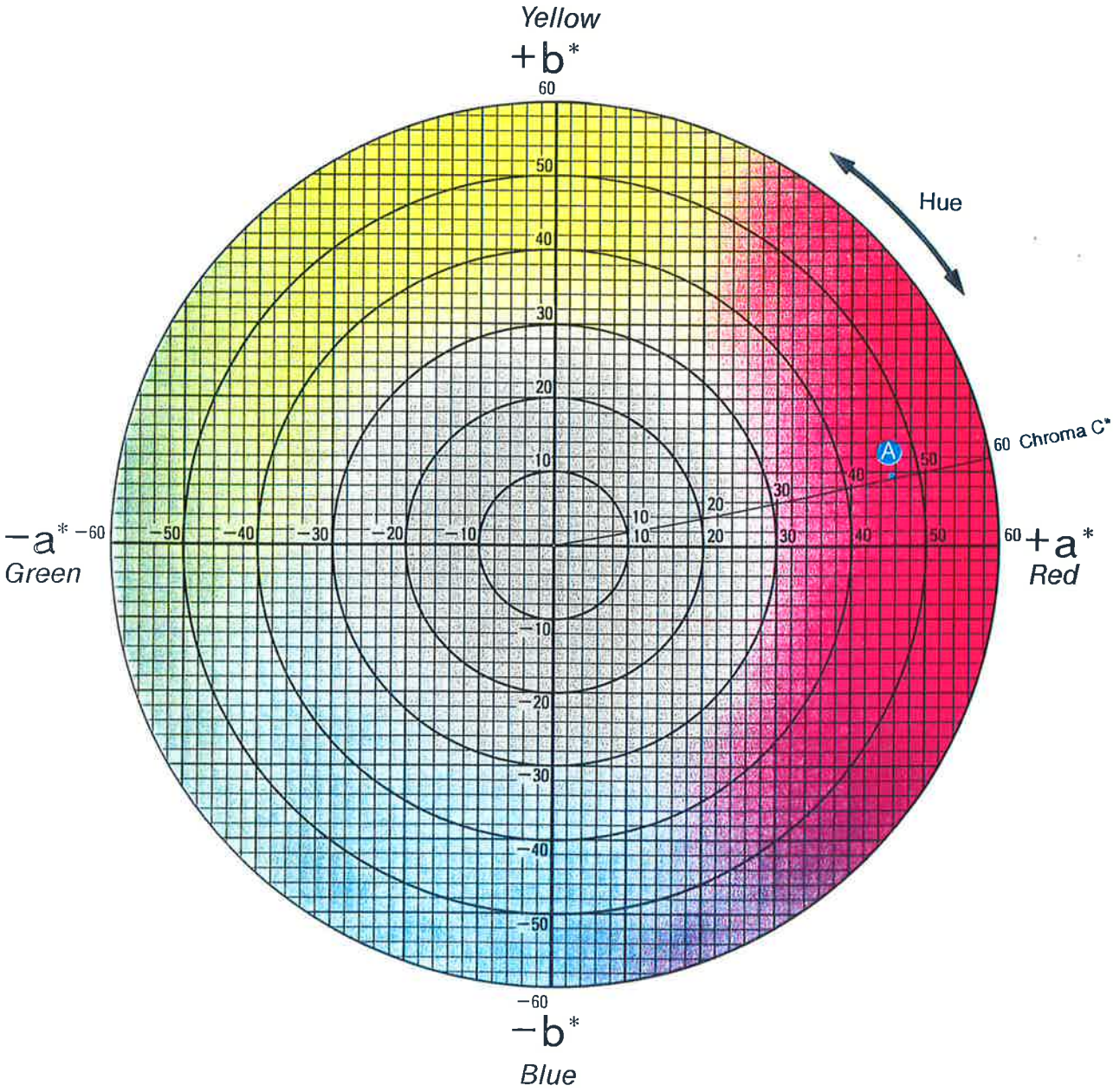

Yellow
+b*
60
50
40
30
20
10
Hue
60 Chroma C*
A
−a* −60
Green
−50
−40
−30
−20
−10
10
20
30
40
50
60 +a*
Red
−10
−20
−30
−40
−50
−60
−b*
Blue

Table 5.3 presents estimates of the number of genes (or if linkage is present, the minimum number of effective factors) that may be involved in controlling the different attributes measured in the Trident/Molineux cross.

Table 5.3. The sample size, range, genotypic variance (σ^2_{DH}), putative number of segregating genes (k), average gene effect (d) and the probability of obtaining at least one DH line containing all (un)desirable genes in the test sample (P)

Character	Sample size	Range	σ^2_{DH}	k	[d]	P	k for P=95%†
Septoria Score, 1995	216	2-5 (RS)	1.1	2	1.0	100	2
Septoria Score, 1996	112	0.8-7.8 (SPS)	3.2	3	1.1	100	3
Coleoptile length							
Double dwarf	34	46.8-91.7 mm	89.3	10	3.0	6.4	4
Semidwarf	82	47.4-102.6	119.4	8	3.9	47.4	5
Tall	39	64.4-118.9	196.1	4	7.2	99.5	4
Plant height I‡							
Double dwarf	34	44.5-67.0 cm	47.1	5	3.1	88.9	4
Semidwarf	82	68.0-100.0	52.0	9	2.7	27.5	5
Tall	39	82.5-122.0	103.1	6	4.2	71.0	4
Plant height II‡							
Double dwarf	52	44.5-67.0 cm	41.6	2	2.9	100	2
Semidwarf	119	10.7-21.3	55.3	8	2.7	60.7	6
Tall	45	82.5-128.5	141.5	6	4.9	76.0	5
Leaf colour	115	12.5-19.7 Minoltab	2.0	9	0.5	36.2	6
Heading date	114	16.1-30.5 (days)	9.6	5	1.5	99.9	5
Yield	114	860-1378 (kg)	10464.9	9	34.3	38.2	6

† The minimum number of genes involved. This is estimated at 95% probability and is affected by sample size.
‡ Plant height I is the same population as that which was used for coleoptile length, Plant height II includes all doubled haploids
RS = Rosielle Scale SPS = Sarri-Prescott Scale

5.3.1. Segregation of Genes Controlling Septoria tritici Blotch

Molineux, the male parent used for producing F_1 plants, is known to be moderately resistant to Septoria tritici blotch under Southern Australia conditions (Rathjen *et al.* 1989). Those lines scored as 2 or less on the Rosielle scale are considered resistant and can be used as donor parents in wheat breeding programs. Those which scored 3 are moderately resistant and this is satisfactory for release as a variety. None of 216 DH lines which were

tested in this study showed a strong resistant reaction to Septoria tritici blotch. Overall, about 46% of the DH lines showed a resistant or moderately resistant reaction to leaf blotch disease, scored as less than 3. The rest which scored higher than 3 were classified as susceptible or very susceptible. The reaction of the parents were consistent with previous observations meaning that Trident (female parent) was susceptible and Molineux (male parent) was moderately resistant in reaction to Septoria tritici blotch.

The frequency distribution of 216 DH lines based on their reaction to the Septoria tritici blotch disease are illustrated in Figure 5.1. Mean scores of two replicates were calculated and because Septoria infection was not evenly distributed across the nursery, probably because of uneven sprinkler watering, each line was rated according to its most susceptible score, providing a conservative approach for selecting for resistance. Based on long experience of the wheat breeders at Roseworthy, plants receiving a score of 3 or less in the 1-5 scoring scale (equivalent to 2 or less in Rosielle 0-5 scale, see Wilson, 1979) should be regarded as resistant. Indeed, all lines less than three carry enough homozygous alleles to be regarded as resistant.

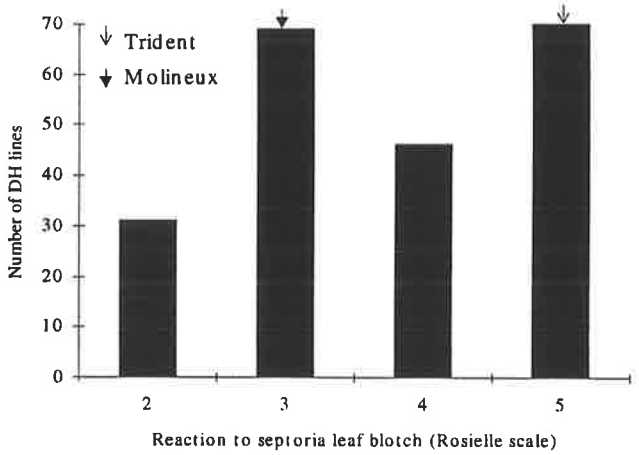


Fig. 5.1. Frequency distribution of DH lines for Septoria blotch reaction in the 1995 Septoria nursery

In 1996 different environmental conditions were experienced so the Saari-Prescott 0-9 scale was used to indicate disease development up the plants. The frequency distribution of

the different rating (Fig. 5.2) shows transgressive segregation for more susceptible and more resistant progeny amongst the DH progeny. It should be noted that the 1996 winter in South Australia was very wet which allowed Septoria progress in the field and so these field based data are considered a more critical test of DH lines for Septoria reaction.

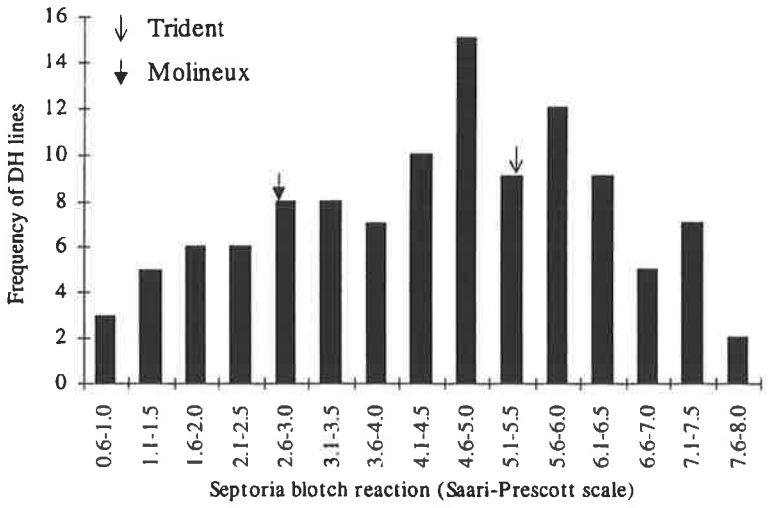


Fig. 5.2. Frequency distribution of DH lines for septoria tritici blotch reaction in 1996 trial, natural infection

The absence of significant skewness by either methods of scoring indicates that additive epistasis is not present and the significant negative kurtosis (flatter shape) in 1995 indicates that the loci affecting Septoria resistance in this cross are not interacting with each other (Choo and Reinbergs, 1982a, see Table 5.2).

The spread of Septoria infection under natural conditions is affected by rain splash, so plant height may play a critical role in the severity of infection on a plant. Figure 5.3 shows the relationship between the severity of infection and plant height. There is a low negative but significant correlation between these characters (r=-0.17*). Similarly, the progress of infection can be affected by heading date (Fig. 5.4). Like plant height the correlation coefficient between infection severity and heading date is significantly negative but low (r=-0.21*).

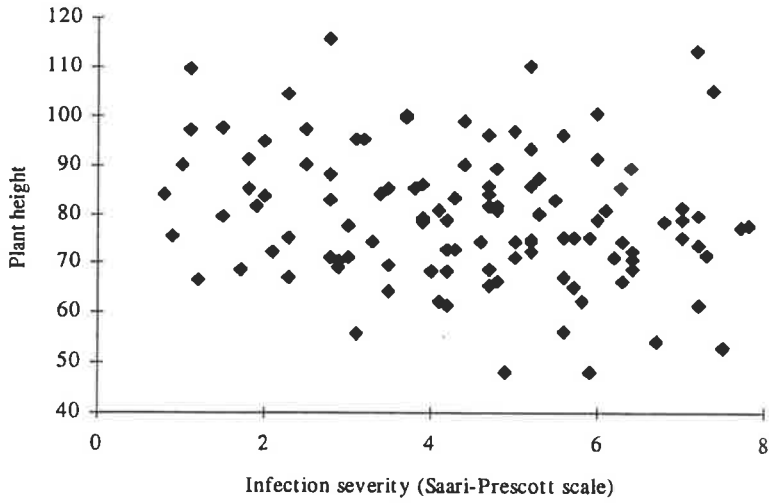


Fig. 5.3. Scatter diagram of Septoria tritici blotch infection in severity reaction to plant height

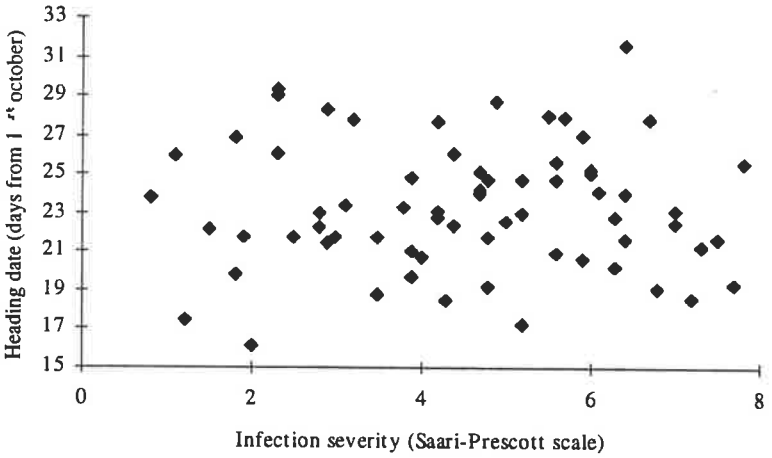


Fig. 5.4. Scatter diagram of Septoria tritici blotch infection in severity reaction to heading date

The number of genes estimated to be segregating for Septoria tritici blotch resistance are 2 and 3 in the 1995 and 1996 trials, respectively. The genetic effects of both trials are very close together. The size of this DH population was enough to be more than 95% certain of containing at least one line homozygous for all desirable or all undesirable alleles (Table 5.3).

5.3.2. Segregation of Genes Controlling Plant Height

In addition to the major genes discussed earlier in Chapter 4, there are other genes of minor effect on plant height which should be considered in this DH population. The doubled haploid lines were classified into three groups based on height and GA_3 insensitivity test so statistics have been calculated separately for these three groups (Tables 5.2 and 5.3).

The data presented in Tables 5.2 and 5.3 for plant height II were analysed because they give a larger sample size than those compared in plant height I.

Within the tall group (genotype *rht1rht1*) and the semidwarf type (genotypes *rht1Rht2* and *Rht1rht2*) the frequency distributions were positively skewed. This indicates complementary gene interactions rather than multiplicative gene effects for minor genes affecting height. It was estimated that there are at least 6 and possibly as many as eight genes causing variation for height within the semidwarf group but only 6 (definitely 5) in the tall group. This difference is probably due to the inability to separate the two semidwarf major genotypes *Rht1rht2* and *rht1Rht2*. If *Rht1* and *Rht2* are quantitatively different as suggested by Gale and Youssefian (1985) then the extra variability caused by combining the data from these two genotypes would give an overestimate of additional genes involved.

Within the very short dwarf group there was no significant skewness and it appears that there are only 2 loci causing the variation observed. This could easily be an underestimate. These additional genes for height might have their effect on plant growth and physiology as a proportional increase in height through greater stem cell size or greater stem cell number. Such effects on very short or dwarf plants will not be as marked as on tall plants and their measurement may be lost in the inaccuracies caused by the actual measurement procedure and environmental variation.

It is important to understand these minor genes for height because tall 'semidwarfs' may be of the same height as short 'talls' and one might argue are of equal value in varieties because both would have the same lodging resistance and heritable height. However, pleiotropic effects of the *Rht1* and *Rht2* genes, for example their GA_3 insensitivity, affects tiller number and spike size (Gale and Yousofian, 1984). These latter effects are more important in South

Australia than lodging resistance and tall semidwarfs have greater yield potential than short talls. Coleoptile length however might be adversely affected by the dwarfing genes.

5.3.3. Segregation of Genes Controlling Coleoptile Length

The coleoptile length of a cultivar is important in determining the maximum sowing depth in the field to still have satisfactory emergence (Whan, 1976). Variation in coleoptile lengths are clearly visible in Plate 5.3. The frequency distribution of DH lines according to their coleoptile lengths is shown in Figure 5.5.

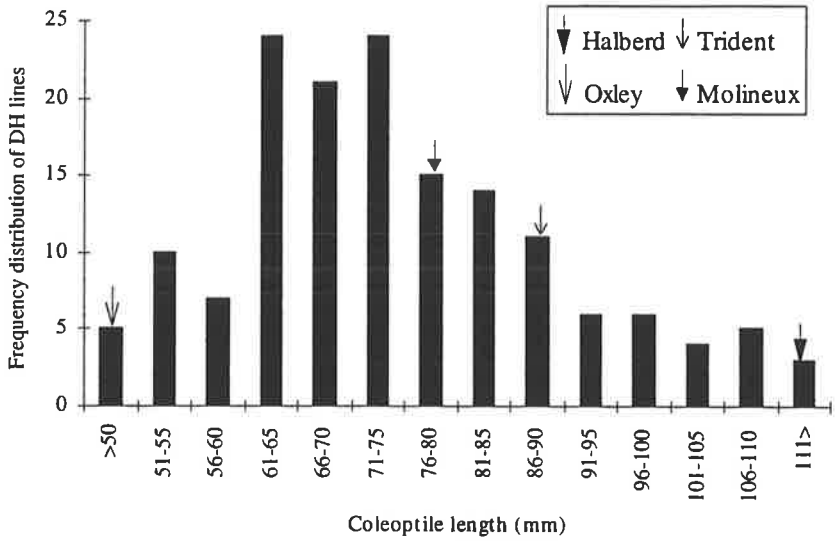


Fig. 5.5. Frequency distribution of DH lines for coleoptile length

Transgressive segregation occurred among the DH population with a high frequency of lines having either longer coleoptiles than Molineux or shorter coleoptiles than Trident. It has been shown that there is a close association between coleoptile length and plant height, especially between shorter coleoptile length and dwarfism (Gale and Marshall, 1975). A similar association was also observed for the Trident/Molineux DH population (Fig. 5.6). Thus, before considering the number of genes involved account must be taken of the *rht* classes to which individual DH lines belong.

Since DH lines were classified into three groups, namely dwarf, semidwarf and tall, coleoptile lengths and plant height are graphed within these three groups in Figure 5.6. An analysis based on univariate and multivariate methods showed that means of the populations

are very different in both coleoptile length and plant height (Tables 5.4 and 5.5). All three groups are different from each other in these attributes. Wilk's lambda were calculated as 0.41, 0.44, 0.17 and 0.26 for the comparison of dwarf with semidwarf, semidwarf with tall, dwarf with tall and dwarf with semidwarf and tall, respectively (Wilks, 1932).

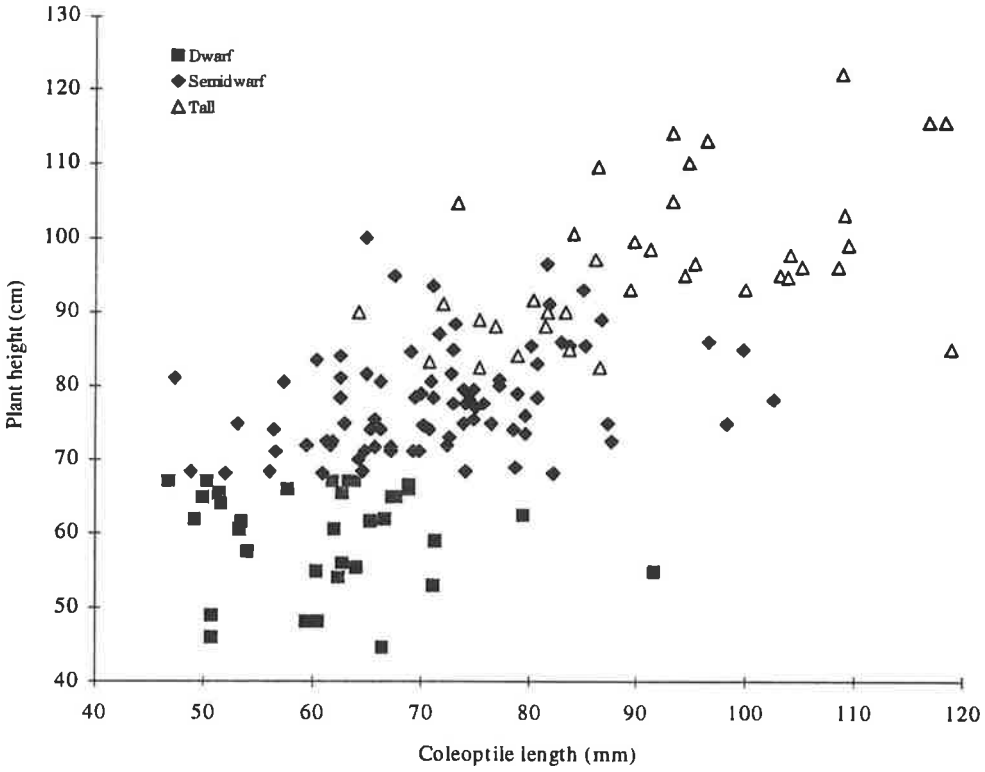


Fig. 5.6. Association between coleoptile length and height in Trident/Molineux doubled haploids

The correlation between plant height and coleoptile length was not significant in the dwarf population, but in the other two populations was highly significant (semidwarf, *r=0.32, df=80* and tall, *r=0.50, df=37*). The growth of most of the individual doubled haploid lines was consistent with the theory that lower plant height reflects shorter coleoptile length (Fig. 5.6, Tables 5.4 and 5.5). Overall, there was no instance were a dwarf DH line produced a long coleoptile nor were there any tall DH lines that produced short coleoptile

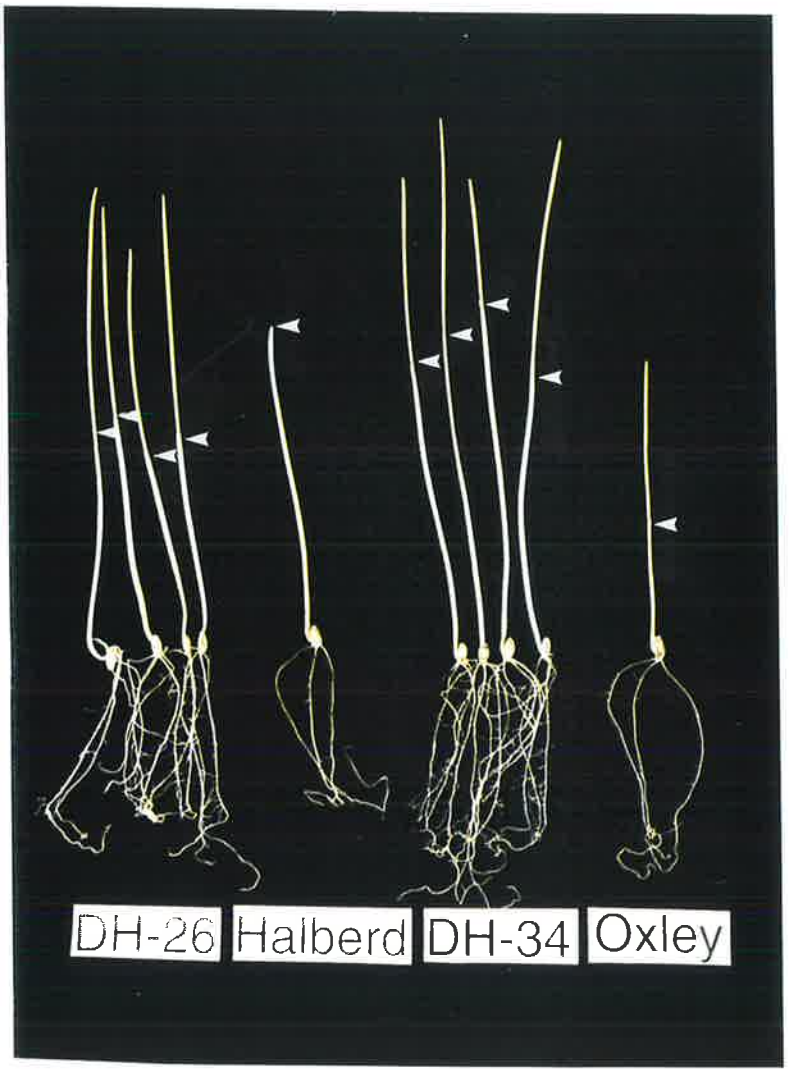
Plate 5.3. Variation in coleoptile length of doubled haploid lines.

DH-26 = 62.8 mm

Halberd = 113.2 mm

DH-34 = 110.4 mm

Oxley = 48.9 mm


DH-26
Halberd
DH-34
Oxley

lengths. The only possible exception is line DH-109 a double dwarf line (55 cm) which produced a longish coleoptile (91.7 mm).

Table 5.4. Multivariate mean matrices for dwarf, semidwarf and tall DH lines for coleoptile length (CL) and plant height (PH)

	Mean	
Class	CL	PH
Dwarf	[61.5	59.8]
Semidwarf	[71.9	78.2]
Tall	[91.6	96.9]

The number of polygenes controlling coleoptile length and plant height in these doubled haploid lines were estimated as eight loci involved for both characters (Table 5.3). It should be noted that the sample size in this study was different for each group than that reported for height (section 5.3.2). Thus, for the available sample size, with the assumption of no or only a loose linkage, only 4, 6 and 4 genes can be determined for coleoptile length for dwarf, semidwarf and tall DH lines, respectively. If long coleoptile are important for crop establishment then in selecting for tall semidwarfs the breeder must be sure to simultaneously select for coleoptile length.

Table 5.5. Univariate and multivariate analysis of DS†, ST† and DT† groups for coleoptile length (CL) and Plant height (PH)

		Univariate MS		Multivariate MS matrices		
Population	df	CL	PH		CL	PH
Dwarf-Semidwarf	1	2618.6**	8048.5**	CL PH	[2619** 4691	8049]
Semidwarf-Tall	1	10247.5**	9319.1**	CL PH	[10247** 9772	9319]
Dwarf-Tall	1	16488.5**	24970.9**	CL PH	[16489** 20291	24971]
All populations	2	9110.2**	12529.4**	CL PH	[18220** 20974	25059]

† DS, SD and ST refer to dwarf-semidwarf, semidwarf-tall and dwarf to tall, respectively
** Significant at 1% level of probability

5.3.4. Segregation of Genes Controlling Leaf Colour

Data for leaf colour of individual doubled haploid lines are detailed in Appendix 1. There were very significant differences in leaf colour, however, there were no doubled haploids whose leaf colour was significantly darker than Trident nor paler than Molineux.

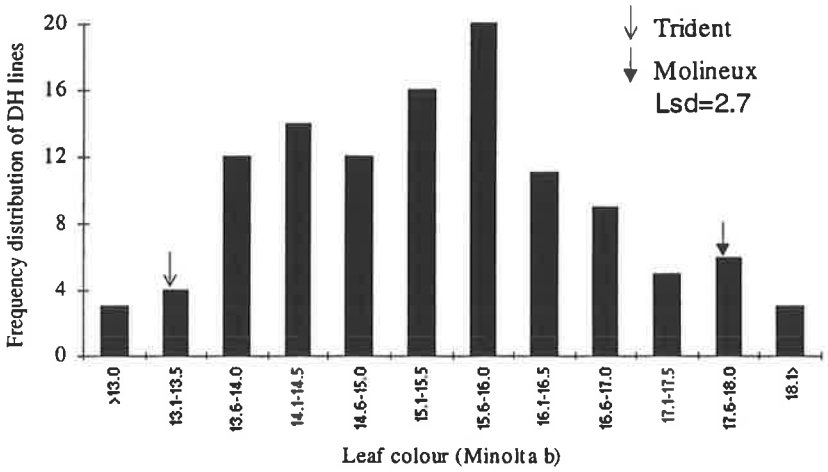


Fig. 5.7 Frequency distribution of DH lines for leaf colour measure based on Minolta b*

In this experiment Minolta parameters a*, b* and L* were closely correlated (Table 5.6) particularly a* and b* so a full analysis is presented for one, b*, only. The frequency distribution for b* is graphically depicted in Figure 5.7 and the population statistics are shown in Table 5.2. The distribution is significantly skewed to the right, that is toward paler leaf colour. There is no kurtosis. It is estimated that there are 9 loci controlling leaf colour (P=36.2%)(Table 5.3).

Table 5.6. Correlation matrix of leaf colour measured by Minolta Chroma Meter

	a*	b*
b*	0.94[1]	
L*	0.56	0.67

[1] All correlations are significant at P<0.05

The association between leaf colour measured by Minolta b* with grain yield was investigated. A significant negative correlation (*r=-0.19, df=111*) was found between these two characters meaning that lines with paler green leaves (higher b* values) tended to have lower yields (Fig. 5.8).

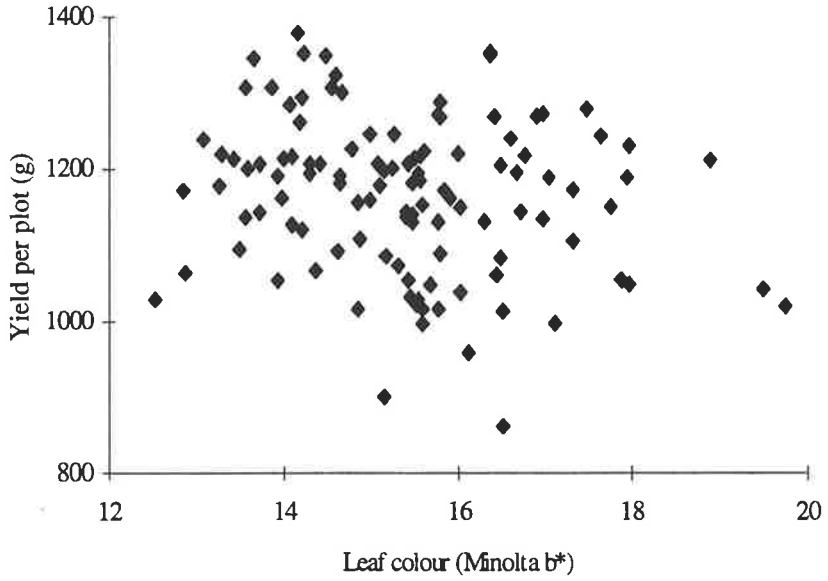


Fig. 5.8. Association between leaf colour and grain yield in different DH lines

5.3.5. Segregation of Genes Controlling Heading Date

The frequency distribution for heading date was normal (Fig. 5.9) and neither skewness nor kurtosis were significant (Table 5.2). Such a normal distribution for heading date indicates additive gene action with no epistasis nor gene interaction.

The number of genes involved in this character is estimated as 5 (Table 5.3). Based on the size of the DH population tested the probability that a line homozygous for five early or for five late genes is included in the population is 99.9%. Additional doubled haploids from these parents is unlikely to result in producing a doubled haploid line earlier or later than the earliest or latest in this population.

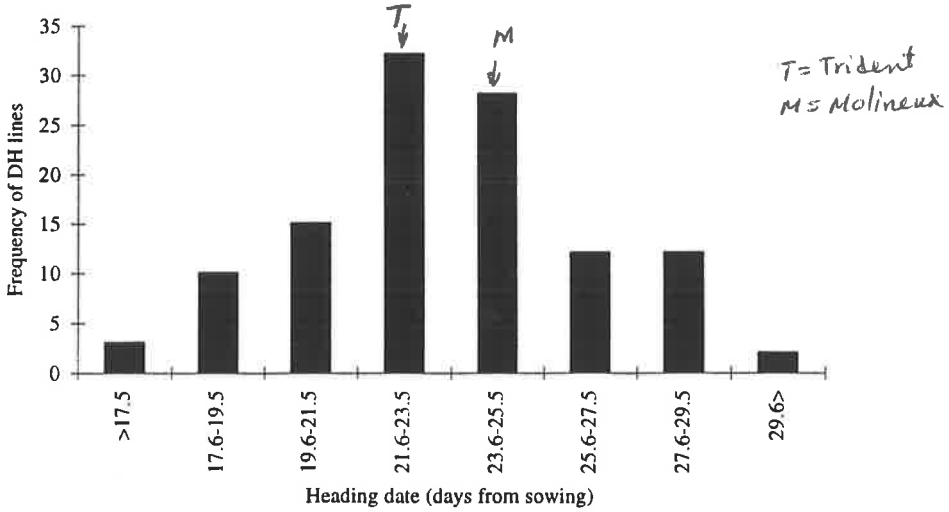


Fig. 5.9. Frequency distribution of DH lines for heading date

5.3.6. Segregation of Genes Controlling yield

Figure 5.10 shows the frequency distribution of doubled haploid lines for grain yield in the 1995 row plot trial. Although the mean is to the right of the median the distribution is not significantly different from a normal distribution. This indicates that additive epistasis appears to be absent for grain yield in this cross (Table 5.3). Therefore, a multiplicative gene interaction may be present among the loci involved in grain yield. At least 6 loci are estimated to be involved in the control of grain yield (Table 5.2), but this is probably an underestimate.

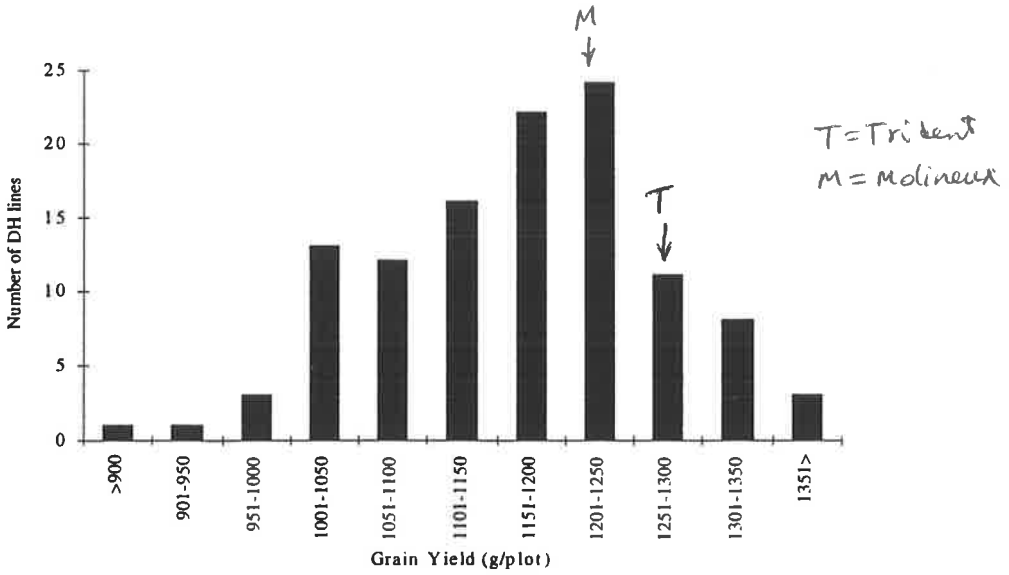


Fig. 5.10. Frequency distribution of DH lines for grain yield in row plot trial

5.4. Discussion

Most field and greenhouse experiments on Septoria tritici blotch have concentrated on the study of presumed major genes. Often a single isolate or a bulk collection of *S. tritici* isolates have used for inoculation. However, van Ginkel and Scharen (1988) utilized eight various isolates separately in their study on durum wheat. They found general combining ability and its associated effects explained their results. Generally, unlike the findings of the present study, this is regarded as the existence of additive gene effects. Studies on *S. nodorum* showed different patterns of genetic control of resistance. Bronnimann (1975) found that the tolerance to *S. nodorum* was inherited mainly additively and polygenically. Laubscher *et al.,* (1966) crossed a resistant variety with a moderately susceptible or a susceptible variety and found that the mode of inheritance of resistance at the seedling stage was recessive. In another experiment the variety Atlas 66 appeared to be resistant at the seedling stage and this resistance is controlled by a single dominant gene (Frecha, 1973). Using different crosses, a similar result was found by Wilson (1979). Kleijer *et al* (1977) found that this dominant gene is located on chromosome 1B in Atlas 66.

Studies on inheritance of resistance to *S. tritici* by various workers have shown different patterns of inheritance, including monogenic control (Narvaez and Caldwell, 1957; Rosielle and Brown, 1979), control through two additive genes (Narvaez and Caldwell, 1957) and at least three recessive genes (Rosielle and Brown, 1979).

In this study 2 to 3 loci are involved but because doubled haploids are completely homozygous it is not possible to decide upon dominance or recessiveness at these loci. It does however give the breeder hope in achieving useful levels of resistance with only small populations. About 24% of the doubled haploid lines in the 1996 trial had a level of resistance considered to be sufficient for South Australian conditions.

The artificial inoculation is a much more straight forward screen than natural infection because confounding effects such as height, maturity and disease progression up the plant (all important in natural conditions) are swamped by the spraying technique. *S. tritici* can be saprophytic even on dead tissues of resistant plants and this may account for high susceptibility scores in such a nursery.

Two types of gene action are involved in controlling height of wheat plants in this cross; genes of major effect and genes with minor effects with a quantitative nature of inheritance. Lines were classified into three height groups according to major genes in which the gene action and their distribution followed Mendelian ratios (see Chapter 4). However, a more complicated behaviour of minor genes resulted in height variation within each group.

In all groups, except the tall the number of estimated minor genes reduced as the sample size increased. Choo and Reinbergs (1982b) found four minor genes for their semidwarf line while in this study it was estimated as 9 at a very low probability and 8 at a low probability. They also found even up to 13 and 9 genes involved with very low probability (2 and 32%, respectively).

A close association between coleoptile length and final plant height in semi-dwarf wheats has been shown frequently (Allan *et al.*, 1961; Allan and Pritchett, 1973; Gale *et al*, 1975; Gale and Marshall, 1975). Production of lines with long coleoptile length and semidwarf height is

of interest to breeders. So far, there has been no success of combining these two traits in bread wheat but in durum wheat some were found by chance (Gale *et al.*, 1975).

It is interesting to note that both characters are polymorphic for eight minor genes and the genetic values for these genes were large. Both parents were semidwarf but differ in dominant alleles for dwarfism. Since additive epistasis was detected for both characters, it is likely that the estimated number of genes could be biased by the presence of additive epistasis. The sample size required to include at least one line homozygous for n desirable alleles with 5% failure is expected to be $ln\ 0.05/ln[(2^n-1)/2^n]$ (Choo *et al.*, 1985). Thus, the least number of doubled haploid progeny to include all eight derived genes controlling coleoptile length is over 766.

If additive epistatic effects do not change the number of genes and a similar number of genes controls both characters then these results agree with the conclusion that adult plant height and coleoptile length are most likely pleiotropic effects of the same gene (Gale *et al.*, 1975). Therefore, with a simple strategy population size can be reduced before field trialling by selection for long coleoptile length followed by testing for GA_3 insensitivity to separate the tall and semidwarf lines at the seedling stage.

The changes in leaf pigments responsible for the colour differences, such as those observed between the parents and among the DH population, have not been investigated. They probably involve pigments of the photosynthetic pathway and may affect photosynthetic efficiency and therefore grain yield potential. As it seems that leaf colour is controlled quantitatively with a considerable number of genes involved, work on this trait for plant improvement would be very difficult and possibly noneconomic. A quick phenotypic visual selection is sufficient to identify this trait.

Ketata *et al* (1976) found that grain yield was positively correlated with the number of days to maturity and was negatively correlated with the number of days to heading while heading date itself was positively correlated with number of days to maturity. Therefore in

South Australia conditions, it is accepted that heading date can be used as an alternative for days to maturity.

The finding that in Trident/Molineux at least five genes are involved in controlling heading date (Table 5.3) demonstrates the polygenic nature of this trait. With DH lines derived from Trident/Molineux crosses this indicates that both parents probably contain the same genes for photoperiod and vernalization which reflects heading date.

Unlike this result Ketata *et al* (1976) recognized that epistasis contributed to heading date and they also observed duplicate epistasis both for heading date and grain yield. Therefore, according to their finding selection for earlier maturity and higher yield could be difficult. In contrast, this study showed the possibility of selection for earlier maturity and high yields. These contrasting findings may relate differences in the length of the growing season and for the grain filling period between the environments studied by Ketata *et al* (1976) versus those in South Australia.

General Conclusion

Overall the inheritance of six more or less quantitative characters were considered in a DH population. Various numbers of DH lines were examined for the different characters (Table 5.3). The number of genes estimated for septoria resistance in 1995 and 1996 and for heading date were 2, 3 and 5, respectively. These estimations could reflect the actual number of genes involved, if one assumes no linkage and no additive epistasis. Cumulants calculated for these characters showed that the coefficient of skewness did not differ significantly from zero in any instances and so it can be assumed that there were no additive epistatic effects. This was especially the case in the 1995 septoria trial where highly negative kurtosis indicated no gene interaction. These results lead to the conclusion that these traits have relatively simple inheritance.

Conversely, the probability of obtaining at least one line homozygous for all desirable or undesirable alleles was low in the rest of traits (namely, plant height, coleoptile length, leaf

colour and grain yield)(Table 5.3). The sample sizes used in coleoptile length, plant height I and leaf colour were enough to contain at least one most desirable or least desirable line for a trait conferred by 6 genes (95% probability). For traits controlled by more than 6 genes additional DH lines are needed to get a better estimation of the gene number involved.

In general, using third and fourth cumulants is useful for understanding the type of gene interactions which might be involved in polygenic traits (Choo, 1981). In the absence of gene interaction, linkage has no effect on the deviation of the most extreme line from the sample mean if the number of segregating genes is small or the number of doubled haploids derived from F_1 plants is large. Since there is no idea of the number of genes involved in a particular cross prior testing the lines, using a larger sample would improve the estimation of the number of genes. For plant height when the sample size increased different results were achieved demonstrating the improvement that a larger sample size would have on accuracy of estimations.

There are still unsolved problems with estimating the number of genes by all formulae presented by various statisticians. Several recent attempts have been made to solve these problems (Jinks and Towey, 1976; Comstock and Enfield, 1981; Zeng *et al.*, 1990) but it appears that at least some of Wright's (in Castle, 1921) original assumptions are still required. However, the common outcome of all of the assumptions is an underestimate of the number of effective factors. Mather and Jinks (1982) explained how to reduce some of assumptions from Wright's formulae. Mayo and Hopkins (1985) also criticised some of these formulae and tried to solve them on theoretical grounds. All of these estimates are inexact and can only give an approximate number of effective factors involved and care should be used in interpreting such data. Irrespective of the formulae used, sample size remains an important factor in the accuracy of the estimation. Such studies and analyses though are still of benefit to breeders in both planning crosses, determining population sizes and selection strategies.

Part III

Cross Prediction Studies with Wheat Doubled Haploids

Chapter 6

6.1. Comparison Between Doubled Haploid and F_5 Lines Derived from Different Wheat F_1

6.1.1. Introduction

The main objective of wheat breeding is to increase yield potential by genetic manipulation. Yield that is realized, however, is the result of complex interactions between genetic, physiological, climatic and edaphic conditions.

Increasing wheat production stimulates a faster turnover of varieties as diseases evolve and disease resistances break down and/or as growers change their farming methods. Similarly buyers demands for quality change. The average life span of a variety is 5 to 6 years while the breeding process takes about 10 to 12 up to 15 years. Breeders are continually seeking new techniques to speed up the breeding process and improve its efficiency.

Conventional selection procedures can be divided into two major stages: early generation selection based on major genes whilst a high level of homozygosity and homogeneity is achieved, followed by extensive field testing to select for the important quantitative attributes, yield and quality. In order to speed up the breeding process breeders need to reduce the time needed in stage one. One such way is to raise several generations per year but an even more effective method would be to reduce the number of generations of selfing needed to get a reasonable level of homozygosity. The production of doubled haploids offers such a technique. With doubled haploids derived from F_1 hybrid plants it is possible to achieve homozygous lines from which to select several years before F_5 lines, which are on average, homozygous at 15/16 loci, would be produced in a conventional pedigree breeding program. Because the number of doubled haploids that can be produced in wheat using current techniques (wheat × maize) is not high then the doubling of F_1 × maize crosses will not produce enough superior homozygotes from each F_1 or within the total F_1 population of many crosses in any one year to provide the full range of genotypes

needed to achieve the same degree of gain under selection as current conventional techniques.

Some workers suggest applying the doubled haploid procedure to selected F_2 plants rather than F_1 thus enhancing the population for attributes recognizable on single F_2 plants and reducing the frequency of undesirable doubled haploid lines (Choo *et al, 1985;* Foroughi-Wehr and Wenzel, 1990). This technique, whilst an improvement, still does not allow selection between crosses.

Selection strategies within different crosses could be improved if there was some method by which the promise of individual crosses for quantitative characters could be predicted. Doubled haploids offer such a method of cross prediction because it is possible to produce doubled haploids, multiply seed and conduct field evaluation on homozygous lines before such lines are available in a pedigree selection program. No reports on comparisons between doubled haploid lines derived from F_1 gametes and F_4 derived F_5 lines from the same F_1 obtained by conventional pedigree methods have been published. In attempting to evaluate a large number of lines which is needed in such comparisons, there are several alternatives. Use unreplicated trials with traditional row plot technique, devote one generation for seed multiplication and then doing actual test in the year after in replicated row plots or, instead of reducing the number of replications or delaying one year for seed supplies, decrease the plot size to some form of micro-plots. By using micro-plots the potential advantages of DH-lines for saving time will not be compromised, unless performance in micro-plots is not a good indicator of on farm performance. This chapter describes an experiment to investigate such a comparison.

Bonnett and Bever (1947) proposed a micro-plot trial as a head to hill method of planting head selections in small grain and Ross and Miller (1955) suggested the use of hills for screening large numbers of early generation lines. Hill- and row-plot performances were then compared for the evaluation of early generation bulk lines of oats (*Avena sativa* L.) at various locations (Jellum *et al.*, 1963; Frey, 1965). They determined that hill-plots were useful for testing yield, plant height and heading date, but not for lodging resistance.

However, others found different results. Burrow and Shands (1964) reported that plot size has little effect on lodging resistance when testing selections from the barley world

collection in row or hill-plots. In wheat Khadr *et al* (1970) found similar efficiencies for both hill and row-plot techniques in discriminating among approximately 100 F_4 lines for heading date, plant height, and kernel weight but not for grain yield. They concluded that the number of entries in common in the extreme 25% portions of the population using each method appeared satisfactory and justified the use of hill plots for testing large populations. The effectiveness of selection in hills for line performance based on the range of the expression, magnitude of error variance and genetic correlations between the two plot types has been discussed by Baker and Leisle (1970). They concluded that hill-plots appeared to be very useful for genetic studies and for early generation selection.

Many other reports occur in the literature on the value of micro-plots and hill plots and comparisons with row plots (Ross and Miller, 1955; Frey, 1965; Fonesca and Patterson, 1968; Baker and Leisle, 1970; Smith *et al.*, 1970; Khadr *et al.*, 1970; Welty and Ramage, 1973; Walsh *et al.*, 1976; Pfeiffer and Pilcher, 1989; St.Martin *et al.*, 1990; Tragoonrung *et al.*, 1990). There is no consensus as to the relative merits of the two plot types, except that selection in micro-plots for highly heritable traits is more successful than selection for traits, such as yield, whose expression is influenced by interactions with neighbouring plots.

The underlying issue, in comparing hill and row plots, is the level of coincident trait expression between the two methods. In general there is a consensus that hill plots provide effective discrimination for highly heritable traits, such as maturity and plant height, but there is considerable disagreement as to their effectiveness for traits such as grain yield. An advantage of hill plots is that their limited spatial requirements minimize field heterogeneity when large numbers of lines are evaluated. However, in an experimental comparison of row vs hill plots, the number of entries must be minimized in order not to unduly bias the performance of the row-plot. Nearest neighbour and other spatial analyses of field trials can minimize the effects of underlying fertility trends and response surfaces so that large number of entries can be compared.

6.1.2. Materials and Methods

6.1.2.1. Plant materials

To fit the time available to this thesis a set of crosses made in the University of Adelaide, Roseworthy wheat breeding program in 1991 were remade to provide F_1 hybrid seed. The F_1 hybrid seeds were grown and pollinated with maize pollen (cv. Illini Gold) to produce haploid embryos which after being rescued and grown into plants were treated with colchicine to produce doubled haploids (protocol described in section 3.4). The selection of the fifteen parental genotypes used was based on their relevance in a commercially oriented wheat breeding program and because F_5 derived F_4 selections would be available for field evaluation at the same time as doubled haploids produced much later from the same crosses. A flowchart of the procedure is detailed in figure 6.1.1.

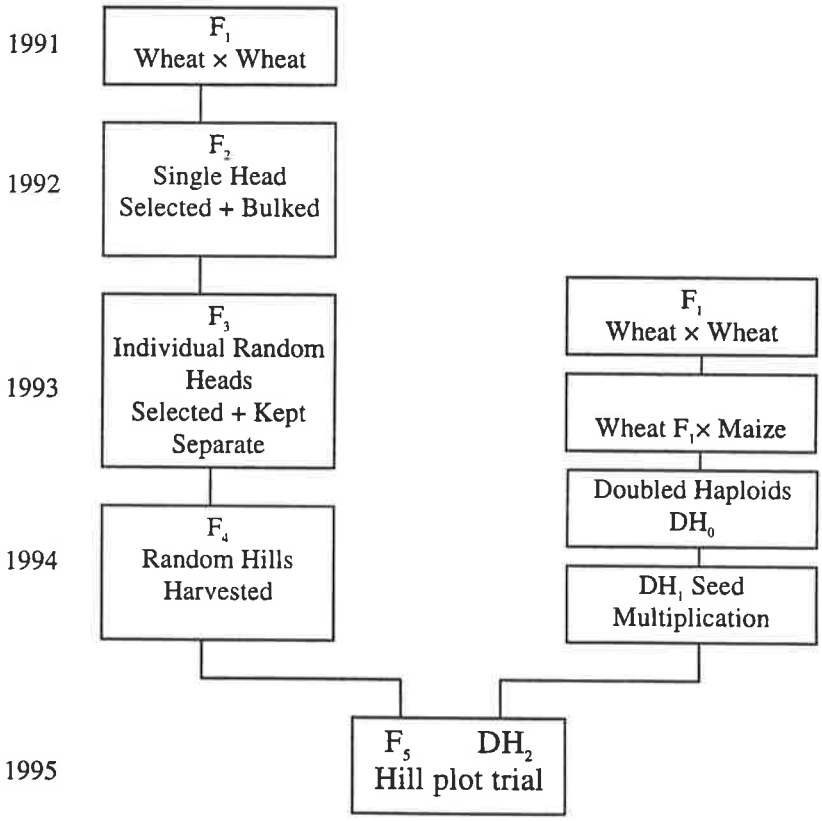


Fig. 6.1.1. Flowchart of pedigree breeding activities (left) and doubled haploid activities (right) leading to simultaneous field evaluation of lines derived from both breeding methods.

Note: F_4's are F_2 derived lines and each relates back to a different F_2 plant.

The fifteen parental genotypes are shown in Table 3.10. There were 16 crosses involved. In order to get more information about the parental genotypes themselves, a group (the first 6 listed in Table 3.10), was used to produce a diallel set of doubled haploids.

The number of doubled haploids produced from each cross ranged from 3 to 46. 114 randomly selected lines from the TRI/MNX doubled haploids used in the experiments of Chapters 4 and 5 were also included. Overall 477 doubled haploid lines were used in this experiment. Not all DH lines produced mature seed of a suitable quality for field trials. This introduced a possibility of bias in the sample lines in some crosses (Table 6.1.1).

Original DH_1 seed was multiplied over summer in 1994/5 in an irrigated field nursery. F_5 derived F_4 seed was grown in the same nursery but in winter 1994 (Plate 6.1.1).

Table 6.1.1. Parental cultivars and the number of DH and F_5 lines included in a hill plot trial

Cultivar	TRI	EXC	FRM	R710	R719
EXC	46-20† *				
FRM	16-24	27-20			
R710	4-30	21 - 0	9-25		
R719	12-22	4 - 0	7-20	11-0	
R702	9-17	13 - 0	3-20	41-0	9-0
BTS	38-20				
MCH	29-0				
MNX	114-20				
R613-46	34-0				
R711	4-28				
R712	5-30				
R745	7-26				
RVS	7-28				
SNF	7-30				

† The first and the second number show the number of doubled haploid and F_5 lines, respectively.

* Highlights are crosses analysed in the F5 vs DH Comparisons.

6.1.2.2. Hill plot layout

The DH versus F_5 line comparison was conducted in a hill plot field trial sown at Roseworthy on the 22 June 1995. The trial was planted as a randomized complete block design with four replications, each replication consisting of 24 main plots one for each parent combination. The main plots consisted of sub plots, one for each of the 20 F_5 lines

and 20 doubled haploid lines. A sub plot was a hill of 10 seeds of the entry concerned planted with a corn jab planter. Hills were planted on a 30 cm square grid pattern. Where there were not 20 doubled haploids of the relevant cross available surplus doubled haploids (ie over the 20 required) from other crosses were sown as 'fillers'.

The spatial layout of a main plot was 8 × 5 hills, of a replicate 32 × 30 and of the whole trial 32 × 120 hills in all covering an area 9.6m × 36.0m (Plate 6.1.1). Two rows of buffer hills were planted around the whole area. The area had been earlier cultivated and fertilized with 150 kg/ha 19:13:0 (NPK). Weeds were controlled by hand pulling.

6.1.2.2.1. Measurements

The following measurements were taken:

i) Yield per hill. Yield per hill (YH) was measured as the weight of grain produced from each hill. All seeds in the hill were separated from the spikes using a simple small seed thrasher.

ii) Spike number. Spike number (SN) was measured by counting the number of spikes in each hill.

iii) One thousand kernel weight. One thousand kernel weight (KW) was measured by weighing 1000 grain from each hill or if the total grain produced was less than 1000 using an adjustment. A seed counter was used for counting 1000 seeds.

iv) Harvest index. Harvest index (HI) was determined on all plots using whole above ground biomass in the hill. It was expressed as the percentage of yield per hill on whole above ground biomass as:

$$HI = \frac{YH}{CW} \times 100$$

Where, YH is yield per hill (g/hill) and CW is above ground biomass (g/hill).

v) Plant height. Plant height (PH) was measured as the height from ground level to the tip of the spikes of the majority of the plants in the plot.

vi) Days to heading. Days to heading (DTH) was measured as days from sowing to heading time when most plants in the hill (more than 75%) had heads completely emerged from the boot.

Plate 6.1.1 Field trials, Roseworthy 1995

6.1.1a Hill plot experiment at early stem elongation

6.1.1b Unreplicated row plot experiment looking across (rather than along) the plots. Plots have been trimmed to 3.2m long using glyphosate herbicide

6.1.2.2.2. Statistical procedures

Restricted Maximum Likelihood (REML) analysis was used to analyse hill plot data. In this analysis only those crosses (main plots) which had populations of both doubled haploid lines and F_5 lines were included. The model fitted for each hill plot was

$$Y_{ijk} = \alpha_i + \mu_{ij} + \beta_k + \varepsilon_{ijk}$$

Where, α_i = fixed effect for the breeding method i, i=1,2 (either DH or F_5),
μ_{ij} = random effect for j^{th} genotype in i^{th} method, (j=1,...,20),
β_k = fixed effect for k^{th} replicate (k=1,...,4), and
ε_{ijk} = random error, $\varepsilon_{ijk} \sim N(0, \sigma^2)$

To test for genetic effects two estimates were calculated:

Firstly, assuming a common genetic variance between the two groups (DH and F_5) for each cross, ie fit $\mu_{ij} \sim N(0, \sigma^2_g)$ by means of ANOVA and equate mean squares with replication to estimate σ^2_g.

Secondly, to assume different genetic variances for each group, that is $\mu_{ij} \sim N(0, \sigma^2_{gi})$, and use REML to estimate σ^2_{gi}.

The deviances of these two models were then compared to test the hypothesis of a common genetic variance.

Knowing this, the appropriate Wald statistic could then be applied to test for difference between the mean of each method for each cross. GENSTAT 5 (Lane *et al.*, 1987) commands were used in these analyses and a worked example is listed in Appendix 2.

Spatial analyses using the TwoD program (Gilmour, 1992) were used to adjust data, to minimize the affects of any underlying trend in the trial area, for all entries in the hill plot trial.

6.1.3. Results

Table 6.1.2. gives the means and standard errors for DH and corresponding F_5 populations for each cross separately for all six measured characters.

In all crosses but one, when the alternative populations had significantly different variances (ie standard errors), the DH population has the greater variance. The exception was plant height in the EXC/FRM cross. The generally higher variance of the DH population could not be attributed solely to small population sizes. In comparisons where there were more than ten DH lines (7 crosses in all and 63 separate comparisons) the same situation holds. Thirteen of the 63 comparisons had significantly different variances. Within this same set there were 16 significantly different means between DH and F_5 lines. Combined, some 23 comparisons were different. In other words, the DH population did not accurately predict F_4 derived F_5 line performance in 23 of 96 individual comparisons.

The poorest DH line prediction was for plant height. In every comparison, whether the means were significantly different or not, the DH populations were on average shorter than their F_5 counterparts. Some of these crosses were segregating at the *Rht1* and *Rht2* loci so that DH populations would have *Rht1 Rht2* and *rht1 rht2* plants (dwarfs and talls) present. The F_2 populations could have been subjected to selection for height with talls not selected in the F_2 or F_3 generations from which these F_5 were derived. Dwarfs too, could have been hidden in the segregating F_2 and F_3 generations and tended to be overlooked during selection. The F_4 selection was random. If this unintentional selection had occurred in early generations the F_5 populations would have few *Rht1 Rht2*, the dwarf genotypes, but could have short *rht1 rht2* the results of this bias giving a taller mean than the random DH populations.

Days to heading showed only two crosses with significantly different heading times between DH and F_5. In both crosses the DH population was earlier by 3 or 4 days. This may seem unimportant, but agronomically this difference is effectively 3 or 4 days longer grain filling period and under harsh high temperatures with desiccating winds, which often happens during the logarithmic grain filling growth stage in South Australia, such a difference has great importance.

The TRI/R710 DH population averaged 3 days earlier to heading than the corresponding F_5 population (Table 6.1.2). There was a severe dry spell during grain filling and this difference may be the cause of the improved grain size in this DH population. The only other significant differences in grain size were in the crosses TRI/BTS and TRI/R711. In these crosses the DH population had the smaller grain.

F_5 lines are still not fully homozygous, on average 93.8% of loci which were polymorphic between the parents will be homozygous, whereas DH lines are fully homozygous. Therefore, one would expect more DH lines than F_5 lines to have extreme genotypes thus leading to a greater variance in the DH population than its corresponding F_5 population. The greater the number of genes affecting a trait the greater the expected difference between DH and F_5.

Table 6.1.2. Means and standard errors of DH and F_5 lines from 16 crosses for some agronomic characters

	YH						SN					
	Mean			Standard Error			Mean			Standard Error		
Cross	DH	F_5	P=0.05†	DH	F_5	P=0.05	DH	F_5	P=0.05	DH	F_5	P=0.05
TRI/BTS	26.9	30.5	ns	17.6	9.5	ns	28.9	27.7	ns	3.5	6.5	ns
TRI/FRM	30.6	33.2	ns	14.7	4.8	*	25.5	26.2	ns	3.4	3.3	ns
TRI/EXC	30.7	31.6	ns	7.6	4.2	ns	26.6	26.4	ns	2.9	3.2	ns
TRI/R702	35.0	31.6	ns	20.5	3.4	*	26.6	27.0	ns	5.4	2.2	ns
TRI/R710	32.4	32.4	ns	11.5	5.1	ns	27.7	27.9	ns	3.7	3.1	ns
TRI/R711	26.2	32.0	*	15.6	3.8	ns	27.4	24.2	*	8.3	1.9	ns
TRI/R712	28.7	32.7	*	6.1	2.6	ns	23.9	28.9	*	4.6	1.9	ns
TRI/R719	29.5	34.4	*	15.0	4.8	ns	25.0	26.8	ns	9.8	2.6	*
TRI/R745	30.6	31.4	ns	23.5	4.1	ns	26.4	27.2	ns	11.3	2.6	ns
TRI/RVS	29.4	32.1	ns	9.2	5.3	ns	28.0	27.9	ns	6.6	3.5	ns
TRI/SNF	28.3	29.8	ns	39.1	5.2	*	28.9	28.0	ns	17.0	1.8	*
EXC/FRM	33.8	27.5	*	18.3	4.9	*	28.9	24.2	*	5.1	3.8	ns
FRM/R702	21.9	32.6	ns	135.3	3.9	*	22.9	25.7	ns	8.9	2.7	ns
FRM/R710	27.7	31.1	ns	26.3	5.3	*	28.8	27.8	ns	5.6	6.0	ns
FRM/R719	33.1	33.5	ns	17.4	4.5	ns	26.9	26.2	ns	14.7	2.1	*
TRI/MNX	31.7	28.5	ns	15.7	4.0	*	25.5	23.6	*	5.5	2.5	*
	1000KW						HI					
TRI/BTS	33.0	36.9	*	8.8	1.4	*	41.4	40.7	ns	13.2	4.4	*
TRI/FRM	37.5	37.9	ns	2.1	1.5	ns	43.9	44.3	ns	3.2	1.1	*
TRI/EXC	35.8	36.0	ns	5.6	3.9	ns	44.5	42.7	ns	3.8	3.5	ns
TRI/R702	38.6	36.2	*	3.6	2.2	ns	45.7	42.3	*	2.5	1.9	ns
TRI/R710	36.6	33.5	*	13.7	2.1	ns	46.2	42.5	ns	13.9	3.3	ns
TRI/R711	28.9	36.5	*	8.6	3.2	ns	41.9	43.6	ns	12.2	2.4	ns
TRI/R712	35.1	34.8	ns	2.4	1.2	ns	41.8	42.0	ns	6.1	1.1	ns
TRI/R719	38.9	38.0	ns	11.6	1.8	ns	44.0	43.6	ns	6.5	1.2	*
TRI/R745	36.5	35.3	ns	11.0	1.8	ns	43.5	42.6	ns	22.0	3.6	ns
TRI/RVS	35.2	37.3	ns	6.5	1.6	ns	40.6	41.7	ns	17.0	1.5	ns
TRI/SNF	31.8	33.0	ns	7.9	3.5	ns	41.0	41.2	ns	6.2	3.7	ns
EXC/FRM	37.4	38.9	ns	6.4	5.0	ns	44.5	42.5	ns	6.2	3.2	ns
FRM/R702	35.5	37.8	ns	3.0	2.9	ns	33.1	44.4	*	69.9	1.8	*
FRM/R710	33.9	35.5	ns	14.0	4.1	ns	39.5	41.6	ns	30.7	4.5	*
FRM/R719	37.7	38.9	ns	2.8	2.7	ns	40.7	43.4	*	6.9	2.2	ns
TRI/MNX	33.1	34.9	ns	5.3	1.5	*	41.3	42.5	ns	3.5	2.0	ns
	PH						DTH					
TRI/BTS	68.5	81.7	*	98.5	33.6	*	96.8	97.4	ns	6.7	3.2	ns
TRI/FRM	79.4	81.5	*	3.5	1.5	ns	99.8	98.6	ns	2.0	1.3	ns
TRI/EXC	72.6	77.8	*	4.9	3.2	ns	94.9	96.6	ns	3.5	2.4	ns
TRI/R702	77.0	80.0	ns	10.4	8.3	ns	98.3	100.4	ns	3.4	2.5	ns
TRI/R710	74.9	78.1	ns	1.6	3.0	ns	96.1	99.2	*	18.5	1.9	ns
TRI/R711	70.9	78.3	ns	139.8	7.2	*	97.8	94.8	ns	19.6	1.4	ns
TRI/R712	79.9	82.7	ns	12.6	2.7	ns	100.4	99.4	ns	0.8	0.9	ns
TRI/R719	74.8	79.7	*	5.6	5.8	ns	95.3	99.2	*	6.5	1.5	*
TRI/R745	74.3	77.4	ns	27.7	4.5	ns	95.6	96.2	ns	9.0	2.9	ns
TRI/RVS	81.0	84.1	ns	32.5	8.4	ns	100.6	101.4	ns	5.3	2.0	ns
TRI/SNF	70.8	79.1	ns	100.2	35.7	ns	100.3	99.7	ns	0.8	1.1	ns
EXC/FRM	75.1	80.5	*	5.2	24.0	*	96.3	97.0	ns	4.5	3.7	ns
FRM/R702	74.9	79.1	ns	4.8	5.6	ns	98.1	97.0	ns	7.2	3.2	ns
FRM/R710	77.4	81.9	*	20.6	8.1	ns	98.1	99.9	ns	6.2	2.2	ns
FRM/R719	81.0	82.7	ns	38.7	2.9	*	98.6	97.7	ns	3.8	4.0	ns
TRI/MNX	76.5	82.5	*	11.3	12.4	ns	99.3	99.4	ns	1.1	1.1	ns

† ns=non significant, *=significant

Correlation matrices of all lines involved in the hill plot trial are shown in Table 6.1.3. The scatter diagrams relating to these correlations are depicted in Figures 6.1.2, 6.1.3 and 6.1.4.

Unlike the parental cultivars which showed only one significant correlation of characters, a positive relationship between 1000 kernel weight and harvest index, DH and F_5 progenies showed many highly significant correlations particularly between yield and other attributes.

Table 6.1.3. Correlation matrices for agronomic attributes measured on hill plots of lines from 16 crosses.

	YH	SN	1000KW	HI	PH
	Parental cultivars (df=13)				
SN	0.30^{ns}				
1000KW	0.18^{ns}	-0.45^{ns}			
HI	0.24^{ns}	-0.38^{ns}	0.78**		
PH	-0.43^{ns}	-0.12^{ns}	0.21^{ns}	0.26^{ns}	
DTH	-0.22^{ns}	0.43^{ns}	0.07^{ns}	-0.21^{ns}	-0.16^{ns}
	F_5 lines (df=410)				
SN	0.54**				
1000KW	0.44**	-0.06^{ns}			
HI	0.44**	-0.03^{ns}	0.50**		
PH	0.35**	0.17**	0.30**	-0.06^{ns}	
DTH	-0.14**	-0.07ns	-0.28**	-0.28**	0.04**
	DH lines (df=475)				
SN	0.49**				
1000KW	0.43**	-0.12**			
HI	0.47**	-0.05^{ns}	0.45**		
PH	0.31**	0.07^{ns}	0.29**	-0.16**	
DTH	-0.13**	0.02^{ns}	-0.28**	-0.35**	0.16**
	All DH and F_5 lines (df=902)				
SN	0.51**				
1000KW	0.45**	0.01^{ns}			
HI	0.44**	0.04^{ns}	0.48**		
PH	0.47**	0.2**	0.39**	0.06^{ns}	
DTH	-0.14**	-0.11**	-0.24**	-0.37**	0.06^{ns}

^{ns,**}non-significant and significant at 1% level of probability

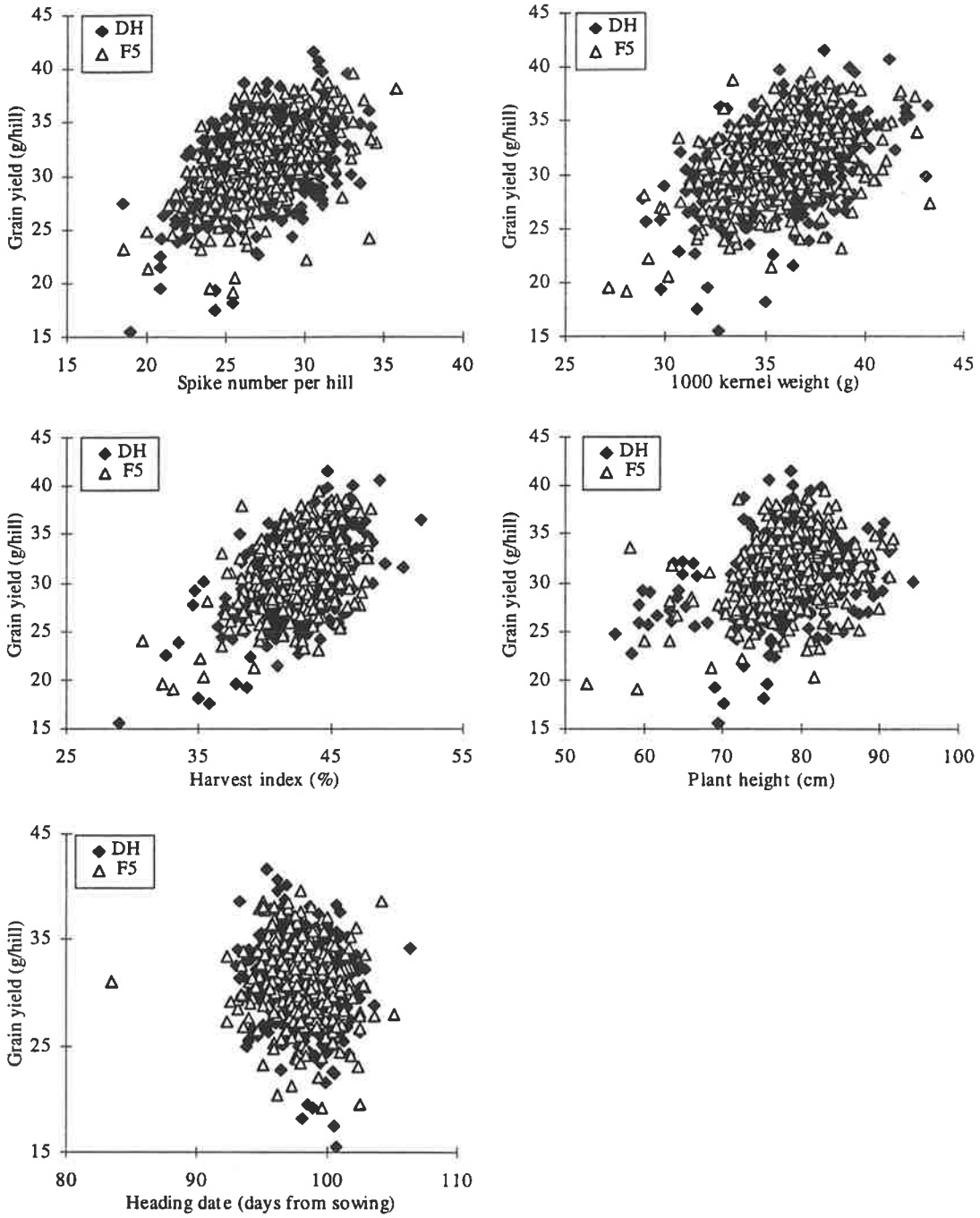


Figure 6.1.2. Scatter diagrams showing the relationships between grain yield in hill plots and other agronomic characters measured in 1995.

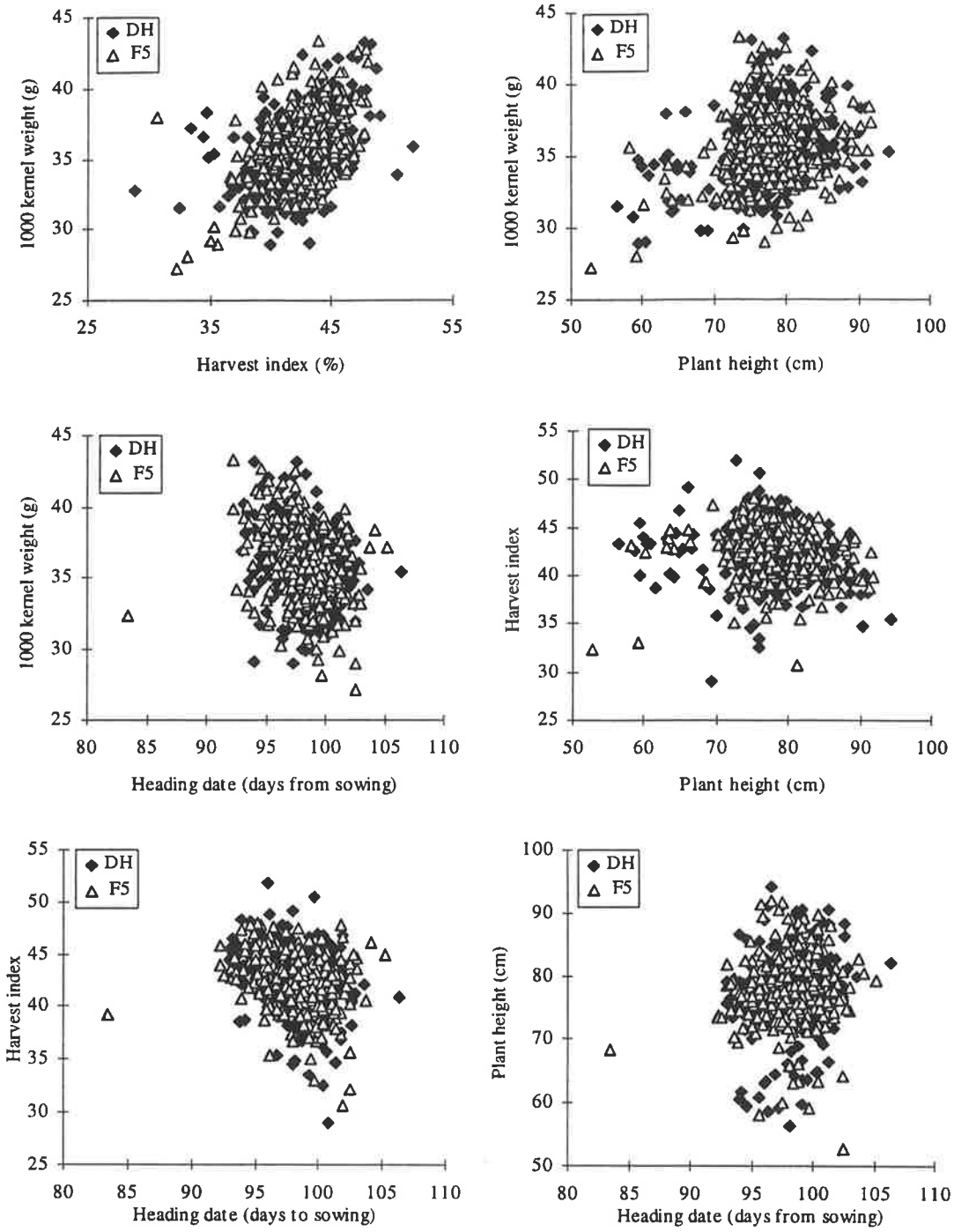


Figure 6.1.3. Scatter diagrams showing the relationships between some agronomic characters measured on hill plots in 1995.

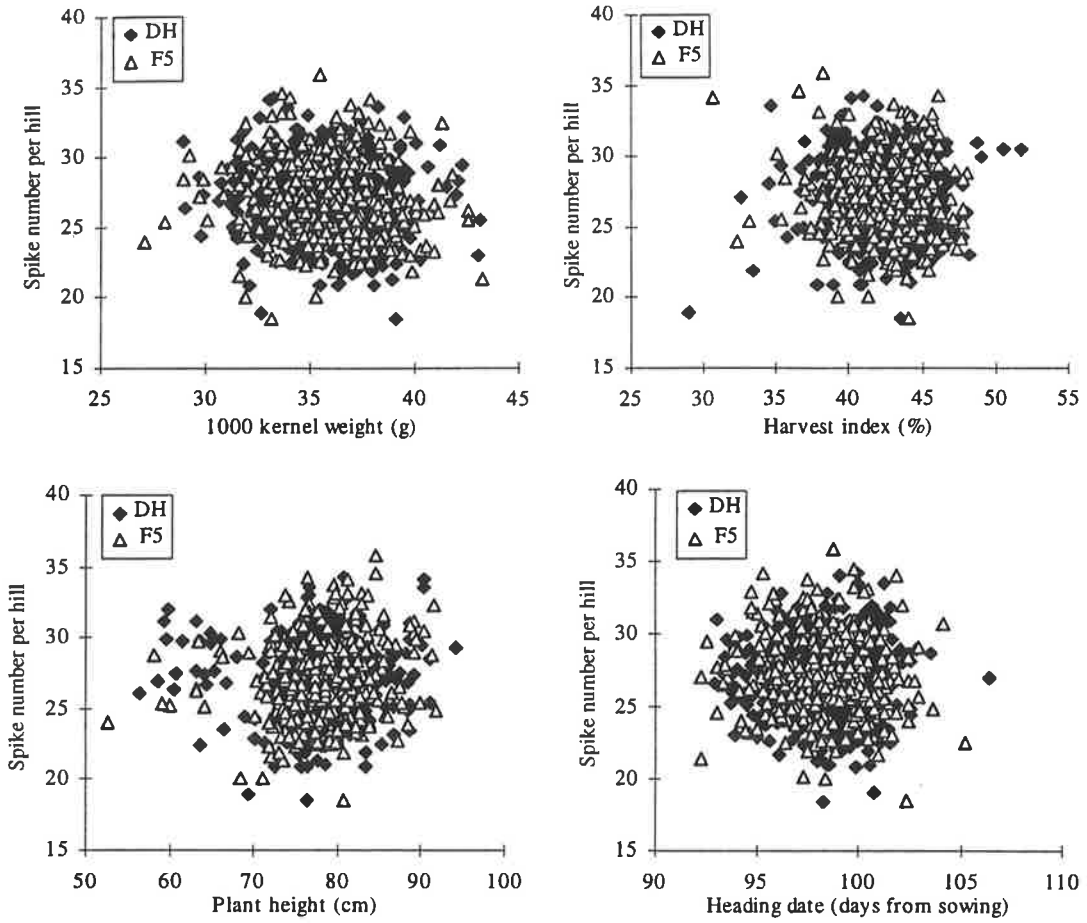


Figure 6.1.4. Scatter diagrams showing the relationships between spike number in hill plots and other agronomic characters measured in 1995

The non significant correlations in parental lines may be partly due to the small number tested. These lines are all adapted to South Australia, although some have not been released as new cultivars because of inadequate disease resistance and /or end product quality deficiencies. Thus they are the result of stabilizing selection for maturity (DTH) and height leaving little variation on which to establish correlations.

The yields of the total F_5 population and the total DH population showed similar associations with other attributes measured (Fig 6.1.2). Spike number (SN) grain size (KW) and harvest index showed strong positive correlations with yields as would be expected.

Plant height was also strongly positively correlated with yield. By examining the scatter diagram though it is noted that the highest yielding plots were by no means the tallest but of intermediate stature and probably semidwarf genotypes. The poor performance of very short plants, presumably of double dwarf genotypes, forced the significant correlation. In the highly competitive, mutual shading conditions that occur in hill plot layouts this may be an artefact of the experimental technique because the dwarfs would be shaded by taller neighbours.

The actual distribution of individual lines of doubled haploids reflects that of F_5 lines very closely (Fig 6.1.2, 6.1.3 and 6.1.4). The variance data suggest that there are more doubled haploid lines in the extremes of the distribution, so that as a total breeding population available for selection there appears equal or even a greater chance of finding desirable transgressants in DH populations than in F_5 populations.

6.1.4. Discussion

In wheat breeding yield improvement is usually the primary aim. To improve yield breeders improve the combinations of different traits. These traits include heading date, plant height, above ground biomass, spike number, grain yield and seed size (kernel weight). Physiologically all of these characters are correlated whether positively or negatively with final grain yield performance. The best combinations will differ according to environment.

Many crosses are made in breeding programs without knowledge of the combining ability or genetic polymorphisms between parents. It is therefore not possible to predict the outcomes from such crosses. Yet, such crosses are important to raising yield potential of new cultivars. Breeders must carry large numbers of progeny from these crosses to a level of reasonable homozygosity and with enough seed of each so that field evaluation for yield can occur. At this stage, F_5, or at the earliest F_4, many crosses are found wanting. The breeder is disappointed but also angered by all the effort and time to find this. As funding reduces, his ability to carry such crosses will decline.

If it was possible to predict the outcome in terms of improved homozygous lines which might eventuate from a cross the breeder would be well informed and guided as to the effort which should be put into each cross. Doubled haploids give such an opportunity because homozygous lines can be produced from each cross and evaluated in the field for yield within 2 years of the F_1 hybrid having been produced and two years before lines become available from conventional breeding through several generations of self pollination where adequate homozygosity and homogeneity is not achieved until F_5.

In most occasions where the variances were significantly different between the two methods, the number of DH lines was much lower than the minimum number of lines necessary for this kind of comparison (Choo *et al.*,1986). If these are ignored then in very few cases were the differences between DH lines and F_5 progenies significant. Therefore, it seems that DH lines can successfully predict the performance of F_5 progenies for most of the characters evaluated and so would be useful as a breeding method especially for those traits controlled by a few genes or those with simple inheritance patterns. As there is little risk of distorted segregation (see Chapter 4) actual numbers of progeny needed can be calculated from probabilities of binomial distributions. The gene number (or the effective factors) (see Chapter 5) segregating in a cross, in conjunction with the information from these experiments, would help breeders to develop new varieties using the DH system.

With respect to plant height, DH lines were shorter on average than F_5 lines, not only in the overall data but also in every cross although the differences were significant in only a few crosses. A GA_3 insensitivity test would correctly classify DH lines into their *rht* groups. The remaining dwarf lines within the doubled haploids led to the average height being less than that of the F_5 lines which had undergone at least two generations of selection in favour of semidwarf genes. This variation in selection history between the two populations resulted in the DH lines demonstrating wider variation and having a shorter average height. Such a difference in the representativeness of the DH predictor group would be overcome if only those DH lines within certain constraints are entered into prediction calculations. That is, in this case dwarfs and very talls should be eliminated from the analysis or not even grown, if recognized beforehand.

Moreover, if DH lines can produce lines superior to their higher yielding parents a DH system can be used directly for improvement of certain characters. Therefore, although it is enough to find that there are no differences between lines produced using these two systems, if the DH system showed better results in many occasions this may encourage the acceptance of this method as an alternative for pedigree methods. Today many new varieties have been released in France (DeBuyser, 1987), China (Hu, 1996) and some in other countries from doubled haploid technology. It is worthwhile building a wheat breeding system based on this methodology.

6.2. Comparison Between Hill- and Row-Plot on Yields of Doubled Haploid and F_5 Lines of Wheat

6.2.1. Introduction

The study of cross prediction (Section 6.1), was arranged as a hill plot trial with four replications. This design was used because there was insufficient seed of many of the doubled haploid lines to include more replicates or to sow rows instead of hill plots. Thus the question as to whether performance in hill plots is a useful measure of performance in farmers fields, as experimentally measured routinely in row plots, needs to be answered.

The experiment described here was to test the adequacy of hill-plot performance for predicting row-plot performance and hence extrapolation to commercial crop performance in the South Australian environment.

6.2.2. Materials and Methods

6.2.2.1. Hill-Plot Experiment

The entries and layout of the hill plot experiment are described in Section 6.1.2.

6.2.2.2. Row-Plot Experiment

712 entries of the 960 entries in the hill plot trial had sufficient seed for an unreplicated row-plot trial. These entries were parent genotypes, F_5-lines and some doubled haploids.

This experiment was planted on the same day as and adjacent to the hill plot trial. Seed was sown at equivalent to 65 kg/ha and 100 kg/ha of 18:13:0 (N:P:K) fertilizer was applied with the seed.

Each row-plot consisted of 6 rows at 18cm spacing and was 5m long. There was a 36cm gap between plots. Plots were trimmed to 3.2m for harvesting. Plots were arranged into 12 bays with 72 plots per bay.

Whilst each entry was unreplicated the trial design was augmented by repeating each of eight check cultivars within each bay. This allowed analysis by the TwoD program (see section 6.1.2) and a statistical adjustment for underlying fertility trends.

Yields only were measured.

6.2.2.3. Field trials 1996

Doubled haploid lines from the TRI/MNX cross were grown in a randomized complete block trial of 3 blocks at two sites, Stow (80km North of the Roseworthy Campus) and Buckleboo (upper Eyre Peninsula) in 1996. The plot layout at Stow was the same as that of 1995 row plots experiment. At Buckleboo plots were 4 rows of 15cm spacing and a harvested length of 4.2m. These plots were arranged in 15 bays. These trials, performed by the Roseworthy wheat breeding group, provided additional data to enable comparisons on the efficiency of hill plots.

6.2.3. Results

The row plot dimensions used in the row plot trials are typical of those used by cereal breeders in most countries for evaluating likely on farm performance. These then are the standard against which to test the efficiency of hill plots.

Figure 6.2.1. shows the relationship of 4 replicate hill plot means to the single replicate 1995 row plot trial for all lines which were common to both trials.

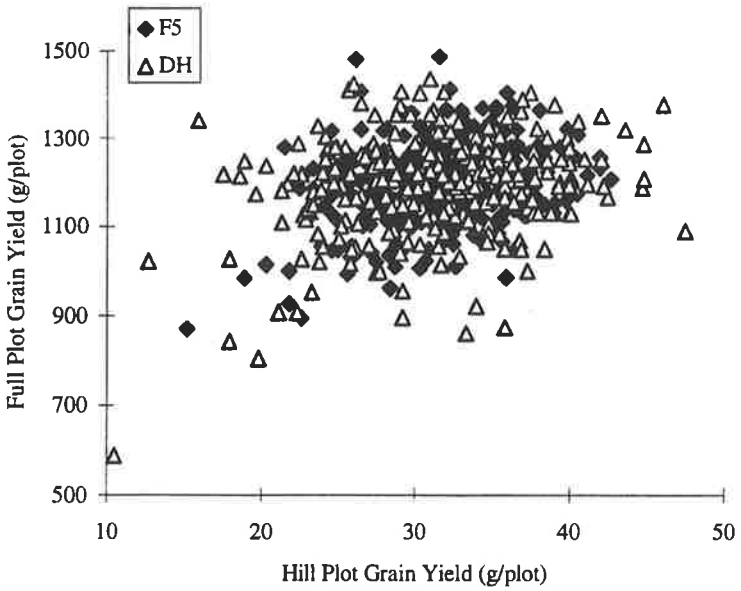


Fig. 6.2.1. Relationship between mean yields of a 4 replicate hill plot experiment and a spatially adjusted single replicate row plot trial 1995 ($r=0.26^{**}$).

The doubled haploid lines are depicted separately from the F_5 lines. Again the spread of each is very similar but there are more extreme DH lines than F_5 lines. The yields of hill plots and row plots are significantly correlated (r=0.26, n=278, P=0.01), but the association is not one that breeders would consider close or very useful.

Using a subset of the above entries, viz. doubled haploid lines from TRI/MNX, it is possible to compare results from 1996 with 1995. Table 6.2.1 shows the correlations between measurements relevant to comparing hill and row plots performances. The bivariate scatter diagrams for these correlations are plotted in figures 6.2.2 and 6.2.3.

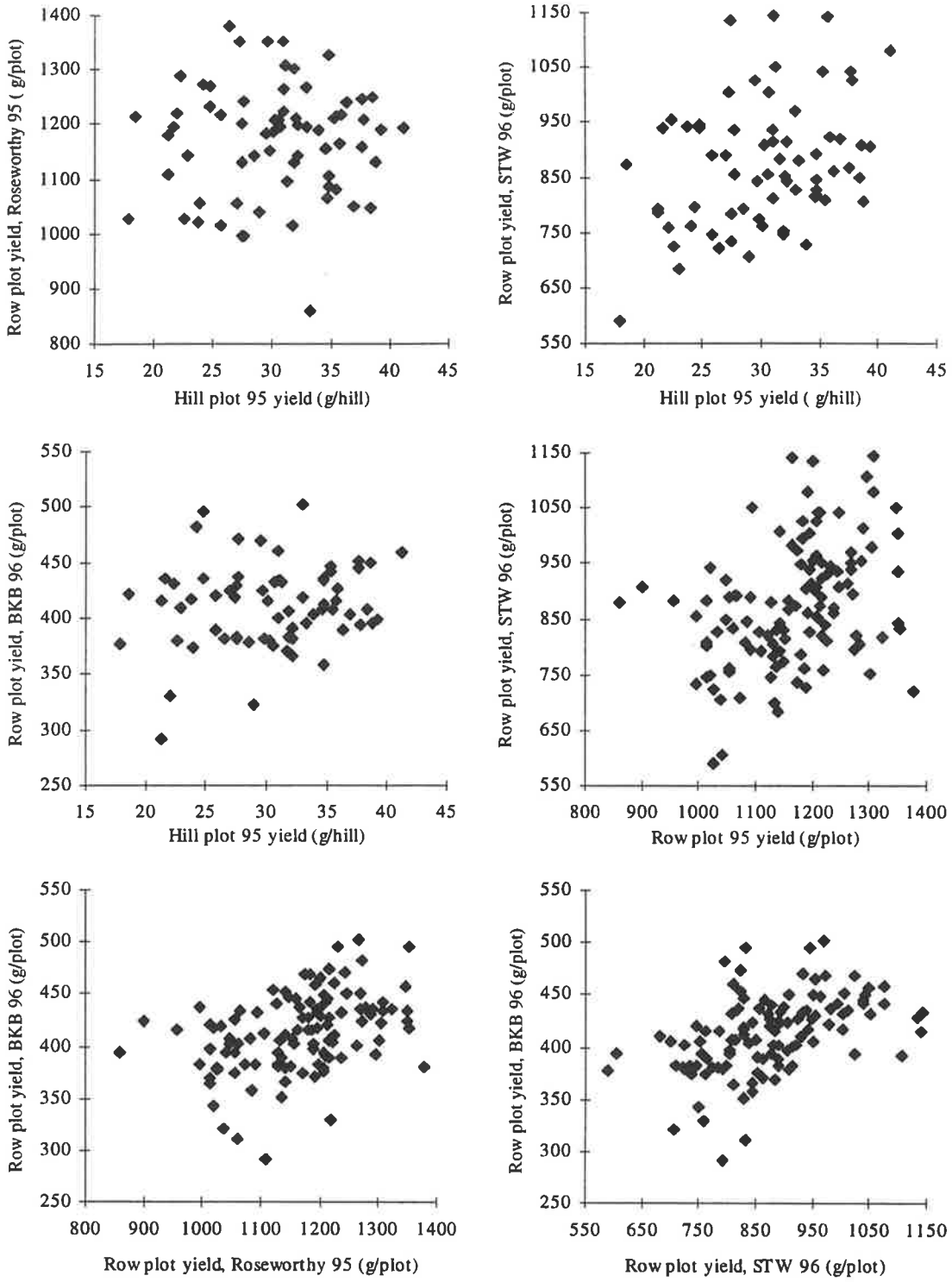


Fig. 6.2.2. Scatter diagrams comparing yield performance of TRI/MNX doubled haploids in different environments.

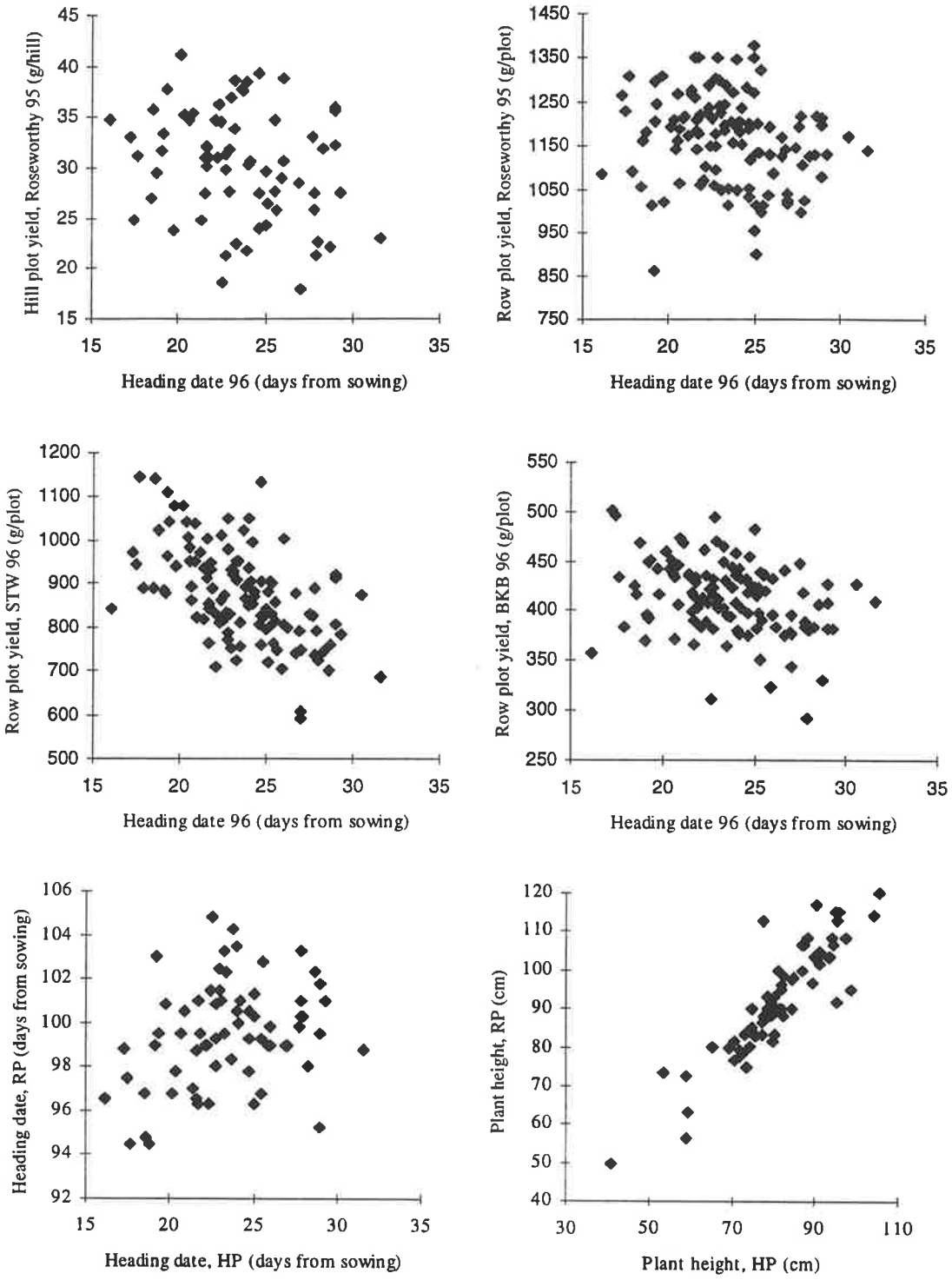


Fig. 6.2.3. Scatter diagrams comparing TRI/MNX doubled haploids for heading date, plant height and yield in three locations and two years for hill plots and row plots

Table 6.2.1. Correlation matrix between characters in hill and row plots at different sites

	HP-Y95	RP,RWY95	RP,STW96	RP,BKB96	DTH,RWY95	PH,RWY95
RP,RWY95	0.04					
RP,STW96	0.35*	0.37*				
RP,BKB96	0.17*	0.41*	0.47*			
DTH,H95	-0.25*	-0.17*	-0.54*	-0.32*	0.31*	
PH,H95	-	-	-	-	-	0.89*
LC,BKB96	0.14	-0.36*	-0.19*	-0.23*		
BT	-	0.01	0.02	0.03		

HP-Y=Hill plot yield, RP= Row plot, RWY= Roseworthy, STW=Stow, BKB=Buckleboo, DTH=Day to heading, PH=Plant height, H95=hill plot 1995, LC= leaf colour, BT=Boron tolerance, *. Significant at 95% probability

Plates 6.1a and 6.1b show the hill- and row-plot trials respectively in the vegetative growth stage. In the row-plot trial differences in vegetative growth among lines are clear (Plate 6.2).

There was no correlation between yield in hill plots (for Trident/Molineux cross only) and in adjacent row plots in the 1995 Roseworthy trial. Examining the scatter diagram (Fig. 6.2.1) shows only one outlier in an otherwise fairly random distribution, so the lack of correlation is real and not due to extremes. However, there was a significant correlation between hill plot yields in 1995 and row plot yields at Stow in 1996 (Fig. 6.2.2). The correlation between hill plot yield 1995 and Buckleboo in 1996 was not statistically significant (Fig. 6.2.3). The correlations between lines for yield in row plot trials in the three environments, Roseworthy 1995, Stow 1996 and Buckleboo 1996 were similar ($r=0.4$, $n=114$, $p=0.01$).

Plate 6.2. Row plot field trial 1995 at Roseworthy. Variation in vegetative growth, height and maturity is evident amongst doubled haploid lines.

6.2.4. Discussion

The generally poor relationships in comparing hill plots at a single site with row plots at several sites raises the questions: 1) is there some difference between the growing environment of the hill plot layout which differentially affects genotypes and makes hill plot performance a poor predictor of on-farm performance? and 2) why should the yields in row plots at Stow 1996 correlate with hill plot yields? There are some agronomical and physiological explanations regarding these contradictory correlations. Hill-plot plants spacing are not representative of on-farm plant spacings as, although seed rate per hectare may be similar, there will be intense competition within each hill especially for those plants in the centre of hills. Shading from neighbours could affect shorter lines. Tall neighbours could knock grains from short genotypes, especially from the upper part of spikes. Compaction of soil between hill plots by trampling during planting, weeding and note taking might affect lines differentially. Fertilizer is not in close contact with the seed in hill plots. None of these problems occur with row plots which, apart from the wider spacing between plots and possible edge (marginal) effects which might result, are very similar to on-farm plant arrangements.

It is not surprising then that hill plot performance does not correlate well or predict row plot performance. At Stow in 1996 plots were planted later than normal, grew under quite wet conditions until just after anthesis but then there was no rain during the whole grain filling period. Plants were very drought stressed and small poorly filled grains resulted. Earliness of anthesis was a distinct advantage under these conditions because the grain filling period was effectively longer. This is demonstrated by the significant correlation between yield and heading date at Stow in 1996 (Table 6.2.1, Figure 6.2.3.c). Heading date was also correlated with hill plot yields (Figure 6.2.3.a). Although this correlation was not as close as Stow it is postulated that intense competition within hills might initiate drought stress because soil exploited below each hill could become depleted of moisture quickly whilst moisture might be left between hills. This depletion of 'available' soil moisture would not have occurred in Roseworthy 1995 row plots when the spring rainfall was greater than in 1996.

In contrast to yield, relative plant height of lines was very closely correlated between hill plots and row plots even though row plot height was measured in 1996 and hill plots in 1995 (Table 6.2.1, Figure 6.2.3.f). Hill plot height is a good predictor of on-farm heights.

Heading time measured in the hill plot experiment 1995 was significantly correlated with that measured on row plots in 1996 (Figure 6.2.3.e). However, the correlation, and therefore prediction, was not as close as for height or as found by other researchers.

Thus, under South Australian field conditions the value of hill plots for estimating on-farm performance was good for plant height, satisfactory for heading time but of no value for yield itself. This agrees with the findings of most other researchers (Khadr *et al.*, 1970; Walsh *et al.*, 1976).

This finding is irrespective of whether the lines under test were F_5 or DH so it does not affect the comparisons between F_5 and DH populations, but greatly effects any extrapolation or selection of individual lines on hill plot yield data both in this experiment and in the adoption of DH technology in a breeding program. Hill plot planting was only used because of limited seed supplies. For yield assessment lines should be tested in row plots managed similarly to the management used on-farm, for seeding rate, fertilizer use, sowing time, weed control and so forth. Moreover, these assessments should be conducted over several sites and seasons to evaluate the extent of genotype-environment interactions.

Therefore, using DH breeding system instead of traditional methods of breeding is worthwhile to release varieties faster with less expense. Also if the breeding program has focused on highly heritable traits controlled by a few major genes then hill plot has advantage to be used for line assessment.

Chapter 7

General Discussion

Doubled haploid technology has received a lot of attention by plant breeders and geneticists for some time. Wheat breeders, however, have not until recently been able to use this technology because the widely used methods of producing haploids, namely, anther or microspore culture and crosses with *Hordeum bulbosum*, have not been efficient with wheat.

The number of haploids produced by anther culture is few relative to the resources spent. Also, many wheat genotypes cannot be successfully induced to produce haploids from crosses with *H. bulbosum* because they carry one of the dominant genes *Kr1* and *Kr2* which suppress crossability with *H. bulbosum* and other species including cereal rye.

When Laurie and Bennett (1986) reported the production of wheat haploids from pollinating wheat with maize and that there was no apparent crossability problems or wheat genotypes which could not be induced to produce haploids, wheat breeders had a new potential tool to speed up their breeding. Since their original work, other researchers have further modified the technique to improve efficiency.

Several aspects have been considered in this current study, including the genotype of the pollinator, possible interactions between the maize pollinator and the female wheat genotype, timing of pollination, use of hormones to promote seed set, culture media for embryo rescue and colchicine chromosome doubling techniques.

Laurie and Bennett (1987) found maize cultivar Seneca 60 to be more efficient in inducing

the formation of haploid embryos than other maize parents tested. Ushiyama *et al.* (1991) found a teosinte accession was better than other maize genotypes but Lefebvre and Devaux (1996) found no differences in effectiveness between the teosinte accession (not the same accession tested by former workers) and some superior genotypes of maize. Morshedi *et al.* (1995) reported the sweetcorn cv. Kelviden Glory to be a highly efficient pollinator. In the present study sweetcorn cv. Illini Gold was equally as efficient as Seneca 60. Other attributes of maize genotypes such as ease of culture in glasshouse or controlled environment conditions, height, tassel size, length of anthesis, availability of seed are just as important as effectiveness as a pollinator *per se* in choosing which to use as a pollinator.

Wheat haploids were recovered for all wheat × maize combinations, although the rate of recovery was different in different crosses and the condition of the haploid embryos also differed. This variability in success between crosses is similar to the experiences of Laurie and Bennett (1987), Snape *et al.* (1980), Suenaga and Nakajima (1989), Laurie and Reymondie (1991) and Lefebvre and Devaux (1996). *Kr* crossability genes may or may not be influencing these crosses but at least they do not cause complete incompatibility as happens in most crosses between Australian wheat cultivars and *H. bulbosum*. The health of the female wheat parent and the effect of intraplant competition and main tiller versus secondary tillers are important.

The cultivar Trident and F_1 hybrids derived from crossing Trident with other wheat genotypes had a higher percentage and efficiency of embryo formation than other wheats used. This may be a real difference or it may be merely because Trident was used more often and over a longer period than other wheats.

Embryo rescue and plantlet regeneration is another step in doubled haploid production where attention to detail is critical for success. Stimulation of embryo growth after pollination by injecting 100 mgl^{-1} 2,4-D into the peduncle first suggested by Suenaga and Nakajima (1988), worked well. Increasing the 2,4-D concentration to 150 mgl^{-1} was more effective than 100 mgl^{-1}. Using this method the average frequency of embryo formation was 38%, higher than that achieved by Laurie and Bennett (1988)(21%), Suenaga and

Nakajima (1989)(25%) and Laurie and Reymondie (1991)(33%). Morshedi *et al* (1996) found no difference between this method and immersing the spike in 2,4-D solution straight after pollination.

The composition of the culture medium onto which embryos are rescued might also affect embryo germination and regeneration. No work has been published where different culture media have been compared. Of the three media most widely used in wheat embryo rescue procedures half strength MS appeared the most satisfactory.

The wheat × maize system of producing doubled haploids needs attention to detail and the development of special skills. Since the protocol described in chapter 3 was developed and used to produce the doubled haploids for this study, 1993, the system has been further improved (Morshedi and Darvey, 1996 pers comm; Howes 1996, pers comm), for example,

a) Use only the main tiller of each female plant, remove all other tillers and use the healthier plants
b) Emasculate the wheat spikes as close as possible to normal anthesis
c) Use only maize pollen that is less than 2 hours old after anthesis
d) Pollinate twice on successive days
e) Immerse the spikes into 2,4-D solution
f) Double using colchicine added to the culture media before plantlets are transplanted to soil
g) Hydroponic growing of the plants treated by colchicine to produce many tillers gives greater chance of getting doubled chimeras

For genetic studies, and to be of maximum value to breeder, it is critical that doubled haploid populations produced are a random sample from the female gametes. That is, there should be no predisposition of some gametic genotypes to form doubled haploids. Only if this is so can the inheritance of traits and the location of genes controlling them be established, and can the breeder determine minimum population sizes to recover certain gene combinations.

Distorted Mendelian segregation patterns were reported by Thomson *et al.* (1991) in a doubled haploid population produced by anther (microspore) culture. The frequency of such distortion is much less in doubled haploid populations produced by the *H. bulbosum* system (Pickering, 1983; Kjaer *et al.*, 1991; Powell *et al.*, 1986, 1990). Because the wheat × maize system is physiologically very similar to the *H. bulbosum* system it is likely that the occurrence of distorted segregation patterns will also be similar, that is, infrequent. There are few reports of distorted segregation patterns in wheat × maize systems. The doubled haploid population from the F_1 between wheat cultivars Trident and Molineux produced in this project was scored for eight loci of major effect known to be polymorphic in this cross, viz. VPM1 segment for *Sr38*, *Yr17* and *Lr37*, *Rht1*, *Rht2*, *Bo1*, *GluA1*, *GluA3*, *GluB3* and CCN resistance gene. These loci are located on different chromosome arms. None of these markers showed any distortion from that expected of 1:1. Suenaga and Nakajima (1993) reported a similar finding for their doubled haploid populations with eight different segregating loci. They did find a discrepancy between their doubled haploids and haploid lines for glume pubescence but they attributed this to factors associated with colchicine treatment.

Thus available data indicate that doubled haploids produced from a wheat × maize system are a random set of unselected female gamete genotypes doubled to produce fertile plants. Any differences between wheats in their ability to form doubled haploids must be due to maternal or external conditions rather than the genotype of female gametes.

The Trident/Molineux doubled haploid population was also used to investigate the inheritance of other traits of unknown inheritance and could be used in searching for molecular markers which may be useful to screen for otherwise difficult to score traits. Pinthus and Levy (1984) suggested a random sample of F_5 and F_6 lines could be used for the same purpose, but considerable heterozygosity still remains in such material. Alternatively, random populations from single seed descent could be used. Doubled haploids are a random population of homozygotes and thus are most appropriate for such studies.

Frequency distributions were established for resistance to *Septoria tritici*, coleoptile length, heading time, leaf colour and grain yield. Applying the methods proposed by Mather and Jinks (1971) in the same fashion as Choo and Reinbergs (1982) the numbers of loci (or effective factors if linkage is present) controlling the above attributes were estimated at 2 to 3, 4 to 10, 5, 9 and 9, respectively . In most cases these would be underestimates.

Recent development in genetic marker technology suggest that an RFLP probe, BCD175, can be used as a rapid screen for the VPM1 chromosome segment bearing *Sr38* (Langridge, pers comm) and another RFLP probe, CDO347, might be closely linked to the cereal cyst nematode resistance from wheat cv Festiguay which is the source of resistance in Molineux (Paul *et al.*, submitted for publication). This Trident/Molineux DH population is an excellent population to test the efficacy of these probes.

There are two major advantages of doubled haploid technology, compared to a pedigree method, as a plant breeding system for self pollinated crops where improved varieties are grown as pure lines. Doubled haploid technology shortens the time taken to achieve homozygosity (and homogeneity), and reduces the population size needed for the selection of favourable gene combinations for those traits whose inheritance is known.

Gene frequencies in doubled haploids are a gametic frequency rather than a zygotic frequency, that is, in doubled haploids from an F_1 hybrid the frequency of the desired combination is the square root of the expected frequency of the same homozygote in the F_2. For example if there are 3 loci polymorphic in an F_1 the desired genotype will be expected in 1 of 8 lines whilst the same derivable homozygote will only occur on average 1 in 64 lines in F_2 population.

Moreover, if the trait is controlled by dominant genes the situation is further complicated using the F_2 system and needs successive selfing generations for identifying the relatively homozygous dominant loci from heterozygous. In the F_2 generation only one out of 64 lines is homozygous at three desired loci but cannot be identified phenotypically from 26 other heterozygotes, while again in DH system like in case for recessive loci one out of 8

lines will carry the dominant alleles at the three loci. Therefore, in both cases the production of DH lines make identifying the genotype easier and this in turn reduces the sample size required to identify the desired genotypes. Additional resources may then be allocated to other objectives or additional desirable lines can be included for evaluation.

Breeders could improve the efficiency of their programs by combining doubled haploid technology with other strategies. For example, it is several generations after a cross has been made before individual lines are field tested for yield and the breeder has a measure of the value of the cross in producing improved lines for potential release. Not many lines are needed to determine the mean and variance of a cross and to be able to compare its potential with other crosses. As few as 20 doubled haploids could be produced from these crosses and be field tested several years before hand, whilst larger numbers are carried through by conventional breeding methods. Thus the breeder would have foreknowledge of cross potential and know what selection pressure to apply to each cross, even to discarding whole populations from poor crosses. The use of such small numbers of doubled haploids for cross prediction was reported in Chapter 6. In very few crosses were there significant differences between each of 16 doubled haploid populations and the corresponding F_3 derived F_5 populations.

Doubled haploid technology combined with a recurrent selection procedure and screening techniques to allow selection for desirable genes prior to doubling haploid plants can further speed the breeding (Howes *et al*, 1996).

While the genetic stocks produced in these experiments were produced to test various facets of a doubled haploid technique as a genetic or plant breeding tool, the parents used and the crosses made were taken from a varietal improvement program and the traits measured are of economic importance. Thus it is of interest to examine these stocks for any lines which could have practical value in wheat breeding programs for Southern Australia, either as parents or for release as cultivars in their own right.

Figure 7.1 shows a recommended flow chart for testing and selection of the genetic stocks from these studies when introduced into the Roseworthy wheat breeding program. The next step is testing at several locations in replicated experiments.

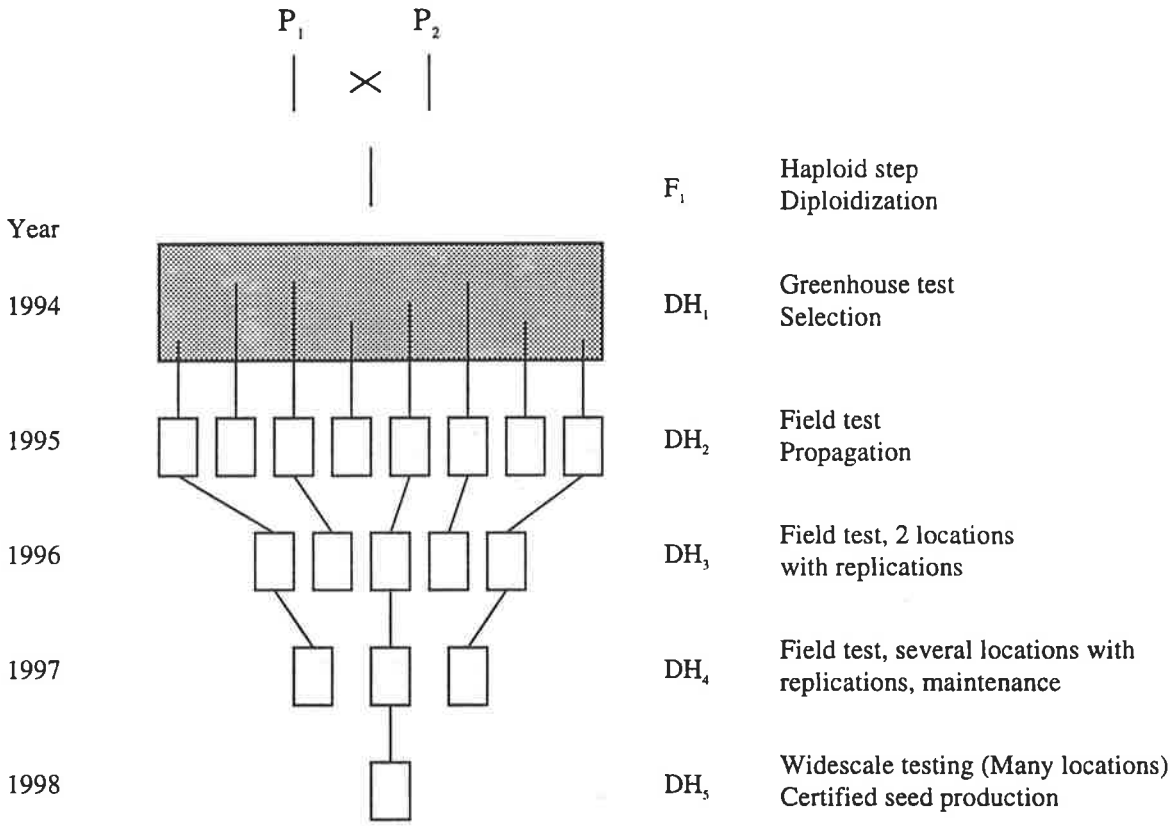


Fig. 7.1. Breeding scheme for doubled haploid lines produced through intergeneric crosses of wheat × maize (modified from Foroughi-Wher and Wenzel, 1990)

Note that the seed produced from F_1 wheat × maize is DH_0 and is generally not enough for doing experiments. DH_1 seed produced by one round of off-season seed multiplication will be used in this scheme.

The Trident/Molineux doubled haploid population is of immediate promise and interest. Because of its size it is a valuable population for genetic mapping studies so samples of each selection will be preserved for others to use by depositing them in the Australian Winter Cereals Collection, Tamworth, New South Wales, Australia, 2340. By reference to the polymorphic traits listed in Tables 4.2.1 and 5.1 and the data collected on each DH line detailed in Appendix 1 it is possible some lines will find a place as new improved varieties for South Australia. Unfortunately because hill plot yields cannot be extrapolated to row

plot or farm yields then one can only select on other attributes and yields will need be determined in subsequent field experiments. The first sorting of these lines should retain all lines with:

1) The VPM1 segment for resistance to Stem, Stripe and Leaf rusts, ie Stem rust reaction type X=, X- or X

2) Useful resistance to *Septoria tritici*, ie Septoria score ≤3

3) Allele *GluA3-c* to give improved dough extensibility over *GluA3-e*

4) Not too tall, nor too short like double dwarf height.

Secondly selection after sorting can then be carried out to further reduce the number of lines for specific situations, such as:

- Districts where Boron toxicity is likely, retain all lines with root length >52 mm
- Situations where cereal cyst nematode a problem, retain lines with CCN score ≤4
- Both the above.

After these screening DH selections 16, 80 and 134 hold the best promise for release.

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Appendices

Appendix 1. Characteristics of doubled haploid lines derived from Trident/Molineux cross scored for *Septoria*, stem rust and CCN reactions, Glutenin protein alleles, height, gibberellic acid insensitivity, heading date, leaf colour, coleoptile length, Boron tolerance and grain yield.

	Septoria Mean		Rust	CCN	Protein			Height					Heading date		Leaf Colour Minulta			Boron tolerance	Yield			
DH No.	1995	1996 field	Infection type	cysts/plant	*Glu B1*	*Glu A3*	*Glu B3*	Glass	Irrigated	Hill	1996 field	GA3	Oct-96	Coleoptile	a*	b*	L*	Root Length	Hill plot	Full plot	Stow 96	Buckle boo
1	3	3.9	2	6.9	7+9	e	h	79.0	82		79.9		21	78.9	13.47	16.77	40.14	39.0		1217	822	473
2	2.5		X=	1.1				81.5														
3	3		x=	1.7	7+9	c	b	88.0	97	87.0	106.5	S	24	81.5	12.30	15.56	42.44	85.6	21.7	1195	938	436
4	2	3.2	2	21.0	7+9	c	b	95.0	110	87.3	106.7	S	27.8	94.3	13.52	17.11	41.32	34.9	27.5	996	734	383
5	4		x=	4.4				80.2		86.3									43.9			
6	5	7.3	3	5.7	7+8	c	h	71.5	72		51.7		21.2	67.3	11.30	12.86	37.30	39.6		1173	972	469
7	4	6.4	x=	1.6	7+9	c	h	89.0	93		100.7	I	24	86.7	11.77	13.66	39.89	37.1		1347	1049	458
8	3		2	19.9	7+9	e	b	78.2														
9	4	5.2	3	19.7	7+8	e	b	93.0	96	93.8	103.3	S	24.7	100.0	13.49	17.04	41.97	59.7	39.3	1189	904	398
10	4		3	16.5	7+8	c	b	90.0		85.0									32.4			
11	3	6	2-	3.0	7+9	e	b	91.0	95		91.5	I	25.2	81.9	12.74	15.16	39.78	37.9		901	906	424
12	5		x=	2.9				80.0														
13	3	7.7	x=	0.9	7+8	c	b	77.0	84		86.6		19.3	75.1	12.27	14.21	39.69	35.0		1295	1108	393
14	5	3.9	3+	3.7	7+8	e	b	86.0	99		93.3	I	24.8	96.6	12.28	14.22	39.73	48.6		1120	821	454
15	3	3.9	2	6.3	7+9	e	b	78.0	83		86.7		19.7	74.6	11.86	13.87	38.62	69.2		1307	1078	443
16	2		x=	0.8	7+8	c	h	83.0	85	73.0		I		80.9				71.2	30.4			
17	5	4.7	x=	3.6	7+8	c	h	96.0	98	94.0	108.4	S	24	105.1	13.87	17.98	42.30	91.6	38.4	1047	850	407
18	2		x	6.8	7+8	c	b	71.5		52.5									19.8			
19	2	2.3	3	5.5	7+9	c	b	67.0	70	75.0	85.0		29.3	63.4	14.41	15.79	40.80	35.4	27.5	1131	783	381
20	3	7	x=	3.4	7+8	e	b	75.0	77		96.7		23.1	74.0	12.53	14.80	40.20			1226	929	411
21	2	3.1	3+	15.4	7+9	c	h	55.5	71	75.8	82.7		23.4	64.2	13.15	15.81	39.56	41.9	22.4	1287	953	431
22	4	1.2	2	17.1	7+9	c	h	66.5	73	70.5	76.7		17.5	68.9	13.78	17.96	40.75	79.2	24.8	1231	945	496
23	3	4.2	3-	1.7	7+9	c	b	78.5	92	82.5	98.4		23.1	80.8	12.92	15.69	39.65	69.2	36.9	1048	920	403
24	4		2	18.2				71.5		85.5									31.2			
25	2	1.8	x=	1.1	7+8	e	b	85.0	90	79.5	91.7	S	19.8	118.9	12.63	15.52	41.19	88.4	23.8	1020	941	417
26	5	1.9	2	17.7	7+9	c	b	81.5		94.8	115.0		21.7	62.8	12.49	15.41	39.87	68.7	32.2	1142	843	366
27	3		x=	13.6				79.0		53.5									32.3			
28	2		3+	15.9				75.0														
29	2		2	15.6				108.3		100.8		S							34.6			
30	2		3+	13.6				92.5		77.0									29.9			
31	3		2	3.2	7+9	e	b	99.0	118			S		109.3				81.5				
32	3	6.3	x=	1.8	7+9	c	h	85.0	93	82.0	95.0	I	22.8	83.9	11.95	13.51	39.61	24.7	31.3	1095	1051	432
33	2	1.5	2	16.5	7+9	e	h	97.5	104	87.0	100.1	S	22.2	104.1	13.91	17.33	40.45	93.9	34.8	1105	828	413
34	2	0.8	3+	4.4	7+9	e	h	84.0		80.3	93.3		23.8	110.4	12.74	15.00	39.68	71.5	37.6	1158	866	445
35																						
36	3		2-	20.1	7+9	c	h	95.0	102	85.0		S		103.1				44.2	23.1			
37	3	7.8	x=	9.8	7+9	e	h	77.5	97	77.8	87.6		25.5	73.0	12.96	15.60	41.42	33.6	27.7	997	856	437
38	3		2-	18.8	7+9	e	b	76.0	92	73.5				79.7				62.4	31.2			
39	2.5	7.5	x=	17.8	7+9	c	b	53.0	72	72.0	79.4		21.6	71.1	12.39	14.24	39.42	37.7	27.4	1351	1003	418
40	4		2	3.6	7+9	c	h	77.5	110	78.5				75.9				81.1	37.0			
41	4	5.5	x=	1.8	7+9	e	b	82.5	109	88.3	108.4	S	28	75.4	12.75	15.54	41.48	67.4	22.6	1027	724	380
42	2.5	4.8	x=	2.0	7+8	c	b	89.0	100	84.3	90.0	S	24.7	75.5	11.88	13.93	40.45	40.2	24.0	1054	761	374

Appendix 1. Continue

	Septoria Mean		Rust	CCN	Protein			Height					Heading date		Leaf Colour Minulta			Boron tolerance	Yield			
DH No.	1995	1996 field	Infection type	cysts/plant	*Glu B1*	*Glu A3*	*Glu B3*	Glass	Irrigated	Hill	1996 field	GA3	Oct-96	Coleoptile	a*	b*	L*	Root Length	Hill plot	Full plot	Stow 96	Buckle boo
43	4		3+	2.2	7+8	e	h	67.0	71	72.0				61.9				56.2	23.6			
44	3	3	x=	13.1	7+9	c	b	71.0	72		88.3		21.8	67.3	11.82	13.26	40.06	53.5		1180	949	428
45	2	5.9	x=	19.5	7+9	c	h	48.0	55	41.0	50.0		27	59.4	11.39	12.54	38.13	76.7	17.9	1027	591	377
46	2		2	0.9	7+9	e	h	84.5	102	80.3		I		69.1				43.4	31.6			
47	3	2.8	2	7.1	7+9	c	b	71.0	75	78.8	90.0		23	64.9	13.86	17.65	40.93	38.2	27.7	1243	934	471
48	4	6.4	3+	21.0	7+9	c	h	70.5		65.0	80.1		31.6		12.98	15.47	39.67	51.9	23.0	1141	683	410
49	4.5	7	x=	2.6	7+9	c	h	81.0	98	81.3	99.8	I	22.5	47.4	13.05	15.59	41.37	81.9	34.6	1153	814	408
50	2	5.2	3-	20.8	7+9	e	h	72.0	80	69.5	79.9		23	59.5	12.50	14.68	39.09	82.4	31.9	1302	751	406
51	4		x=	7.8	7+9	e	h	71.5	89	75.3				65.8				46.6	33.1			
52	4		3-	12.1	7+9	c	h	67.0	62	55.5				50.4				41.4	26.5			
53	3.5	4.7	3-	2.9	7+8	e	h	65.5	61	53.3	73.3		25.1	51.5	12.47	14.17	38.91	74.0	26.5	1378	722	381
54																						
55	2	6.4	x=	21.0	7+8	e	h	68.5	85	80.3	83.3		21.6	64.8	12.10	14.18	39.54	50.1	31.0	1262	913	400
56	3	2.3	2	18.5	7+8	c	b	104.5	105	97.5	108.3	S	26	73.5	12.64	15.47	40.52	35.5	38.8	1130	805	395
57	3	1.8	2	10.5	7+8	c	b	91.0	98	78.8	93.4	S	26.9	72.1	12.02	13.73	40.19	56.2	28.5	1142	792	379
58	5		x=	0.5	7+8	c	b	91.5	107	92.0		S		80.5				60.7	29.5			
59	4	4.4	x=	18.8	7+8	c	b	98.5	113	104.3	114.0	S	22.4	91.2	13.30	16.62	42.51	51.7	36.3	1238	861	389
60	5	2.9	3	3.7	7+9	c	h	70.0	79	73.5	75.0		21.4	64.3	12.92	15.81	39.83	55.0	24.8	1269	938	436
61	5	7.2	x=	3.1	7+8	e	b	73.5	86	79.3	88.4		18.6	79.7	12.97	15.92	42.04	68.1	35.8	1163	1141	416
62	4.5	5.2	2	13.1	7+8	e	h	74.5	83	75.8	83.3		17.3	70.2	13.40	16.92	40.69	37.5	33.0	1267	970	502
63	3	2	2	21.0	7+8	e	h	94.5	131	94.5	106.7	S	16.1	103.9	12.34	15.18	40.36	44.4	34.8	1085	844	358
64	3	2.8	2	17.2	7+8	e	b	82.5	105	89.7	96.7	S	22.3	86.6	12.76	15.63	40.83	37.9	31.0	1224	811	461
65	2	3.5	2	1.6	7+8	c	h	64.0	65	78.5	87.7		18.8	51.6	11.97	14.65	39.92	73.2	29.5	1183	1025	469
66	5	4.9	x	0.4	7+8	c	b	48.0	63	58.8	56.7		28.7	60.6	11.81	13.28	40.30	66.0	22.1	1219	758	330
67	3	4.7	3+	7.8	7+8	c	b	81.5	97	77.5	83.3	I	24.2	72.8	12.40	15.43	40.54	36.4	30.6	1207	854	376
68																						
69	3	1.1	x=	4.0	7+8	e	h	109.5	132	105.3	120.0	S	25.9	86.4	12.97	16.04	41.30	57.5	29.0	1039	705	322
70	2		x=	8.8	7+8	e	h	56.5		52.0									23.9			
71	3		3+	19.1	7+8	c	h	92.0		80.8									24.6			
72	4	4	3+	3.5	7+8	e	h	68.0	91	91.3	101.7		20.7	82.3	12.22	14.38	39.50	29.4	34.7	1065	893	434
73	3		2	18.0	7+9	c	h	75.0	110	94.8				76.5				50.7	30.3			
74	4		3+	18.7				66.5		86.0									31.4			
75																						
76	3		2-	7.8				71.0		95.8									27.5			
77	2		2-	18.9				70.5		73.3									30.7			
78	2.5	5.9	x	14.4	7+8	c	h	75.0	85		90.0		20.6	53.3	13.47	16.42	41.57	70.1		1269	952	450
79	2	4.8	3	1.0	7+9	c	h	81.5	89	70.5	81.7	I	21.8	65.0	12.04	14.48	38.99	78.9	31.0	1350	934	434
80	3	6.1	x=	4.8	7+9	c	h	80.5	105	84.5	97.7	I	24.1	66.4	13.06	15.09	40.34	66.0	30.3	1208	907	380
81	5	4.4	3+	17.0	7+9	c	b	90.0	119	95.5	91.7	S	26	64.4	13.31	16.68	41.92	66.7	30.7	1194	1003	432
82	5	4.8	3+	19.3	7+9	c	b	80.8	91	77.5	112.7	I	19.2	77.3	13.04	16.53	41.03	72.4	33.3	860	879	395
83	3	4.3	3+	17.5	7+9	e	b	83.2	115	91.8	104.4	S	18.5	70.8	12.06	15.43	41.65	79.0	27.0	1055	890	425
84	4	4.2	3+	14.4	7+8	c	h	72.5	106	90.0	103.3		22.8	87.6	12.57	15.12	39.46	62.6	21.3	1180	786	416
85	5		3+	19.3	7+8	e	h	79.0	91	74.3				70.0				46.6	29.4			
86	5	5.7	x-	10.5	7+9	c	b	75.0	87	72.8	78.4		27.9	63.0	12.32	14.88	40.78	27.7	21.3	1109	792	291
87	3		x-	10.8	7+8	c	b	65.0	73	68.3				67.4				64.7	33.0			
88	4	5.6	x=	9.3	7+9	e	b	67.0	92	77.5	86.6		24.7	63.9	11.51	13.59	40.25	64.4	27.5	1202	1134	429

Appendix 1. Continue

	Septoria Mean		Rust	CCN	Protein			Height					Heading date		Leaf Colour Minulta			Boron tolerance	Yield			
DH No.	1995	1996 field	Infection type	cysts/plant	*Glu B1*	*Glu A3*	*Glu B3*	Glass	Irrigated	Hill	1996 field	GA3	Oct-96	Coleoptile	a*	b*	L*	Root Length	Hill plot	Full plot	Stow 96	Buckle boo
89	4	5	x=	1.0	7+9	c	h	71.0	104	81.3	100.0		22.6	69.3	12.90	15.51	40.19	19.0	18.6	1213	873	421
90	2.5	2.5	x=	4.5	7+9	c	h	90.0	118	95.8	115.1	S	21.7	83.4	12.89	15.58	40.87	52.9	30.2	1186	762	415
91	2	2.9	3+	16.6	7+9	e	h	69.0	93	77.3	83.3		28.3	78.9	12.98	16.31	42.02	43.5	31.9	1129	745	383
92	4	6.8	2-	15.9	7+9	c	h	78.0	113	90.5	116.7		19.1	102.6	12.26	15.77	41.55	69.5	31.7	1014	884	370
93	5	5.6	x=	0.9	7+9	c	h	96.0	102	91.3	104.8	S	25.6	108.5	12.19	14.85	41.46	83.6	25.8	1015	747	420
94	5		3+	18.3	7+9	c	h	72.5	77	65.3				61.9				77.7	24.3			
95	4.5	6	3+	16.4	7+9	c	h	78.5	90	73.3	83.4		25	71.2	13.54	16.97	40.04	80.0	24.3	1273	795	482
96	4	2.3	x=	14.4	7+8	e	h	75.0	92	82.0	96.4		29	98.3	13.30	16.50	41.53	61.6	35.5	1081	807	408
97	3		2-	17.4				71.5		88.3									30.8			
98	3	6.3	x=	8.0	7+8	c	b	74.0	90	81.8	89.9		20.2	56.6	11.82	13.93	40.42	78.5	41.2	1193	1078	459
99	4		3+	4.9	7+9	e	h	59.0	86	73.0				71.3				86.6	30.1			
100	3	3.8	2	19.8	7+8	c	b	85.0	108	93.3	103.4	S	23.3	99.7	13.96	17.94	41.24	48.5	33.9	1188	726	403
101	3	5.6	x=	10.3	7+8	c	b	75.0	89	77.8	88.3		20.9	87.3	11.97	14.00	40.82	77.4	35.4	1213	1040	446
102	4		x=	0.5				74.0		99.8									37.3			
103	3	3.5	x=	7.9	7+9	c	b	85.0	90	84.8	98.2	I	21.7	73.1	11.54	13.72	40.93	29.9	32.1	1209	852	391
104																						
105																						
106			3+																			
107			3-																			
108	3	4.2	4	2.8	7+8	c	b	61.5	70	59.0	72.5		27.7	65.4	12.37	14.31	39.25	31.9	33.0	1196	828	418
109	5		2	16.6	7+9	c	h	55.0	95	79.8				91.7				36.9	33.8			
110																						
111	3.5	6.7	3+	2.0	7+8	c	h	54.0	70	59.3	63.3		27.8	62.5	13.03	15.58	39.47	35.4	25.8	1217	889	389
112																						
113																						
114	3	4.6	x=	14.6	7+9	e	h	74.0	90	82.3	88.3		25.5	70.8	12.43	14.61	40.08	38.9	34.8	1324	816	436
115																						
116	3		2	5.3	7+8	c	b	65.5		82.5									31.1			
117	3		2	5.9	7+8	e	b	103.0	111	95.3	112.7	S	22.8	109.0	13.74	17.77	41.88	88.9	29.9	1150	773	381
118																						
119	3		x-	2.6				53.5		60.0									29.2			
120	4	5	3	21.0	7+8	e	b	96.5	88		78.3	I	23.3	81.8	12.26	15.24	41.04	66.2		1200	954	465
121	4.5		x=	9.4	7+8	e	h	85.5	97			I		80.2				52.6				
122	5		x=	13.6	7+9	e	h	76.5														
123	3			15.8	7+9	e	b	94.0														
124	5	6.2	x=	3.5	7+8	c	h	71.0	80	72.0	77.7		17.7	69.8	11.70	13.57	39.82	45.5	31.2	1308	1145	434
125	3		x=	14.0				50.5														
126	4	4.7	2	5.3	7+8	c	h	85.5	95	81.5	90.0	I	29	85.4	12.36	15.15	41.41	34.7	32.2	1198	913	382
127																						
128	3	3	2	21.0	7+8	e	b	77.5	75	80.0	81.6		23.3	74.2	13.02	14.99	39.63	29.7	38.6	1247	908	450
129																						
130	2		x=	4.8	7+8	c	h	93.0	83	86.0		S		89.3				44.0	39.4			
131																						
132	3		x=	16.3				75.0		70.5									30.9			

Appendix 1. Continue

DH No.	Septoria Mean 1995	Septoria Mean 1996 field	Rust Infection type	CCN cysts/plant	Protein *Glu B1*	Protein *Glu A3*	Protein *Glu B3*	Height Glass	Height Irrigated	Height Hill	Height 1996 field	GA3	Heading date Oct-96	Heading date Coleoptile	Leaf Colour Minulta a*	Leaf Colour Minulta b*	Leaf Colour Minulta L*	Boron tolerance Root Length	Yield Hill plot	Yield Full plot	Yield Stow 96	Yield Buckle boo
133																						
134	3	5.3	x=	0.4	7+9	c	h	87.0	90	75.0	90.1	I	23.7	71.8	12.04	14.30	40.02	60.5	37.8	1207	1025	394
135	4		x=	13.8	7+9	e	h	88.5		84.0					12.08	14.48	39.90		42.3			
136	4	6	2	17.8	7+9	c	h	100.5	123	98.5	94.9	S	20.4	84.1	14.60	18.89	41.52	79.1	35.3	1210	1041	442
137	5		x=	0.0				95.0		86.8									35.4			
138	2		x=	1.3				91.0		86.8									20.6			
139	4		3+	1.2				52.5		48.5									22.5			
140	5		x=	0.2	7+8	c	h	54.0		56.3									32.5			
141	3		x=	18.3	7+8	e	h	69.5		68.3									34.4			
142	5		x=	0.7				61.5														
143																						
144																						
145	2.5	5.3	2-	1.5	7+8	c	b	80.0	85	81.0	90.0	I	19.4	77.4	12.29	15.28	40.10		37.7	1246	1042	451
146																						
147	4.5		x=	20.0	7+8	e	b	65.0	65	57.8				67.9				33.9	27.8			
148	4.5		3+	16.5	7+9	e	h	71.0	85	65.5				56.7				37.1	11.5			
149	2		x=	1.0	7+9	c	h	94.5		87.3									39.5			
150	2	1.5	3-	1.7	7+8	c	b	79.5	83	79.8	89.9		29	73.9	11.95	14.10	39.74	50.7	35.9	1216	922	427
151	4		x=	0.9	7+9	c	b	55.0	53	43.5				60.4				75.6	20.8			
152																						
153																						
154	4	0.9	2	3.5	7+8	c	b	75.5	60		93.3		30.5	74.9	13.82	17.32	41.65	77.9		1171	873	427
155	2	5.8	3+	17.6	7+9	e	b	62.0	72		58.3		24.3	66.8	11.59	13.08	39.29	53.5		1238	871	432
156	5		x=	6.0	7+9	e	b	65.5														
157	2	5.2	3+	14.8	7+8	e	b	85.5	82		76.7	I	23.8	83.8	12.89	15.77	39.11	90.6		1272	894	424
158	3			12.8	7+9	e	b	60.5	65					62.0				31.4				
159	2	2.8	2	3.4	7+9	c	b	115.5	142		98.4	S	20.7	118.3	12.42	14.66	40.74	66.2		1191	862	371
160	3	2	x=	12.4	7+9	e	h	83.5	75		89.9	I	25.2	60.4	12.71	15.60	41.63	69.2		1014	803	397
161																						
162	4		3+	5.6				79.5														
163	5	3.5	3+	14.2	7+9	e	h	69.5		74.3	80.1		25.0		13.44	16.38	40.66	40.6	29.7	1350	843	424
164	4.5		x=	5.8	7+8	c	b	72.5														
165	5	5.6	2	2.9	7+8	e	b	56.0	67		58.8		26.7	62.9	12.04	14.10	39.19	50.4		1128	879	441
166			3+																			
167																						
168																						
169	2	4.1	3+	2.2	7+8	e	b	62.0	58		96.7		25.3	49.2	13.24	16.49	41.70	46.4		1204	900	439
170	3	6.3	3+	21.0	7+8	c	b	66.0	72		59.9		27.5	68.9	13.10	16.03	40.31	71.4		1149	830	448
171	3	3.7	x=	16.6	7+8	c	b	100.0	98		111.6	I	21.9	65.1	12.98	16.02	42.45	68.6		1221	838	404
172	3	4.8	x-	12.7	7+9	e	h	66.0	67		57.5		24.6	57.9	11.58	14.07	40.31	52.1		1285	806	432
173	5	7.2	2	2.3	7+8	c	b	79.5	75		99.9		20.5	74.9	13.54	16.72	41.89	74.2		1143	1006	452
174																						
175	3		x=	4.3	7+8	e	b	68.0	77					61.0				28.9				
176	3	2.8	x=	4.5	7+9	c	h	88.0	95		111.6	S	22.1	76.9	12.74	15.33	40.38	36.7		1073	709	383

Appendix 1. Continue

	Septoria Mean		Rust	CCN	Protein			Height					Heading date		Leaf Colour Minulta			Boron tolerance	Yield			
DH No.	1995	1996 field	Infection type	cysts/plant	*Glu B1*	*Glu A3*	*Glu B3*	Glass	Irrigated	Hill	1996 field	GA3	Oct-96	Coleoptile	a*	b*	L*	Root Length	Hill plot	Full plot	Stow 96	Buckle boo
177																						
178	4.5	4.7	x=	12.4	7+9	e	h	68.5	65		78.2		24.2	48.9	13.57	15.49	40.39	32.1		1183	994	443
179																						
180																						
181	5		3+	21.0				54.5														
182	3		2	6.9	7+9	e	b	71.5														
183	2	2.5	2	2.2	7+8	e	b	97.0	110		114.9	S	27	86.2	15.19	19.74	43.54	61.6		1019	748	343
184	3		3+	4.9	7+8	e	b	88.5	95			I?		73.2				77.0				
185	5	5.7	x=	10.7	7+9	c	h	65.0	70		78.5		20.9	50.0	12.28	13.43	39.10	86.4		1215	951	406
186	2		3+	4.5	7+8	c	b	60.0										23.2				
187	4		2-	5.7	7+8	c	b	67.0	65					46.8				38.4				
188																						
189	5	7.2	x=	1.2	7+9	e	h	113.0	113		109.9	S	17.9	96.4	12.08	14.63	41.00	98.2		1091	889	383
190	3		2-	7.8	7+9	e	h	81.5														
191	3	1.1	x=	5.1	7+9	e	h	97.0	108		106.0	S	25.4	86.3	12.75	15.41	41.99	37.2		1137	763	390
192	3	1	2-	15.8	7+9	c	b	90.0	102		116.7	S	27	81.8	14.76	19.50	43.51	47.2		1042	606	395
193	5	6.4	x=	12.1	7+8	c	h	72.0	86		80.1		19.3	61.8	12.10	14.42	40.13	65.0		1208	964	449
194	3	7.4	2	18.3	7+9	c	h	105.0	118		100.0	S	26.2	93.3	13.08	15.80	39.51	38.5		1090	797	383
195	4	4.3	x=	0.0	7+8	e	b	72.5	92		87.6		21.9	61.4	11.34	12.88	40.26	26.0		1062	891	403
196	5	4.2	3+	4.5	7+8	e	h	68.0	75		66.7		22.7	52.0	13.48	16.45	41.63	93.5		1060	833	311
197	4	3.4	x=	1.7	7+9	c	b	84.0	111		108.4	S	23.5	79.0	13.95	17.89	42.04	91.7		1054	756	395
198	4.5	2.1	3+	14.3	7+9	e	h	72.0	80		71.7		28.6	72.4	13.52	16.98	40.85	64.9		1133	699	406
199	5		x=	5.9	7+8	c	b	70.0														
200	5		3-	2.7	7+9	e	b	68.5	85					74.1				44.1				
201	5	5.2	x=	5.1	7+9	e	h	110.0	120		113.4	S	23.5	94.8	13.20	16.53	42.63	64.2		1013	809	365
202	5		x=	0.6	7+8	e	h	86.0	86			I		83.1				71.4				
203	2.5		x=	0.4	7+8	e	b	122.0	141			S		108.9				31.9				
204	5		2	4.0	7+9	c	b	65.5	76					62.9				36.1				
205	5		x=	0.2	7+9	c	b	59.5														
206	5		3+	4.2				72.5														
207	5	5	x=	7.7	7+9	e	b	74.0	85		86.8		26.7	65.4	13.23	15.84	41.68	61.6		1172	738	374
208	5		3+	2.4	7+9	e	h	93.0	98			I		85.1				79.6				
209	4		3+	9.8	7+9	c	b	115.5	146			S		116.9				63.9				
210	2.5		2	14.9	7+8	e	b	60.5	55					53.4				76.1				
211	3.5	3.3	x=	14.4	7+9	e	b	74.0	85		86.1		22.8	78.6	12.47	14.57	40.66	92.4		1306	980	423
212	2		3+	1.9				90.0														
213	3.5		x=	2.4				121.0														
214	4.5	5.2	x=	4.0	7+8	c	b	74.0	85		98.3		22.4	66.4	12.98	15.81	40.81	76.9		1289	1012	436
215	4		3+	16.0				50.0														
216	5	7.2	3-	17.9	7+9	c	h	61.5	71		78.4		22.9	53.6	13.37	16.39	39.20	69.0		1353	832	495
217	5		2-	18.5				96.5														
218	5		x-	19.0				52.0														
219	3.5		2-	3.3	7+9	e	h	125.0														
220	5	7	3+	5.4	7+8	e	b	78.5	72		83.4		25.1	62.6	13.11	16.13	40.95	23.0		956	883	415

Appendix 1. Continue

	Septoria Mean		Rust	CCN	Protein			Height					Heading date		Leaf Colour Minulta			Boron tolerance	Yield			
DH No.	1995	1996 field	Infection type	cysts/plant	*Glu B1*	*Glu A3*	*Glu B3*	Glass	Irrigated	Hill	1996 field	GA3	Oct-96	Coleoptile	a*	b*	L*	Root Length	Hill plot	Full plot	Stow 96	Buckle boo
221	5	4.7	2	2.2	7+8	e	h	84.0	87		81.7	I	20.6	62.6	11.69	13.99	40.04	38.7		1164	982	437
222	5		3+	4.1	7+8	e	h	114.0	117					93.2				104.1				
223	5		2	7.3	?	c	b	49.0	56					50.8				72.6				
224	5		x=	10.8	7+8	e	b	59.0		94.3												
225	5		3+	3.8	7+8	e	h	59.5														
226	5	1.7	3+	3.2	7+9	e	h	68.5	70		62.7		25.3	56.2	12.32	13.57	38.36	26.3		1136	830	351
227	5		x=	12.0	7+9	e	b	57.5	53					54.1				62.7				
228	3.5	4.1	3+	3.1	7+9	e	b	80.5	72		83.3	I	21.4	57.4	13.80	17.49	41.08	76.9		1277	819	438
229	4.5	3.1	3+	7.5	7+9	c	h	95.0	98		78.4	I	24.3	67.6	12.59	14.85	40.88	64.6		1157	882	403
230	3		3+	4.2	7+8	c	b	73.0	84					72.6				36.1				
231	4	3.7	x-	17.3	7+9	c	h	99.5	112		103.5	S	24.7	89.8	12.67	15.45	41.73	37.5		1032	828	419
232	4.5		x=	13.9	7+9	e	b	80.5	82			I		71.0				41.7				
233	3.5		2	13.5	7+9	e	b	51.0														
234																						
235	5		x=	2.8	7+9	e	b	46.0	53					50.8				36.6				
236	3		x=	0.7	7+8	e	h	128.5														
237	3.5		x=	0.7	7+8	e	h	81.0	86			I		62.6				41.6				
238	4.5		x=	0.2	7+8	e	b	78.5										73.7				
239	3		x=	2.4	7+8	e	b	78.5	82					69.5				49.7				
240	4.5		x=	0.7	7+8	e	b	96.5	119			S		95.2								
241	4		x=					93.5	90			I		71.3				38.6				
242																						
243	4.5		3+	16.3				61.5														
244	5		x=	2.7				44.5	75					66.5				49.6				
245																						
246			x=																			
247																						
248			x=																			
249																						
250	3		2	5.1	7+9	c	b	75.5	83					65.9				38.7				
251					7+8	c	b															
252	4			1.6	7+9	e	h	62.5	85					79.4				41.0				
253	5			11.1	7+9	e,c	h,b	93.0														
254	5							79.0														
255	4			0.4				75.0														
		T 5.5 M 2.6	T X- M 3	T 12.8 T 18.2							T 89.6 M 92.9	T I M I	T 21.8 M 24.1	Ox 48.9 Hbd 113.2	T 11.51 M 13.99	T 13.35 M 17.63	T 39.73 M 41.45	T 86.0 M 33.3		T 1260 M 1218	T 1099 M 811	
		lsd 3.9		M 6.2							lsd 10.3		lsd 2.3		lsd 1.47	lsd 2.71	lsd 1.95	Ox 35.9		lsd 56	lsd 113	
				M 6.3														Hd 87.3				

Appendix 2. REML program in GENSTAT 5 to do separate analysis of characters of DH and F_2 lines within each individual cross.

```
Job 'dhf5'
open 'dhf5.out';cha=3;wid=132;file=output
open 'cross.dat';cha=2;wid=132;file=input
copy[pri=o] 3
unit [140]
factor [levels=2] method
&      [levels=40] geno,geno1,geno2
&      [levels=4] rep
read[cha=2] rep,cross,geno,method,DtoHead,height,culmweight,spike,\
Yperhil,Grnweight,HI,Yperhead,GrnNhead
"Create separate genotype factors for the two methods"
scalar mv
restrict geno ; condition=method.eq.2 ; saveset = g1
restrict geno
restrict geno ; condition=method.eq.1 ; saveset = g2
restrict geno
calc geno1=geno
&    geno2=geno
calc elem(geno1;g1) = mv
calc elem(geno2;g2) = mv
"print rep,method,geno,geno1,geno2,DtoHead,height,culmweight,spike,\
Yperhil,Grnweight,HI,Yperhead,GrnNhead; Fieldwidth=7
""Fit model with different genetic variances for each method.Print out method
means and genotype effects (adjusted for method)."
For x=DtoHead,Height,culm weight,spike,Yperhil,Grnweight\
,HI,Yperhead,GrnNhead
vcomponents [fixed=rep+method] random=geno1+geno2
reml [pterms=method;              \
mvinclude=yes;prin=model,components,means,dev,wald]x
vdisplay[pterms=geno1+geno2;print=effects]
"Fit model with the same genetic variance for both methods.
Print out method means and genotype effects (adjusted for method)."
matrix [rows=3;columns=3;values=0,1,0,0,1,0,0,0,1] gmat
vcomponents [fixed=rep+method;relationship=gmat] random=geno1+geno2;\
constraints=positive
reml [pterms=method;     \
mvinclude=yes;prin=model,components,means,dev,wald]x
vdisplay[pterms=geno1+geno2;print=effects]
endfor
stop
```

2.7. Doubled Haploids as a tool in Barley Breeding

Doubled haploids have great potential in cereal breeding programs. Their use is new in wheat breeding but they have been used in barley breeding for some years. Similar experiences to those which have occurred with barley can be expected in wheat improvement, hence it is important to examine these experiences.

Some workers found that the 'bulbosum' method was more efficient for haploid production (Huang *et al.* , 1984) and others had greater success with anther culture (Friedt *et al.*, 1984). Regardless of the method it is concluded that both methods could be effectively applied to the breeding of new barley varieties. Because of the very rapid production of populations of homozygous lines, the technique compares favourably with single seed descent and bulk breeding methods which are employed for the same end. (Park *et al.*, 1976: Choo *et al.*, 1985).

Doubled haploid lines have shown similar phenotypic stability to conventionally bred control cultivars over a range of environments (Reinbergs *et al.*, 1978). Friedt and Foroughi-Wehr (1983) obtained no differences in mean yield between populations of doubled haploids and their mid-parent values and conventionally produced populations, but there was a shift in the frequency distribution towards lower yields in the doubled haploids. In another study identical means, variances and frequency distributions were obtained for yield from 'bulbosum' derived and anther culture derived doubled haploids (Friedt *et al.*, 1984). In all comparisons it has been possible to identify doubled haploid lines equal in performance to those bred and selected by conventional means (Rossnagel *et al.*, 1987).

Although doubled haploid production from small numbers of F_1 hybrids offers great savings in time over conventional breeding programs, to increase efficiency (frequency of desirable genotypes) and to break repulsion linkages it may be better to make haploids from selected F_2 or F_3 plants (Simpson and Snape, 1981). However to date most doubled haploid barleys released have been produced using the 'bulbosum' technique on F_1 hybrids or selected F_1s from a first backcross, BC_1F_1. This may just reflect the usual practice in breeding programs of using F_1s as starting material (Foroughi-Wehr *et al.*, 1982: Foroughi-Wehr and Friedt,

1984: Kao, 1988: Chen and Hays, 1989: Devaux, 1989). A new variety, Tantangara, was released in Australia from an anther culture doubled haploid by Ballantyne and Smithard (1995). It has considerable yield advantages over older but conventionally bred varieties.

Plant breeders need to predict the potential of crosses in their breeding programs at the earliest opportunity and to discard inferior combinations. As few as 20 doubled haploids produced from early generations are required for this and after their assessment in hill plots more doubled haploids could be produced from superior combinations (Reinbergs *et al.*, 1976). Bjornstad and Aastveit (1990), however, point out that larger numbers of doubled haploids would be needed to give a proper estimate in crosses where there are repulsion linkages and/or negative pleoitropic effects on a character.

Doubled haploids are very valuable for detecting and measuring genetic linkages associated with quantitative characters. Pleiotropic effects of major genes on quantitative characters can be studied by separating a population of doubled haploids, produced from heterozygous F_1s, into two groups according to which allele is present. Any significant differences between the two groups for quantitative attributes can be considered to be due to a pleiotropic effect of the major gene.

Many genes governing morphological characters and isozymes have been mapped on the barley genome (Tsuchiya, 1986), and multiple marker stocks have been produced for assigning genes to particular chromosomes (Franckowiak, 1987). The location of polygenes controlling quantitative characters such as yield has not been easy but can be aided by extracting doubled haploids from crosses between parents with contrasting marker genes (Choo, 1983). There is increasing interest in constructing a genome map based on molecular markers. Doubled haploids are proving an invaluable aid in this form of mapping.

In addition to their use in linkage studies haploids have beed used in breeding for resistance to barley yellow mosaic virus (Foroughi-Wehr and Friedt, 1984). In vitro screening of haploid embryos or microspore callus produced from F_1s derived from crosses between resistant and susceptible lines was not good. However, diploid embryos have proved to be suitable explants for selecting biochemical mutants (Bright *et al.*, 1979).

Can the rate of production of doubled haploids from wheat reach the level that it has in barley and then do the same phenomena experienced with barley which have led to the widespread adoption of doubled haploids as a breeding tool exist for wheat? These are the subjects of this study.

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