Genetic Analysis of Tissue Culture Responses in Barley (*Hordeum vulgare* L.)

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In Dedication

To Mr. Khalid Abdul-Hadi Tahir, who gave not to be praised for his generosity but in the true spirit of "not letting his left hand know what his right hand gave". It was only after his passing that his anonymous sponsorship of my study fees was revealed. My opportunity to further study would not have been possible without his generosity. I offer my deepest thanks and gratitude to his family.

TABLE OF CONTENTS

Abstract

The overall objectives of the work described in this thesis were:

- To screen barley genotypes for *in vitro* culture response and
- To use the doubled haploid (DH) lines derived from the cross between responsive and unresponsive parental lines to confirm the location of previously reported QTL that control barley tissue culture response and search for additional QTL in a different DH population.

In testing the responsiveness of barley genotypes in culture, twenty barley varieties were grown in a glasshouse, and three plants of each variety were sown in 8-inch pots. About 10 to 14 days after the plants reached anthesis, 50 immature grains were harvested and 50 embryos were isolated from them. These embryos were placed in Petri dishes (10 embryos/ Petri dish), which contained solidified MS medium, and incubated in the dark at 24 ℃ for three weeks. Individual embryos were scored for somatic embryo production after this incubation period. The Petri dishes were exposed to the light for a further week after which time the callus cultures were scored for shoot production. This experiment demonstrated a good response for the variety Chebec and a poor response for Harrington. Therefore, the Chebec x Harrington DH population was used to seek Quantitative Trait Loci (QTL) that affect shoot regeneration in barley callus cultures.

The Chebec x Harrington DH lines were grown in a controlled environment growth room. The 115 lines were divided into two separate experiments, and embryo isolation and culture were performed. A log-likelihood (LOD) score threshold of 3 was used to identify regions containing putative loci associated with callus growth

and shoot regeneration. However, this experiment revealed no significant QTL as the generated LOD scores were less than 3.

Since no significant QTL were detected, replicate experiments were performed to demonstrate differences in the shoot regeneration capacity of the parent lines, Chebec and Harrington, and 10 other DH lines. This experiment was designed in randomised blocks. Five blocks of 12 pots (each pot contained a single plant of the twelve different lines) were grown in a growth room. Twenty immature grains were harvested from each line, and embryo isolation and culture response were performed. Statistical analyses of these data revealed no significant differences in the tissue culture performance of Chebec and Harrington, with respect to the average number of somatic embryos and regenerated shoots. Interestingly, three DH lines (128, 48, and 25) demonstrated tissue culture responses significantly better or worse than Chebec or Harrington. These outcomes may be explained by transgressive segregation of the genes responsible for somatic embryo production and shoot regeneration in Chebec and Harrington.

In conclusion, the initial screening experiment of the 20 barley cultivars demonstrated that Chebec responded well in tissue culture compared with Harrington, which responded poorly. When no statistically significant QTL were detected in the DH population produced by the Chebec x Harrington cross, replicate experiments were performed, and demonstrated that there was no significant difference between the parental lines in terms of tissue culture response. These results may be explained by changes in the growth conditions used for donor plants or alterations to the experimental design.

STATEMENT OF AUTHORSHIP

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

I give consent for this thesis being made available for photocopying and loan.

Marei Salem Al-Nahdi

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
$C1_3$ POP	2,4,5-trichlorophenoxypropionic acid
PAA	phenylacetic acid
Dicamba	3,6-dichloroanisic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
SSR	Simple Sequence Repeat
RFLP	Restriction Fragment Length Polymorphism
AFLP	Amplified Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
QTL	Quantitative Trait Loci
DH	Doubled Haploid

List of Publications

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Chapter One

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

Emerging technologies for the genetic manipulation of crop quality and productivity often include tissue culture as an integral component of the procedure. Insertion of foreign or modified genes into commercially-important cereal crops by protoplast transformation, microprojectile bombardment or *Agrobacterium tumefaciens* mediated transformation, involves the growth of the transformed material in tissue culture before the regeneration of fertile, transgenic plants. The overall objective of the work described in this thesis was to compare the performance of a range of barley varieties during tissue culture and regeneration with a view to select potentially useful varieties for transformation and the identification of genetic locations that determine tissue culture performance in barley.

1.1 Background

Barley (*Hordeum vulgare* L.) belongs to the tribe Triticeae in the grass family, Poaceae, which is the largest family of monocotyledonous plants. It was known to exist at least 17,000 years ago in the Nile River valley of Egypt (Wendorf *et al.*, 1979) and can be divided into two-rowed and six-rowed types, according to spike morphology. In two-rowed barley, the lateral spikelets are female sterile, while in six-rowed barley all spikelets are fertile (Briggs, 1978).

Barley is one of the world's five major cereal crop species, which also include wheat, rice, oat and maize. In developed countries, barley is mainly used to prepare malt and to feed farm animals but in developing countries large quantities of barley are also used for human nutrition (Manninen, 2000). Statistics show that the principal producers of barley are Russia, the European Union, and Canada (World Barley Production, 2004) with world barley production in 2004 of approximately 155 million tonnes. Australia's contribution to world production in 2004 is about 8 million

tonnes, with production chiefly confined to the southeastern states of the country (World Barley Production, 2004).

To date, the main strategies for improving barley production have relied on increasing yield through conventional breeding methods and through the expansion of cultivated areas. However, the implementation of these strategies faces a number of limitations that are imposed by biological, environmental and practical problems (Fry *et al.*, 1998), such as climate hazards (drought, floods), farm management issues (salinity) and urban growth (Sharma *et al.*, 2002).

Doubled haploid (DH) production is one of a series of technological innovations which, along with molecular markers and genetic transformation, has become an important tool in plant breeding programs leading to an improved rate of genetic gain. DH production is becoming increasingly important as a mainstream component of conventional barley breeding programs.

1.2 Production of DH in barley

The term haploid is used to describe a cell that carries half (n) the normal diploid chromosome number (2n). DH production is important in plant breeding as it serves to generate true breeding, homozygous lines in a single generation. This procedure has the advantage of accelerating cultivar development by eliminating the 10 to 12 generations required to produce homozygous lines following segregation in conventional breeding techniques (Kasha and Maluszynski, 2003).

Barley DHs were originally generated by chromosome elimination in an interspecific cross between *Hordeum vulgare* and *Hordeum bulbosum* (Kasha and Kao, 1970). More recently, DH production has been achieved by the use of anther and isolated microspore culture techniques. Chemical treatment of the haploid plants recovered from the *H. bulbosum* method using colchicine is required to duplicate the

haploid genome and produce DHs. Alternatively, the genomes of approximately 70% of the plants derived from anther culture or isolated microspore cultures are spontaneously doubled, and chemical treatment is unnecessary (Davies, 2003).

1.3 Barley tissue culture

Tissue culture is a general term used to describe the growth of cells, tissues or organs in a nutrient medium under aseptic conditions. The first plant tissue culture methods were developed in the early 1900s (Raghavan, 1976). Today, tissue culture techniques are used for micropropagation, for the production of doubled haploids through anther or microspore culture, *in vitro* selection, embryo rescue, somaclonal variation, somatic hybridisation and genetic transformation.

Tissue culture methods have become important tools for plant improvement, particularly through the development of DH techniques. These techniques also provide systems that allow the investigation of physiological, biochemical, genetic and structural mechanisms relating to plants (Lazzeri and Shewry, 1993). Generally, tissue culture techniques used for plant improvement require a capacity of the tissue-cultured cells to regenerate fertile plants. Regeneration response *in vitro* is influenced by numerous factors, including explant type, plant growth regulators and plant genotype. These factors are discussed below.

1.3.1 Explant sources

The production of embryogenic callus is strongly influenced by the type of explant used to initiate the culture. The term "callus" refers to the proliferation of de-differentiated cells, which arise from cultured explants, and embryogenic callus has the capacity to form an embryo that can develop into an intact, fertile plant under appropriate conditions (Dodds and Roberts, 1995).

A variety of barley explants has been used to produce embryogenic callus capable of plant regeneration. These include the shoot meristem (Cheng and Smith, 1975; Weigel and Hughes, 1985), the mature embryo (Lupotto, 1984), the immature ovary (Orten, 1979), the immature inflorescence (Thomas and Scott, 1985), and the seedling mesocotyl (Jelaska *et al.*, 1984; Rengel and Jelaska, 1986). In general, however, the immature embryo has been the most widely used explant type for the production of embryogenic callus in barley. This was demonstrated in early reports where highly regenerable callus could be easily produced from the immature embryo (Kasha and Kao, 1970; Dale and Deambrogio, 1979). More recently, the immature embryo of different barley cultivars has been employed to generate embryogenic callus suitable for the regeneration of fertile plants (Lührs and Lörz, 1987; Satoh *et al.*, 1991; Dahleen, 1999; Barro *et al.*, 1999).

1.3.2 Plant growth regulators

Growth regulators also have a strong effect on tissue culture response. The most frequently used growth regulator in plant tissue culture is the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) (Basri, 2000, Slater *et al.*, 2003). In the case of barley, there have been numerous reports of the use of this auxin for the production of embryogenic callus cultures (Lührs and Lörz, 1987; Koprek *et al.*, 1996; Barro *et al.*, 1999; Dahleen, 1995 & 1999; Chang *et al.*, 2003). Other studies have combined 2,4-D with other auxins such as Cl_3 POP (2,4,5-trichlorophenoxypropionic acid) (Goldstein *et al.*, 1986), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), (Baillie *et al.*, 1993) and PAA (phenylacetic acid), (Bregitzer *et al.*, 1995) to establish embryogenic callus cultures of barley. Furthermore, some barley cultivars require cytokinins in combination with synthetic auxins like 2,4-D (Kachhwaha *et al.*, 1997) and 3,6 dichloro-o-anisic acid (Dicamba) (Castillo *et al.*, 1998) for callus production. For previously untested barley

cultivars, careful optimisation of plant growth regulator requirements is needed to achieve plant regeneration from embryogenic callus cultures.

1.3.3 Plant genotypes and growth conditions for donor plants

The frequencies of callus induction and growth and plant regeneration are strongly dependent on genotype and are general features of tissue culture for a diverse range of cereal species, including wheat (Maddock *et al.*, 1983, Mathias and Simpson, 1986; Fennell *et al.*, 1996, Basri , 2000; Przetakiewicz *et al.*, 2003), maize (Rhodes *et al.*, 1986, Songsted *et al.*, 1992; Przetakiewicz *et al.*, 2003), rice (Christou *et al.*, 1991, Rout and Lucas, 1996) and barley (Walmsley *et al.*, 1995, Ruiz *et al.*, 1992, Bregitzer *et al.*, 1998; Dahleen and Bregitzer, 2002, Chang *et al.*, 2003). The effect of barley genotype on tissue culture response has been demonstrated in several previous studies. In a study conducted by Hanzel *et al.* (1985), the frequency of plant regeneration was generally low for the 91 barley cultivars examined. Seventy percent of the regenerated plants were recovered from the callus cultures of two genotypes, Akka and X2387-3. Lührs and Lörz (1987) identified two barley cultivars, Golden Promise and Dissa, which demonstrated superior tissue culture response compared with the 41 tested lines. Baillie *et al.* (1993) studied the tissue culture response of ten Canadian barley cultivars and regenerated the highest number of plants from the immature embryo-derived callus of cultivar Abee. Bregitzer (1992) compared 15 genotypes of barley and demonstrated that high frequencies of embryogenic callus were produced from the immature embryos of Golden Promise, Hector, Piroline and Klages.

Walmsley *et al.* (1995) screened eight Australian barley cultivars for the initiation of embryogenic callus and plant regeneration. The immature embryos of

two cultivars, Clipper and Bandulla, demonstrated rates of plant regeneration similar to the tissue culture-responsive cultivar, Golden Promise.

In addition, it has been demonstrated that *in vitro* culture response for barley explants is strongly influenced by the conditions used for the growth of donor plants (Hanzel *et al.,* 1985; Goldstein and Kronstad, 1986). The time of planting can significantly affect the regeneration response from greenhouse-grown donor plants. Dahleen (1999) observed a low frequency of plant regeneration in two barley cultivars, Morex and Golden Promise, grown in the greenhouse during summer. The high temperature and the long day length affected embryo development, and these factors contributed to the poor quality of the callus produced by the cultured immature embryos. Conversely, the callus produced by immature embryos isolated from grains harvested from donor plants maintained in growth chambers, (20ºC during the day and 16ºC at night with 16 hours of light) resulted in the highest frequency of plant regeneration. Studies have shown that plant regeneration from cultured barley cells is controlled by a number of genes (Komatsuda *et al.*, 1993; Mano *et al.*, 1996; Mano and Komatsuda, 2002). Molecular marker technology has the capacity to identify loci associated with tissue culture response. In the section below, the various molecular marker techniques currently utilised are described.

1.4 Molecular markers

Molecular markers are DNA sequences that can be used to track loci of interest in segregating populations (Liu, 1998). For example, a marker genetically linked to a disease resistance locus can be used to predict the presence of the resistance or the susceptibility allele. The reliability of the prediction increases when marker DNA sequences are closer to the gene that codes for the trait of interest.

Commonly used molecular markers include restriction fragment length polymorphisms (RFLP), microsatellites or simple sequence repeats (SSR), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) (Karakousis *et al.,* 2003c; Langridge *et al.*, 2001).

1.4.1 Restriction fragment length polymorphisms (RFLP)

RFLP analysis is based on the generation of DNA fragments formed by the cleavage of DNA at a particular site by restriction enzymes. The fragments released by the restriction enzymes are subjected to gel electrophoresis, transferred to a nylon membrane and hybridised with a labelled DNA probe (Botstein *et al.*, 1980; Tanksley *et al.*, 1989).

RFLP occur when a DNA probe hybridises with DNA fragments of differing size in DNA from different plants digested with the same restriction enzyme. The different DNA fragments arise through random changes in the DNA sequence of individuals, and RFLP loci are generally inherited in a simple Mendelian fashion. Consequently, the restriction fragments can be used to track genetic loci in a manner analogous to the use of morphological and biochemical markers in plant breeding (Yang, 1999).

Tanksley *et al.* (1989) and Stuber *et al.* (1992) summarised the relative advantages of RFLP markers over morphological markers for most genetic and breeding applications as follows:

1) Abundance: the number of DNA probes used to construct linkage maps is almost unlimited, and several naturally occurring alleles are available at most RFLP marker loci. Thus, natural variation in existing populations can be used without the need to construct special genetic stocks.

2) Neutral phenotype: RFLP markers have no known negative effect on plant phenotype whereas morphological markers frequently cause major alterations in the phenotypes, which may be undesirable.

3) Codominance: alleles at most RFLP loci behave in a codominant manner whereas dominant-recessive interactions frequently hinder identification of all genotypes associated with morphological traits.

4) Epistasis: there are no unfavourable epistatic interactions.

5) Stability: the environment or the specificity of tissues does not influence RFLP markers.

To date, RFLP markers have been used for the construction of genetic maps, QTL mapping and comparative mapping (Williams *et al.*, 1994; Hackett and Weller, 1995; Nelson *et al.*, 1995a). As there are several hundred commercially available restriction enzymes, the number of RFLPs that can be used as markers very high. The main advantage of this system is that it overcomes the limited availability of morphological and biochemical markers that demonstrate measurable polymorphism (Laurie *et al.*, 1992). In addition, RFLP analyses are highly reproducible and with an inherently high level of co-dominance, heterozygotes can be distinguished from either homozygote, therefore providing the maximum amount of genetic information in segregating populations. Furthermore, they are readily transferred between different crosses and different species, since hybridisation will still occur between imperfectly matched DNA sequences. However, the disadvantages of RFLP are that the methodology is time consuming and analysis often produces limited polymorphisms for mapping.

1.4.2 Polymerase chain reaction (PCR) based methods

The polymerase chain reaction (PCR), first described in 1985 by Kary Mullis, is a DNA synthesis technique that amplifies specific regions of DNA that lie between two sites defined by the complementary sequences of two specific primers (Langridge *et al.*, 2001).

The principle of PCR is the two primers flank a target DNA sequence to be amplified. The primers are short single stranded DNA molecules that anneal to the denatured DNA strands at sites with complementary sequences. For the first cycle of amplification, two DNA strands extend from each of the primers along the original template without defined termination sites. In cycle 2, the primers initiate synthesis on the products of the first cycle and two new strands are synthesized. The second strand must terminate at the original primer sites, where the new template ends. Further amplification is therefore restricted to the sequence between sites defined by the primers. As further cycles proceed, the number of DNA fragments increases exponentially. At the end of 30 to 50 cycles, 2^{30} to 2^{40} copies of DNA molecules having sequences identical to the target segment are produced (Snustad *et al.*,1997; Glick and Pasternak, 2003).

PCR is the basis of many marker systems used for constructing molecular linkage maps. These include AFLP, SSR, Single Nucleotide Polymorphisms (SNP) and RAPD moleculare markers (Pejic *et al.*, 1998; Jones *et al.*, 2002; Rafalski, 2002).

1.4.2.1 Randomly Amplified Polymorphic DNA (RAPD)

This method was discovered by Williams *et al.* (1990) and relies on short DNA primers of arbitrary sequence to amplify a large number of random genomic sequences. This type of molecular marker is simple and inexpensive, as no prior knowledge of genomic DNA sequences is required, and there is no requirement for

radioactively labelled probes. This method can be used for genetic mapping, but because of the short primer length and the random nature of DNA fragment generation, they cannot be easily transferred between species (Jones *et al.*, 1997). The other disadvantages of this method are its poor reliability and reproducibility, and its sensitivity to experimental conditions (Karp *et al.*, 1996).

1.4.2.2 Microsatellites or Simple Sequence Repeats (SSR)

Microsatellite markers or Simple Sequence Repeats (SSR) are sequences of repeated nucleotides 1-6 base pairs in length (Langridge *et al.*, 2001, Gupta *et al.*, 2002). SSRs consist of di-nuceotide (AC) n, (AG) n, (AT) n or tri-nucleotide (TCT) n, (TTG) n or tetra-nucleotide repeating units within a SSR (Langridge *et al.*, 2001, Gupta *et al.*, 1999).

There are several advantages of using an SSR molecular marker system (Powell *et al.*, 1996; Langridge *et al.*, 2001; Liu *et al.*, 1996). SSR are abundant in the plant genome, and this robust marker assay system requires a small amount of DNA (compared with RFLPs), is PCR based and amenable to high throughput analysis. The outcomes are mainly co-dominantly inherited, multi-allelic, information rich and transferable between populations.

SSR markers have been used in all major cereal crops including barley (Liu *et al.*, 1996, Hamza *et al., 2004*), wheat (Röder *et al.*, 1998; Stephenson *et al.*, 1998) and maize (Smith *et al.*, 1997; Pejic *et al., 1998*). Several hundred SSR markers have been developed for barley (Ramsay *et al.*, 2000; Struss and Plieske, 1998; Ablett *et al.*, 2003; Karakousis *et al.*, 2003c).

Two hundred and thirty SSRs have been placed on eight Australian mapping populations (Karakousis *et al.*, 2003d), including Alexis x Sloop (Barr *et al.*, 2003a),

Chebec x Harrington (Barr *et al.*, 2003b), Clipper x Sahara (Karakousis *et al.*, 2003b), and Galleon x Harura Nijo (Karakousis *et al.*, 2003a).

1.4.2.3 Amplified Fragment Length Polymorphisms (AFLP)

The AFLP methodology is one of the most powerful tools in molecular marker technology as it provides a reliable technique for the generation of DNA polymorphisms in the plant genome (Mackill *et al.*, 1996; Janssen *et al.*, 1996; Hongtrakul *et al.*, 1997). The technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for all types of DNA, irrespective of its origin or complexity. The DNA is cut with restriction enzymes, after which double stranded adaptors are ligated to the ends of the fragments to generate templates for DNA amplification. The sequences of the adaptors and the adjacent restriction sites serve as primer binding sites for amplification of the restriction fragments. Thomas *et al.* (1995) found that AFLP has the capacity to identify greater numbers of loci compared with the RAPD marker system.

AFLP are well characterised in cereals (Qi and Lindhout, 1997; Powell *et al.*, 1997; Qi *et al.*, 1998) and are used with RFLP markers to create linkage maps (Becker *et al.*, 1995). In barley, the diversity index using AFLP is three times higher than RFLP (Russell *et al.*, 1997), which means that AFLP provide more polymorphism information than RFLP. AFLP markers are used together with RFLP to better saturate the regions between RFLP markers. Becker *et al.* (1995) mapped 118 AFLP markers to an existing barley RFLP map, which mapped to all the barley chromosomes and filled the gaps on chromosomes 2H, 4H and 6H which had not been extensively covered with RFLP markers.

1.4.2.4 Single Nucleotide Polymorphisms (SNP) markers

SNP markers in human genetics are at the forefront of investigating gene linkages to specific disease conditions (Weiss, 1998), but have had relatively little application in plants. In plants, SNPs are likely to be a useful source of polymorphisms for utilisation in the future (Langridge *et al.*, 2001).

Technically, SNPs are PCR-based and identify single base-pair changes between specific target sequences to expose the polymorphism. Estimates establish that the human genome contains 1 SNP every 1058 base pairs (Durrett and Limic, 2001). The advantages of SNPs are that specific genes can be targeted from Expressed Sequence Tag (EST) databases and SNPs can be automated for high throughput (Langridge *et al.*, 2001).

High gene order and gene sequence similarities in barley and rice make it possible to use rice sequences to design SNPs for barley (Langridge *et al.*, 2001). Synteny with rice allows targeting of gaps in genetic maps by the use of the rice genome sequence or the isolation of markers for multiple genes from specific regions of the genomes.

Technologies available for detection and screening of known SNPs in populations are:

1) Taqman (Morin *et al.*, 1999)

2) Molecular beacons (Marras *et al.*, 1999)

3) Fluorescent ddNTPs (Dye-tagged dideoxynucleoside triphosphate terminators) (Fortina *et al.*, 2000)

4) Restriction enzyme polymorphisms (Neff *et al.*, 1998)

5) MALDI-TOF (Matrix-Assisted Laser Desorption-Ionization-Time-Of-Flight) mass spectroscopy (Griffin and Smith, 2000)

6) Microarray hybridisation (Hirschorn *et al.*, 2000)

7) DHPLC (Denaturing High Performance Liquid Chromatography) (Gross *et al.*, 1999)

8) SNAP (Single-Nucleotide Amplified Polymorphisms) (Drenkard *et al.*, 2000)

There is much hope in the investigation of SNPs in plants using the molecular techniques listed, which has the potential to produce rapid analysis. Such research probes will further future developments in diagnostics and nucleic acid research (Langridge *et al.*, 2001; Rafalski, 2002).

1.5 Genetic Map Construction

Jones *et al.* (1997) defined the mapping of genes as "putting markers in order, indicating the relative genetic distances between them, and assigning them to their linkage groups on the basis of the recombination values from all their pairwise combinations." The steps involved in genetic map construction are as follows (Langridge *et al.*, 2003):

1) Identify parents that differ for the trait of interest.

2) Produce a segregating population of plants. Mapping populations of fixed lines (DH or Single Seed Descent) or segregating lines (F2 mapping) may be used.

3) Screen the population for the trait of interest, such as disease resistance, malting quality, or grain size.

4) Construct the linkage map of the population using molecular marker data techniques such as AFLP, RFLP or SSR. The steps involved in map constructing are:

a) Identify markers that are polymorphic between the parents, using techniques such as RFLP, and PCR based markers such as SSR.

b) Screen the population with the polymorphic markers and score the alleles.

c) Carry out linkage analysis using appropriate software such as Map Maker and Map Manager.

d) Place the markers into linkage groups and arrange into chromosome order.

A number of genetic maps have been constructed, including those of importance to Australian agricultural research. The major Australian mapping populations are: Alexis x Sloop (Barr *et al.*, 2003a), Chebec x Harrington (Barr *et al.*, 2003b), Clipper x Sahara (Karakousis *et al.*, 2003b), Galleon x Harura Nijo (Karakousis *et al.*, 2003a) and Mundah x Keel (Long *et al.*, 2003)

1.6 Mapping populations

Selection of a population for genomic mapping involves choosing parents and devising a mating scheme. Parents of a mapping population must have sufficient phenotypic variation for the trait(s) of interest. If the parents are greatly different at the phenotypic level, there is a reasonable chance that genetic variation exists between the parents, although uncontrolled environmental effects could create large phenotypic variation without any genetic basis for the effects. However, lack of phenotypic variation between the parents does not mean that there is no genetic variation. Different sets of genes could result in the same phenotype. In some cases, variation within individuals, based on a high level of heterozygosity, may be exploited in controlled crosses. A highly heterozygous individual may be treated as an F1 in some experimental designs (Liu, 1998). The variation at the genetic level may reflect the phenotypic variation present.

The more DNA sequence variation that exists, the easier it is to map loci controlling a particular trait. Genetic variation of the trait between the parents is important, so that progeny of the population will have a correlation of the phenotypic and genotypic information.

1.7 Quantitative Trait Loci (QTL) as tools for mapping complex traits

QTL mapping is the correlation between DNA sequence polymorphism and phenotypic variation. In 1923, Sax reported the first linkage between a quantitative and qualitative trait in common bean. Following this initial investigation, many studies have been undertaken to detect and map QTL. With the rapid development of biotechnology, linkage maps based on molecular markers are now available for most important crop species, and several reports have established genetic linkage between known molecular markers and QTL (Zhang *et al.*, 1992; Hackett, 2002). Different types of molecular markers can be used to resolve the chromosomal location of the genes that interact to give quantitative control of complex characters, e.g. malt quality, disease resistance and yield (Mohan *et al.*, 1997).

The polymorphic and co-dominant nature of the DNA variation detected with RFLP has made this molecular marker system ideal for the mapping and dissection of QTL (Blanco *et al.*, 1996). To date, there have been several reports of QTL mapping in cereals, including maize (Goldman *et al.*, 1993; Consoil *et al.*, 2002; Tuberosa *et al.*, 2002), wheat (Blanco *et al.*, 1996), barley (Backes *et al.*, 1995; Larson *et al.*, 1996; El Attari *et al.*, 1998) and rice (He *et al.*, 1998; Price *et al.*, 2002).

Many of the traits that contribute to a superior barley cultivar are quantitative. These include yield, maturity, height, and various agronomic and quality traits. For example, Barua *et al.* (1993) used a DH population and a number of molecular marker systems to generate a genetic map and detected seven markers linked to the *denso* locus on chromosome 3H, and a QTL associated with heading time on chromosome 6H. Quantitative traits display continuous phenotypic variation and are determined by:

1) Simultaneous segregation of multiple genetic loci and gene interaction, and

2) Environmental factors that influence expression of the trait (Falconer and Mackay, 1996)

Another consideration for QTL mapping is genotype x environment interaction. Hayes *et al* (1993) reported that the QTL x environment interaction would be expressed as:

1) Significant effects detected only in a subset of the total number of environments;

2) Changes in the magnitude of significant effects of QTL across environments;

3) Opposite favourable alleles at a QTL in distinct environments.

The significance of QTL x environment interaction can be studied by analysis of variance or by comparing the frequency of identification of significant marker-QTL associations in different environments. Several reports in this area (Zehr *et al.,* 1992; Stuber *et al.,* 1992; Bubeck *et al.,* 1993; Paterson, 1995) indicate that differing results may be a function of traits studied or the method of identifying QTL x environment interaction (Liu, 1998). Today, there are several methods and associated computer software available for QTL analysis, including Mapmaker/QTL (Lander *et al.,* 1987; Lincoln *et al.,* 1992a), Map Manager/QTL (Manly, 1997; Manly and Olson,1999; Manly *et al.*, 2001), Q-gene (Nelson, 1997), QTL Cartographer (Basten *et al.,* 1999), Plab QTL (Utz and Melchinger, 1996) MQTL (van Ooijen and Maliepaard, 1996). Also many other software programs for QTL analysis can be found at www.stat.wisc.edu.

The power of QTL detection can be improved through repeated phenotype measurements of progeny in order to account for the variation caused by environmental and genetic factors (Lander and Botstein, 1989). In addition, QTL detection also depends on the type and number of progeny studied. According to computer studies, a population size from a few hundred to a thousand progeny is

required to detect QTL (Manninen, 2000). Population size is especially important if a minor effect QTL is to be detected (Manninen, 2000).

In barley mapping studies, different population structures (DH, Recombinant Inbred Line (RIL) populations) and population sizes (from 100 to 200 lines) have been used, and average chromosome lengths are 200cM (Langridge, 1999). Upwards of 300 markers are mapped, aiming to get evenly spaced markers of 5cM to detect QTL. However, a map saturated with more molecular markers can help to locate QTL more precisely (Darvasi *et al.*, 1993).

1.8 Mapping of Plant Regeneration

Plant regeneration from cultured cells is controlled by genetic factors, and genetic markers associated with plant regeneration have been identified in cereal species, including barley (Komatsuda *et al.*, 1995; Mano *et al.*, 1996), wheat (Ben Amer *et al.*, 1997), rice (He *et al.*, 1998) and maize (Armstrong *et al.*, 1992).

In barley, shoot regeneration is a polygenic trait controlled by multiple genes (Komatsuda *et al.*, 1989; Mano *et al.*, 1996). Following tissue culture of DH lines derived from the cross of Steptoe and Morex, Bregitzer and Campbell (2001) detected QTL controlling the regeneration of green and albino plants. The QTL for the regeneration of green plants were located on chromosomes 1H, 2H, 3H, 4H, and 7H, and the QTL for the regeneration of albino shoots were mapped to chromosomes 5H and 3H. Using the same DH lines, Mano *et al.* (1996) identified multiple loci controlling tissue culture response, with two QTL detected for callus growth on chromosomes 2H and 3H and four QTL responsible for shoot regeneration on chromosomes 2H, 3H, 6H and 7H. The combined evidence from these two studies confirm there were at least three QTL responsible for the regeneration of shoot from barley callus cultures. These QTL were located on chromosomes 2H, 3H and 7H. In

addition, both studies identified other QTL located on chromosomes 1H, 4H and 6H, which strongly suggested that further research is needed to fully determine the importance of these other QTL in barley tissue culture response. Furthermore, wide variation in tissue culture traits was observed in both studies, indicating transgressive segregation in the DH lines for shoot regeneration. Transgressive segregation is a term used to describe the appearance, in progeny, of characters, which quantitatively fall outside the boundaries defined by the phenotypes of the parents in the cross (Xu *et al.*, 1998). In addition, plant hormones incorporated in the nutrient medium, the plant genotype and the combined effects of these two variables significantly influenced the QTL responsible for barley tissue culture response (Hanzel *et al.*, 1985; Lührs and Lörz, 1987; Bregitzer and Campbell, 2001).

1.9 The aims of this thesis

The specific aims of the current study were to:

1) Evaluate twenty barley cultivars for tissue culture response with respect to vigour of growth and their capacity to regenerate fertile plants from tissue-cultured cells.

2) Confirm the location of previously reported QTL that control tissue culture response in barley and search for additional QTL in a different DH mapping population generated from the crossing of a tissue culture recalcitrant parent and tissue culture responsive parent.

Chapter Two

SCREENING OF 20 BARLEY VARIETIES FOR TISSUE CULTURE RESPONSE

2.1 Introduction

There have been numerous investigations to develop protocols for an efficient regeneration system that can be successful applied to genetic transformation in barley. Varieties differ in their ability to regenerate in tissue culture and this trait is under complex genetic control. Identifying suitable genetic material using molecular markers may improve the ability to identify genotypes that are responsive to tissue culture, but to do so an appropriate population is needed to allow genetic mapping of traits. The purpose of this experiment was to identify a cross that would be suitable for mapping genetic traits associated with tissue culture response in barley. This experiment assessed the tissue culture response of 20 barley cultivars.

Historically a number of different barley explants have been used to produce embryogenic callus capable of plant regeneration. These include the shoot meristem (Cheng and Smith, 1975; Weigel and Hughes, 1985), the mature embryo (Lupotto, 1984), the immature ovary (Orten, 1979), the immature inflorescence (Thomas and Scott, 1985), and the seedling mesocotyl (Jelaska *et al.*, 1984; Rengel and Jelaska, 1986). In general, however, the immature embryo has been the most widely used explant type for the production of embryogenic callus in barley. This was demonstrated in early reports where highly regenerable callus could be easily produced from the immature embryo (Kasha and Kao, 1970; Dale and Deambrogio, 1979). More recently, the immature embryos of different barley cultivars have been employed to generate embryogenic callus suitable for the regeneration of fertile plants (Lührs and Lörz, 1987; Satoh *et al.*, 1991; Dahleen, 1999; Barro *et al.*, 1999).

The scutellum tissue (immature embryonic tissue) appears to be the most suitable choice of tissue to maximise shoot generation potential, but for mapping of this trait, suitable parents need to be identified. The regeneration capacity was examined in an experiment that assessed the tissue culture response of 20 barley

cultivars. The purpose of this experiment was to identify parents that may enable a population to be developed suitable for mapping genes associated with tissue culture response in barley.

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

Professor A.R. Barr (Department of Plant Science, University of Adelaide, Waite Campus) kindly provided the seeds of the 20 cultivars used in this experiment. The cultivars were Grimmett, Skiff, Harrington, Gardiner, WABAR 2030, Franklin, Schooner, Sloop 2976, Clipper, VB 9623, WB 190R, VB 1904, Arapiles, Vic 8604 SB, Sloop 2875/22, Tallon, Galleon, VB 9524, Stirling and Chebec. Three seeds per pot were sown in 20 cm diameter pots that contained Horsham soil mix (Appendix 1).

The plants were grown in a greenhouse located in the South Australian Research and Development Institute (SARDI) Plant Research Centre at the end of the winter season of 1998. The temperature in the greenhouse ranged from 12°C to 30°C with a 10-14 hour photoperiod. The plants were fertilized with Aquasol fertilizer (23:4:18 for N-P-K, respectively, Hortico Pty Ltd, Australia). The fertilizer was applied every two weeks until flowering. At the time of harvest in November, the temperature in the glasshouse ranged from 15°C (night) to 30°C (day) with 14-hour photoperiod. Plants infected with mites were sprayed with miticides, Omite (2 g/L) or Pyranica (1g/L), while plants infected with fungi were sprayed with fungicides, sulphine (3 g/L) or Barfidan (0.4 ml/L).

2.2.2 Isolation and culture of immature barley scutella

Barley spikes were harvested 10-15 days after pollination when the embryo was about 1-2 mm in length. On the same day, the grains were surface sterilised

with 70% (v/v) ethanol for 30 seconds and washed with sterile distilled water. The immature embryo was excised from the grain and the embryo axis was carefully removed from the immature embryo. Between 8 and 50 immature scutella were prepared for each cultivar and DH line. Details for individual experiments are given below.

Fifty immature scutella were prepared for each cultivar. A malfunction in the air-conditioning in the glasshouse caused the temperature to reach 35°C on one day. Consequently, many of the embryos in some genotypes were unsuitable to use and only thirty embryos within the standard 1-2mm range were found. Although more embryos would have been desirable, it was decided to proceed with a smaller number.

Ten scutella were cultured in five separate 90 mm-plastic Petri dishes (Sarstedt Australia, Pty Ltd Technology Park, SA). The callus induction medium was based on the protocol of Murashige and Skoog (1962) (MS) (Appendix 2) and contained 30 g/L sucrose, 3 mg/L 2,4-D and 8 g/L agar (DIFCO, Detroit, MI, USA). The pH of the medium was adjusted to 5.7-5.8 with sodium hydroxide prior to autoclaving. The medium was sterilised for 20 min at 121°C and dispensed into plastic Petri dishes. The scutella were cultured cut side-down on the callus induction medium. The Petri dishes were sealed with Parafilm® (American National Can™, Menasha, WI, USA), covered with aluminium foil and incubated in a tissue culture growth room at 24-26°C for three weeks.

2.2.3 Callus induction, somatic embryo production and plant regeneration

After incubating in the dark for 3 weeks, the Petri dishes were transferred to the light (16 h light/8 h dark photoperiod at 24-26°C) for one week. The diameter of

the individual callus cultures was measured, and each explant was scored for the number of somatic embryos and number of shoots.

After recording these data, somatic embryos and emerging shoots were transferred to hormone-free MS medium for four weeks to induce plant regeneration. After four weeks, the total number of plants produced per callus was recorded. Plants with well-developed root systems, were removed from the Petri dishes, washed with sterile water to remove traces of agar, and planted in three-inch diameter pots containing Horsham soil mix. The pots were placed in a 37.0 x 25.0 cm tray covered with a clean plastic lid and transferred to the glasshouse. For two weeks, the plastic lid on the top of the tray was closed to protect the plants from desiccation.

During the third and fourth weeks, the vents on the top and the sides of the plastic lid were opened completely to harden off the plants. At the end of the fourth week, the plastic lid was removed, and the trays were placed on the benches in the glasshouse. During the next four to five weeks, the plants that grew were transplanted to eight-inch diameter pots and grown to maturity. At the same time, seed-derived control plants were also grown in the greenhouse. The phenotypic characteristics and fertility of three of the tissue culture-derived plants were recorded and compared with three of the control plants.

2.2.4 Statistical analyses

The immature embryos were scored for the production of somatic embryos and shoot regeneration, and the data were analysed using the GENSTAT for Windows (5^{th} Edition) statistical program.

2.3 Results and Discussion

The scutella were isolated (Fig 2.1 A) and cultured on callus induction medium in the dark. After five to seven days of culture, the isolated scutella started to swell and cell proliferation commenced. Callus was produced approximately 10 to 14 days after scutella isolation, and the cultures were incubated in the dark for a further 7 days to allow callus growth (Fig 2.1 B). In week 4, all the callus cultures were transferred to the light (Fig 2.1 C). At the end of that week, the numbers of somatic embryos and shoots were recorded for each callus. Shoots were transferred to hormone-free MS medium to induce plant regeneration (Fig 2.1 D).

In general, all the genotypes produced more somatic embryos than regenerated shoots (Table 2.1). The exception was WABAR 2030, which produced the same average number of somatic embryos and shoots per cultured scutellum (Table 2.1). Chebec was significantly better than all other genotypes in terms of the production of somatic embryos and the regeneration of shoots (Table 2.1). There were other significant differences between genotypes.

For example, Skiff showed higher levels of somatic embryo and shoot regeneration than Clipper, Gairdner, Grimmett, Stirling and WABAR 2030 (Table 2.1). In addition, VB 9623 and VIC 8604 SB produced more somatic embryos and regenerated more shoots compared with Gairdner and WABAR 2030 (Table 2.1). All the tissue culture regenerated plants survived transfer to soil (Fig 2.1 E), with the exception of genotype VB 9104, which died in soil. Many of the tissue culture regenerated plants produced more heads and set more seed than their corresponding control plants, while plant height was greater for control plants compared with tissue culture-derived plants (Table 2.2). These phenotypic characteristics of tissue culture derived plants could be the direct result of the *in vitro* culture process. The tissue culture induced variation has previously been observed

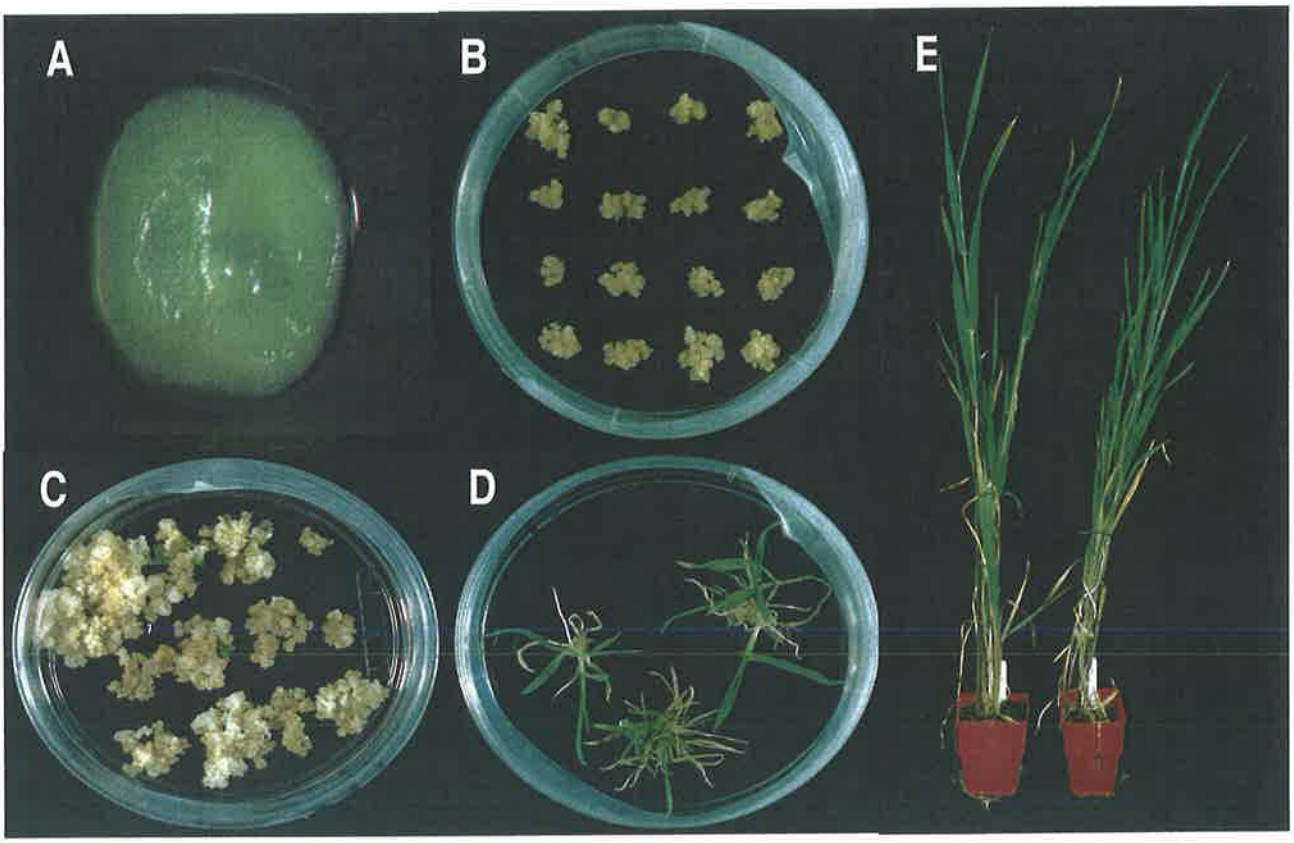

Figure 2.1: Steps in tissue culture and regeneration of fertile barley plants.

A. Immature scutellum.

B. Callus growth after 3 weeks incubation in the dark.

C. Somatic embryo production and shoots formation.

D. Plant growth on hormone-free MS medium.

E. Plants established in soil

Table 2.1 Tissue culture results for 20 barley varieties under analysis

Variety	Total No. calli produced from 50 scutella	No. embryogenic calli produced from 50 scutella	% embryogenic callus from 50 scutella	Mean No. somatic embryos per callus	Mean No. shoots produced per callus	Mean size of callus after 28 days in culture (mm)
Chebec	50	50	100	3.22a	2.280 a	13.0
Skiff	22	20	40	1.70b	1.120 b	8.0
VB 9623	40	35	70	1.68b	0.920 bc	9.7
Schooner	50	39	78	1.58bc	0.740bcd	13.3
Sloop 2976	34	32	46	1.26bcd	0.720bcd	10.5
Vic 8604 SB	24	24	48	1.18bcd	0.920bc	7.5
VB 9524	50	37	74	1.04bcde	0.800bcd	12.0
Arapiles	26	25	50	0.96bcde	0.720bcd	7.0
Franklin	14	14	28	0.90bcde	0.820bcd	6.6
VB 9104	19	11	22	0.82bcde	0.640bcd	6.6
Sloop (2875/22)	36	26	52	0.78bcde	0.660bcd	8.0
WB 190R	24	22	44	0.66bcde	0.460bcd	6.1
Harrington*	30/30	25	84	0.62bcde	0.560bcd	10.6
Galleon	50	47	94	0.56cde	0.340bcd	9.0
Tallon	15	12	24	0.40de	0.320bcd	5.4
Stirling	8	7	14	0.34de	0.200cd	6.2
Grimmett*	30/30	22	73	0.24de	0.240cd	11.8
Clipper	7	6	13.4	0.22de	0.120cd	5.2
Gairdner*	30/30	26	87	0.08e	0.040d	16.2
WABAR 2030*	30/30	26	87	0.04e	0.040d	12.2

*Only 30 scutella cultured
Means with the same letter are not significantly different at the 5% level

among plants regenerated from callus cultures of cereals including barley (Bregitzer *et al.*, 1998) and wheat (Vasil *et al.*, 1992).

This experiment demonstrated that there were differences among the barley genotypes in terms of the production of somatic embryos and the regeneration of shoots. Using standard tissue culture conditions, isolated scutella of 19 of the 20 genotypes tested produced embryogenic callus, which could be induced to regenerate fertile plants (Table 2.2). The majority of the cultivars used in the current study had not been tested for tissue culture response previously (Chebec, Gairdner, WABAR 2030, Franklin, VB 9623, WB190R, VB 1904, Arapiles, VIC 8604 SB, Galleon, VB 9524, Sloop 2976, and Sloop 2875/22). On the other hand, previous studies have reported different *in vitro* culture response for Harrington, Clipper, Grimmett, Stirling, Tallon, and Schooner compared with the present study (Baillie *et al.*, 1993; Walmsley *et al.*, 1995, Nobre *et al.*, 1996; Singh *et al.*, 1997; Bregitzer *et al.*, 1998; Barro *et al.*, 1999; Dahleen and Bregitzer, 2002). The current study demonstrated the wider application of the tissue culture methodology to untested barley genotypes and revealed that Chebec was the best performing line and that Harrington was one member of a group of genotypes that responded poorly in culture (Table 2.1). The results here demonstrated that the average numbers of shoots per embryo for Clipper, Grimmett, Schooner, Stirling, and Tallon were 0.12, 0.24, 0.74, 0.20, and 0.32, respectively. On average, callus from the same genotypes produced 3.1, 1.1, 1.6, 0.4, and 1.6 shoots respectively, in an earlier study (Walmsley *et al.*, 1995). Not only were the number of shoots different, but also the varieties were ranked in a different order with respect to tissue culture response.

Table 2.2 Phenotypic characteristics and fertility of tissue culture-derived and control plants

Cultivar	Source of explants	Mean no. of heads per plant	Mean plant height (cm)	Signif.	Mean no. of grains/ plant	Signif	Mean grain weight (g)	Signif
Tallon	Grain (control)	14	70.0	n.s	293.5	n.s	14.1	n.s
	Tissue Culture	34	82.7		424.7		15.7	
Clipper	Grain (control)	21	73.5	n.s	408.0	n.s	19.8	n.s
	Tissue Culture	20	81.3		320.3		14.4	
Wabar 2030	Grain (control)	13	74.0	n.s	257.0	n.s	7.8	n.s
	Tissue Culture	18.6	71.0		336.3		13.8	
Schooner	Grain (control)	22	90.6	n.s	362.7	n.s	18.7	n.s
	Tissue Culture	30.75	85.5		433.3		19.6	
Chebec	Grain (control)	16.75	77.5	*	232.8	n.s	12.1	n.s
	Tissue Culture	30.75	59.3		412.0		17.3	
VB 9524	Grain (control)	21.3	86.0	*	323.7	n.s	15.6	n.s
	Tissue Culture	12.3	67.3		201.3		8.6	
WI 2875/22	Grain (Control)	25.75	93.5	n.s	310.3	n.s	15.5	n.s
	Tissue Culture	30	83.3		467.3		22.5	
WI 2976	Grain (Control)	18.5	67.5	n.s	267.0	n.s	15.8	n.s
	Tissue Culture	39.3	65.0		483.0		25.0	
VB 9623	Grain (Control)	22	80.7	*	396.3	n.s	16.5	n.s
	Tissue Culture	16.33	66.0		183.7		8.8	
Gallon	Grain (Control)	30.33	63.3	n.s	417.0	n.s	24.5	n.s
	Tissue Culture	36.6	66.0		412.3		20.6	

Continued

Table 2.2 (contd)

Cultivar	Source of explants	Mean no. of heads per plant	Mean plant height (cm)	Signif.	Mean no. of grains/ plant	Signif	Mean grain weight (g)	Signif
VIC 8604 SB	Grain (control)	24.8	83.0	n.s	348.0	n.s	14.5	n.s
	Tissue Culture	45	76.5		489.5		22.6	
Gardener	Grain (control)	14.8	70.0	n.s	206.7	n.s	8.6	n.s
	Tissue Culture	22.5	76.5		342.0		17.9	
Skiff	Grain (control)	34.25	80.8	*	311.7	*	16.6	*
	Tissue Culture	37	65.8		719.0		28.1	
Stirling	Grain (control)	22	93.0	n.s	355.5	*	16.8	n.s
	Tissue Culture	43	80.0		695.7		22.9	
Grimmett	Grain (control)	13	78.0	n.s	237.3	n.s	7.1	n.s
	Tissue Culture	22.33	68.3		273.0		10.0	
Araples	Grain (control)	28.33	72.0	n.s	441.0	*	20.4	*
	Tissue Culture	22	51.3		221.3		8.6	
Harrington	Grain (Control)	23	94.0	n.s	456.0	n.s	22.7	n.s
	Tissue Culture	24	91.3		525.7		19.7	
WB 190R	Grain (Control)	22	94.3	n.s	399.3	n.s	22.1	n.s
	Tissue Culture	34.3	85.7		541.7		24.5	
Franklin	Grain (Control)	24	82.5	n.s	461.0	n.s	23.9	n.s
	Tissue Culture	38	78.3		380.3		15.4	

n.s = not significant
* = Significantly different at the 5% level ($P<0.05$) based on ANOVA test.

Although the same basic MS medium was used in both studies, the final sucrose concentration was different (30g/L sucrose in the current study compared with 20g/L sucrose in Walmsley *et al.* 1995). Carbohydrates are very important for the culture of immature embryos because they are the dominant energy source for non-photosynthesising plant cells and they regulate medium osmolarity, which is a critical factor in the *in vitro* development of the explants (Ślesak and Przywara, 2003; Scott and Lyne, 1994a). For *in vitro* culture of plant tissues, sucrose is generally regarded as the best and therefore the most widely used carbon source for culture medium (Scott and Lyne, 1994b). The high concentration of sucrose used in the current study could have negatively affected plant regeneration. It was noted that 40 mM sucrose (14g/L) caused barley microspore death through the accumulation of ethanol in the cells (Scott *et al.*, 1995). It is possible that plant regeneration from immature embryo-derived barley callus is also affected in a similar manner when exposed to 88 mM (30g/L) sucrose in the culture media employed here. On the other hand, the hydrolysis of sucrose produces high levels of glucose, which increases the osmolarity of the culture medium and could reduce the capacity of the *in vitro* cultured cells to differentiate into plants. Therefore, an alternate carbon source or a lower sucrose concentration could be important for the improvement of plant regeneration in cereal callus cultures. González *et al.* (2001) evaluated the *in* vitro culture response of twelve durum wheat cultivars and determined that plant regeneration was strongly dependent on the type of culture medium used to induce embryogenic callus.

The frequency of plant regeneration was higher for embryogenic callus induced in the presence of maltose compared with sucrose. Walmsley *et al.* (1995) compared sucrose and maltose-containing MS medium for the production of embryogenic barley callus. Although maltose was better for the production of

somatic embryos, shoot regeneration was similar for both carbon sources. Other factors including the concentration of 2,4-D and the type of explant could also explain the differences observed between these two studies. Walmsley *et al.* (1995) produced embryogenic callus on culture medium supplemented with 5 mg/L 2,4-D, while the callus produced here was achieved with 3 mg/L 2,4-D.

In the current study, the average number of regenerated shoots per scutellum-derived callus was 0.56 for Harrington (Table 2.1). Two previous studies reported 1.5 and 9.3 shoots/embryo, respectively, for this tissue culture responsive genotype (Bregitzer *et al.*, 1998; Dahleen and Bregitzer, 2002). These differences could be explained by the substitution of maltose for sucrose and the alteration of the media sterilization process (Bregitzer *et al.*, 1998), and the modification to the H_3BO_3 and $FeSO_4$ concentrations and the addition of BAP to the callus maintenance and shoot regeneration media (Dahleen and Bregitzer, 2002).

In conclusion, plant genotype strongly influenced barley tissue culture response. The study identified barley genotypes that demonstrated different behaviour with respect to the production of somatic embryos and the regeneration of shoots (Table 2.1). The different tissue culture responses of Chebec and Harrington should make the DH population based on these parents suitable to investigate the genetic mechanisms that control somatic embryo production and shoot regeneration in barley at the molecular level. The outcomes of this experiment are described in the next chapter and include statistical analyses to identify QTL associated with shoot regeneration in barley callus cultures.

Chapter Three

GENETIC ANALYSIS OF SHOOT REGENERATION IN THE CHEBEC X HARRINGTON DOUBLED HAPLOID POPULATION

3.1 Introduction

The results presented in the previous Chapter demonstrated that there was a significant difference in tissue culture response between Chebec and Harrington, which are the parents of a mapping population. A second experiment was conducted using an initial map of 115 Doubled Haploid (DH) lines to identify QTL (Moan and Komatsuda, 2002), which affect shoot regeneration in barley callus cultures in DH population lines derived from the Chebec x Harrington cross.

Harrington was bred by the University of Saskatchewan and was released in 1981. Its key features include spring growth habit, tall stature, wide adaptation, long basic vegetative period, high malt extract, low wort viscosity, and very high diastatic power (Eglinton *et al.,* 1998). The other parent was the Australian variety Chebec, which was bred by D.H.B.Sparrow and R.C.M. Lance in South Australia and released in 1992. Chebec has some desirable features for malting, including moderate levels of malt extract, good free amino nitrogen, low wort viscosity and low wort beta-glucan but it was not approved by the Australian malting industry because of its low diastatic power and low fermentability. Chebec has moderately tall straw, plump grain, short basic vegetative period, early maturity, adult plant resistance to net form net blotch and resistance to the cereal cyst nematode (Kretschmer *et al.*, 1997). The initial cross Chebec x Harrington was made at Waite Campus in 1990, and doubled haploid plants were produced from F1 donors using anther culture to produce a full genetic linkage map for this population (Barr *et al.,* 2003b). Professor. P. Langridge (Department of Plant Science, University of Adelaide, Waite Campus) kindly provided seed of these lines.

3.2 Materials and Methods

3.2.1 Plant material and tissue culture techniques

One hundred and fifteen DH lines from a Chebec x Harrington cross were used for this experiment (Barr *et al.*, 2003b) The 115 Chebec x Harrington DH lines were grown in a controlled environment growth room, and were maintained using the temperature and light conditions described in Table 3.1. The 115 lines were divided into two batches of 53 and 62 lines, as a result of space constraints in the growth room. Seven lines were included in both batches and served as controls to enable a statistical comparison of tissue culture response and thus ensure that the two batches were considered as a single experiment. For each line, a single grain was sown in a 15 cm diameter plastic pot. The plants were maintained according to the procedures described in Section 2.2.1. The parental lines were not included in this experiment.

Thirty-two immature scutella explants were isolated from each DH line, and eight scutella were cultured in four separate 90 mm plastic Petri dishes (Sarstedt Australia) containing the callus induction medium described in Section 2.2.2. The scutella were scored for the production of somatic embryos and the regeneration of shoots after three and four weeks of culture, respectively (Section 2.2.3).

3.2.2 Statistical analysis

Analysis of the data collected in this experiment was performed using the methods described by Eckermann *et al.* (2001) and used a linear mixed model in the S-plus software with a function called SAMM (Spatial analysis of mixed model). A total of 351 markers covering all barley chromosomes of the Chebec x Harrington cross were used for simple and interval regression analysis (Barr *et al.*, 2003b).

Table 3.1 The growth room conditions for donor plants used in this experiment

N=Night, D=Day, H=Hours

Growth Period (week)	Temperature (ºC)	Photoperiod (H)
1	10 (N)_____12 (D)	8
2	11 (N)_____13 (D)	8.5
3	12 (N)_____14 (D)	9
4	12 (N)_____14 (D)	9.5
5	13 (N)_____15 (D)	10
6	14(N)_____16(D)	10.5
7	14(N)_____16(D)	11
8	15(N)_____17(D)	11.5
9	16(N)_____18(D)	12
10	16(N)_____18(D)	12.5
11	16(N)_____20(D)	14

Relationships between trait expression of the number of somatic embryos and the number of shoots were examined and compared to molecular marker alleles from Chebec and Harrington. A marker locus thought to be associated with a gene or chromosomal region conferring tissue culture response was tested for two-way interaction, and a minimum logarithm of odd ratio (LOD) threshold of 3.0 was used to detect statistical significance. Three methods were used to analyse the data:

1) Individual embryos from each DH line were treated as a unique experimental unit. The number of somatic embryos and the number of shoots for each embryo were analysed using the following linear mixed model:

a) Number of somatic embryos for each embryo = mean + left marker + right marker + number of experiment (either 1 or 2) + number of plate (1,2,3,4) + line + error

b) Number of shoots for each embryo = mean + left marker + right marker + number of experiment (either 1 or 2) + number of plate (1,2,3,4) + line + error

These data were transferred to S-plus software with the SAMM function. This computer programme scans the genetic map of each chromosome of the Chebec x Harrington population to detect marker associations using interval mapping and maximum likelihood statistics.

2) All 32 embryos of each line were treated as an experimental unit. The average number of somatic embryos and regenerated shoots were analysed using the following linear mixed model:

a) Average number of somatic embryos for each line = mean +left marker + right marker + number of experiment (either 1 or 2) + number of plate (1,2,3,4) + line + error

b) Average number of shoot for each line = mean + left marker + right marker + number of experiment (either 1 or 2) + number of plate (1,2,3,4) + line + error. These data were transferred to S-plus software programme to detect QTL.

3) The final method tested used the procedure described by Mano *et al.* (1996) to successfully detect QTL that control shoot regeneration in the cross Steptoe x Morex.

The proportion of embryos of each line that regenerated at least one shoot were determined to calculate the probability (P) that an embryo produced one or more shoots. Then Log [P/(1-P)] was taken as the tissue culture response, and these values were transformed to a normal distribution using the following formula:

Log (P/1-P)= mean + left marker + right marker + experiment (1 or 2) + line + error.

Finally, the data from log (P/1-P) were transferred to software S-plus to find QTL.

3.3 Results and Discussion

Initially, the cultured embryos of the DH lines were scored for the production of somatic embryos and the regeneration of shoots (data not shown). These numbers were subsequently used for the three methods of QTL analysis previously described in Section 3.2.2. This was achieved by looking for associations between molecular markers, the number of somatic embryos and the number of shoots in the DH population produced by the cross Chebec x Harrington (Barr *et al.*, 2003b).

Three methods were used in this experiment to detect QTL that control the production of somatic embryos and the regeneration of shoots from barley callus cultures. Method one was used to investigate QTL for the total number of shoot and somatic embryos produced. Method 2 was used in addition to method one because it was not certain whether each embryo could be treated as an experimental unit. Hence all 32 embryos were treated as one unit. Method three was also to investigate the probability that an embryo produced one or more shoots, rather than investigating the total number of embryo and shoots produced. The outcomes of these analyses are presented below.

Method One:

The results demonstrated that approximately 40% of the cultured embryos produced no somatic embryos or shoots (Figure 3.1 A and B). Also, the results indicated that QTL were present for the production of somatic embryos and the regeneration of shoots. The largest LOD score obtained was 1.274 on chromosome 3H (Table 3.2).

Table 3.2: LOD score of number of somatic embryos and shoots produced for each individual embryo in the Chebec x Harrington DH population. Table only includes data with a LOD score ≥ 1.0

Trait	Chromosome	Left Marker	Right Marker	LOD Score
No. Embryos	3H	WG940	BCD298	1.274
No. Embryos	UMP	ABC151	PSR162	1.051
No. Shoots	UMP	ABC151	PSR162	1.176

UMP= Unknown Map Position

Method Two:

The result from this method of analysis demonstrated that the average numbers of somatic embryos and shoots for each line ranged between zero and six (Figure 3.2 A and B). The LOD score was 1.57, also on chromosome 3H, which was higher than the value achieved with method one (1.274), but again it was not statistically significant (Table 3.3).

Table 3.3: LOD score of number of somatic embryo and number of shoots produced where all the embryo for each DH line were treated as a single experimental unit. Table only includes data with a LOD score ≥ 1.0

Trait	Chromosome	Left Marker	Right Marker	LOD Score
No. Embryos	3H	WG940	BCD298	1.570
No. Embryos	7H	BCD15a	ABG320	1.022
No. Embryos	UMP	ABC151	PSR162	1.035
No. Shoots	3H	WG940	BCD298	1.091
No. Shoots	UMP	ABC151	PSR162	1.162

UMP = Unknown Map Position

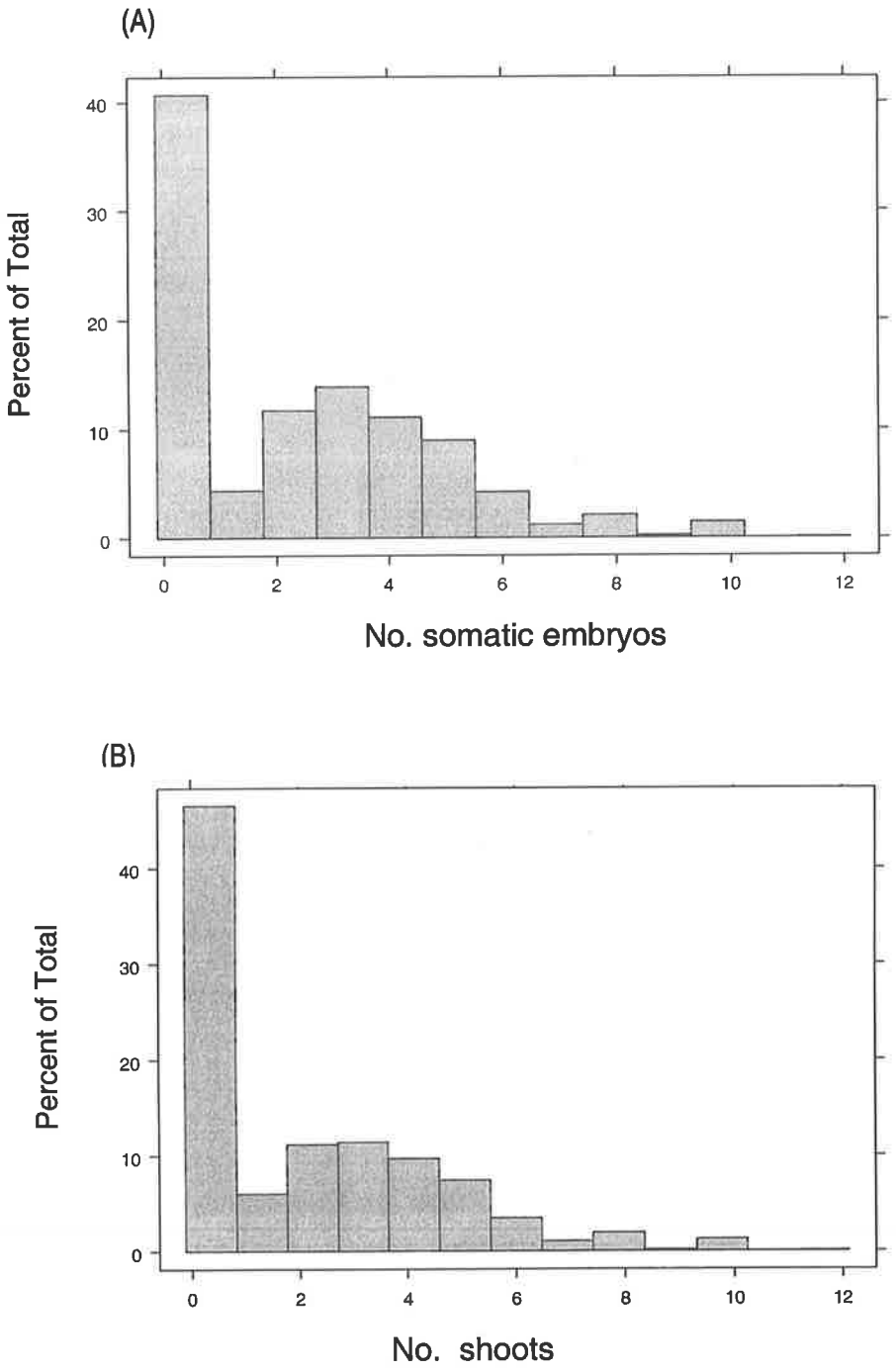

Figure 3.1: Ferquency distributions of the **(A)** number of somatic embryos **(B)** number of shoots in the Chebec x Harrington DH population. Each embryo was treated individually.

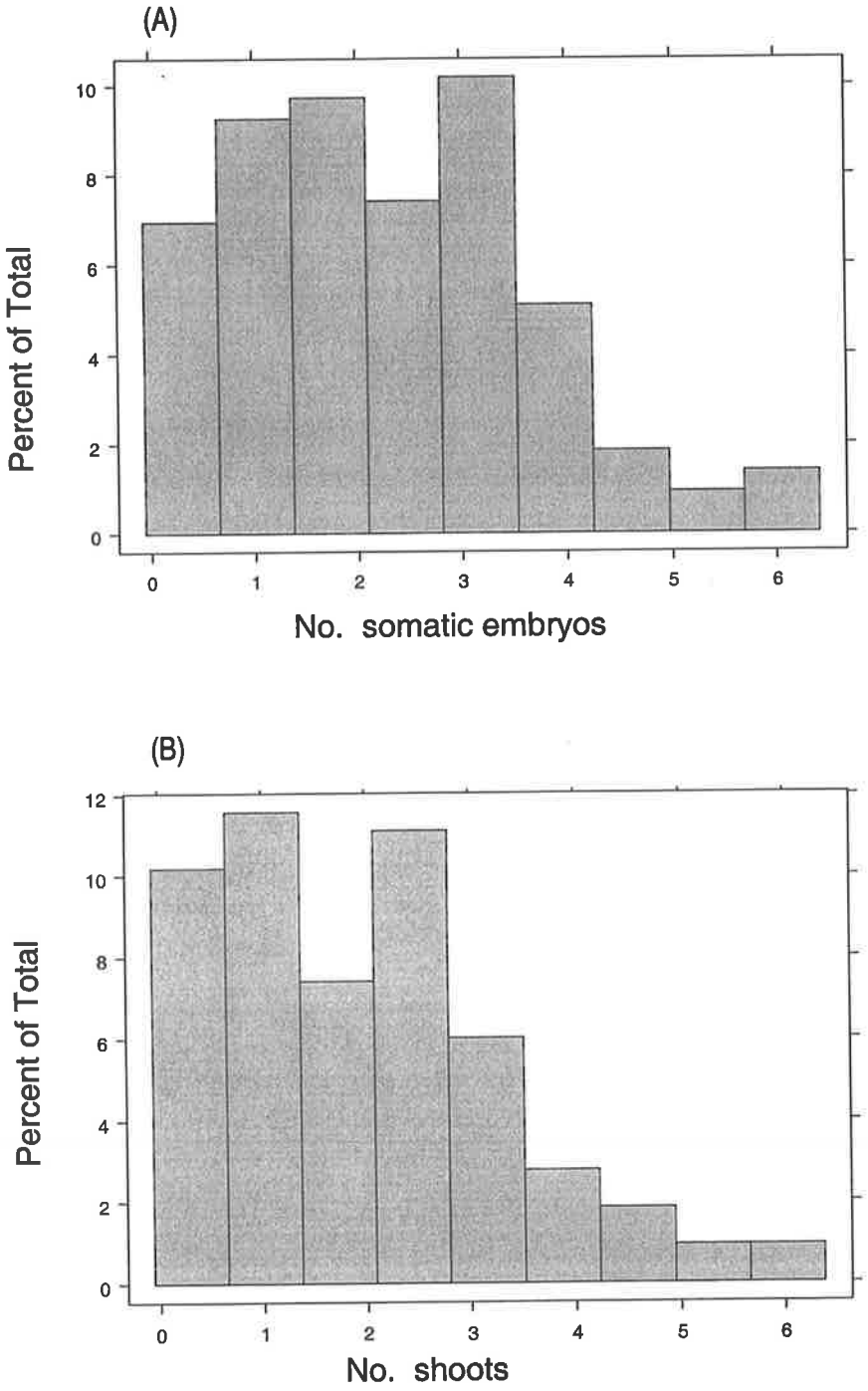

Figure 3.2: Frequency distributions of the **(A)** number of somatic embryos from each line **(B)** number of shoots from each line in the Chebec x Harrington DH population. All 32 embryos were treated individually.

Method Three: The results from this method showed no significance and are summarised in Table 3.4

Table 3.4: LOD score of shoots produced where only those embryos that regenerated at a single shoot were analysed. Table only includes data with a LOD score ≥ 1.0

Trait	Chromosome	Left Marker	Right Marker	LOD Score
Proportion	3H	WG178	CDO718	1.049
Proportion	7H	ABG476	AWBMA15	1.139

Although the LOD scores detected in the current study were not significant with any method of analysis, there was a weak association with genetic markers WG940 and BCD298 (Table 3.3), which maps within the same region of chromosome 3H (interval ABC171 and ABG4) that has been shown previously to affect shoot regeneration in barley tissue culture (Komatsuda *et al.*, 1993 and 1995; Mano *et al.*, 1996; Manninen, 2000; Bregitzer and Campbell, 2001; Mano and Komatsuda, 2002). The outcomes observed here could be the result of a number of factors or a combination of different factors such as environmental effects, genetic similarities between the parents and problems with the linkage map.

1) Environmental effects

Two types of environmental variation were detected in this study. There was evidence of variation in embryo response both within and between plates, and the lines themselves also influenced the degree of variation. Therefore, the variation detected here could be attributed to significant environmental effects rather than real genetic differences. In this case, it would be difficult to detect QTLs since genetic variation is insignificant in comparison with variation from other sources. Other potential sources of environmental variation include medium components, number of explants per plate, growth conditions of donor plants and culture conditions for

immature embryos (Walmsley *et al.*, 1995; Bregitzer *et al.*, 1998, Dahleen and Bregitzer, 2002, Chapter Two in the current study). Of these, all were standardised to minimise variation as much as possible, but there was some variation in the growth of the donor plants due to the space constraints in the growth room (Section 3.2.1).

2) Genetic similarities between Chebec and Harrington

In the first experiment of this study, significant differences were detected between Chebec and Harrington in tissue culture response (Table 2.1). However, no QTL were detected through the analysis of doubled haploid lines derived from this cross. This observation could be explained if both parental lines carry the same alleles at the locus/loci that control shoot regeneration and the initial detection of significant variation was due to an error factor relating to non genetic parameters such as environmental variables.

3) Problems with the linkage map

Although the linkage map derived from DH Chebec and Harrington lines has been used successfully to identify QTLs associated with cereal cyst nematode resistance, malt extract and early flowering (Barr *et al.*, 2003b), it is possible that known markers are not closely linked to QTLs for shoot regeneration in this DH population.

Therefore, a number of problems with this experiment were identified which can be investigated in future experiments. A third experiment was conducted to address the issues relating to the random location of donor plants in the growth room and the genetic differences between the parental lines.

Chapter Four

VARIATION IN GENOTYPIC DIFFERENCES FOR TISSUE CULTURE RESPONSE

4.1 Introduction

While randomization often ensures that each experimental unit is treated as similarly as possible, when applied to a few objects or subjects, unreplicated experimental results cannot be accepted because of the inability to assess the level of variability. Replication, the repetition of an experiment on large groups of subjects, is required to enable differences in treatment means to be assessed with confidence. The experimental worth can be seen if a treatment is truly effective, the average long-term effect of replication will reflect its experimental merit. Replication is a tool for the reduction of variability in experimental results, increasing the result's assurance level and confidence with which arrival at conclusions over experimental factors can be made by researchers. (M Lorimer, pers. comm.; Anderson *et al.*, 1994).

The first two experiments were unreplicated: the grains from which the scutella were excised were derived from a single plant or pot. Environmental influences during the growth of the donor plant can affect plant regeneration (Section 1.3.3), and the use of replication may improve the precision of the experiments. In the final experiment, replicates were used to examine differences in the shoot regeneration capacity of Chebec and Harrington and a representative sample of poor, moderate, and highly responsive DH lines. The ten DH lines were selected on the basis of the number of somatic embryos produced and the number of shoots regenerated from the cultured embryos of each individual line. The allele on the chromosome 3H locus (Tables 3.2, 3.3) was checked using Mapmanager QTX (Manly *et al.*, 2001), and the two allele classes could distinguish the phenotypic classes

Table 4.1: The ten DH lines used for the replicate experiment and their tissue culture response compared with parental lines Chebec (High) and Harrington (Poor)

DH lines	Regeneration Capacity of DH lines	3H Chromosome
128	Poor	Harrington
35	Poor	Harrington
14	Poor	Chebec
92	Poor	Harrington
119	Poor	Chebec
113	Moderate	Chebec
58	Moderate	Harrington
27	High	Chebec
48	High	Chebec
25	High	Chebec

4.2 Materials and Methods

In this experiment ten DH lines and their parents were used (Table 4.1). The DH lines were separated into three categories- poor, moderate and good tissue culture responses. Poor responding lines were defined as those that regenerated less than 1 shoot per cultured embryo. Moderate responding lines regenerated between 1-2 shoots per embryo, while lines that responded well gave values greater than 2 shoots per embryo.

A single grain of each line was individually sown in a 15 cm pot on five different sowing dates, the plants were arranged in five randomised blocks (Appendix 3), and were grown using the same conditions described in Section 3.1.1. The five individual blocks contained a single plant of each tested line, and sowing times were staggered at 1-week intervals to facilitate data collection.

When the plants reached anthesis, spikes were harvested and grains were surface-sterilised using the protocol described previously (Section 2.1.2). Twenty immature scutella were cultured from each single plant (20 immature scutella x 5 plants for each line = 100 immature scutella) and each scutellum was cultured in a separate 25 mm plastic Petri dishes containing callus induction medium (Section 2.1.2). The cultures were handled in a similar manner to the procedure described in Section 2.1.3, and each explant was scored for the production of somatic embryos and the regeneration of shoots (Section 2.1.3). The data were analysed using the GENSTAT for Windows (5th Edition) statistical program.

4.3 Results and Discussion

The first component of this experiment was to demonstrate differences in the tissue culture response of parental lines, Chebec and Harrington. ANOVA indicated Chebec and Harrington did not differ significantly from each other with respect to the average number of somatic embryos and the average number of regenerated shoots (Table 4.2). Since this analysis demonstrated that the parents were not significantly different, it was decided to test for difference in the number of somatic embryos and regenerated shoots between individual DH lines and that of the average effect of Chebec and Harrington.

The results demonstrated that two DH lines (128 and 25) differed significantly for the average number of somatic embryos and regenerated shoots for the parents (Table 4.2). DH line 128 produced significantly fewer somatic embryos and regenerated fewer shoots than the parents, while line 25 generated significantly more somatic embryos and produced more shoots compared with the parents (Table 4.2). In addition, the lines themselves showed significant differences in the number of somatic embryos and the number of regenerated shoots (Table 4.2). The replicated

Table 4.2: Table of means for somatic embryos and shoots.

Line	Mean number of somatic embryos	Mean number of shoots
128	0.85 a	0.56 a
35	1.91 ab	1.49 ab
14	2.30 bc	2.28 bc
Harrington	2.38 bcd	2.35 bcd
92	2.50 bcd	2.41 bcd
Chebec	2.96 bcde	2.69 bcde
113	3.14 bcde	2.92 bcde
119	3.27 cde	2.92 cde
27	3.27 cde	3.18 cde
58	3.63 def	3.32 def
48	3.80 ef	3.59 ef
25	4.73 f	3.96 f

Means with the same letter are not significantly different at the 5% level based on ANOVA.

experiment demonstrated that only four lines (128, 27, 48 and 25) behaved as expected according to their characterisation in Table 4.1. The other six lines (35, 14, 92, 113, 119 and 58) demonstrated improved plant regeneration capacity (Tables 4.1 and 4.2).

The purpose of this experiment was to use replicated studies to demonstrate differences in tissue culture performance of Chebec and Harrington and ten DH lines derived from this cross. The results indicate that the tissue culture response of the two parental lines is not statistically different (Table 4.2). This outcome suggests that

the alleles are similar at the gene locus on chromosome 3H that control tissue culture response in both parents.

The study also identified DH lines with levels of somatic embryo production and plant regeneration beyond the range of Chebec and Harrington (Table 4.2). *De novo* mutation induced by hybridity, gene complementation from the two parental lines and the unmasking of recessive genes normally maintained in a heterozygous state in the parental plants are three mechanisms used to explain the occurrence of intraspecific transgression (Rick and Smith, 1953). The most plausible explanation for the results obtained here is that transgressive segregation for somatic embryo production and shoot regeneration in the Chebec x Harrington cross resulted in new allelic combinations at the loci that control *in vitro* culture response leading to additive and subtractive effects in the measured traits of the DH lines. It should be noted that the differences in tissue culture response observed here between Chebec and Harrington compared with the outcomes obtained in Chapter 2 could be due to environmental factors that have previously been discussed in Section 3.2.

Finally, the results identified large variability in line response indicating that in the current experiment only five replications of each line were not sufficient to detect differences in the tissue culture response of Chebec and Harrington (Table 4.2). It has been suggested that more replication of donor plants is needed and that the number of embryos isolated from each plant is reduced to increase the accuracy of the experiment (M Lorimer, pers. comm.; Anderson *et al.*, 1994). This could be the subject of future experimental work to establish that genotype influences tissue culture response

Chapter Five

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The experiments conducted in this thesis detected variation in the tissue culture response of barley. This variation could be attributed to different factors such as genotype, the composition of the different culture media, the growth conditions used for the donor plants and the experimental design. The influence of these factors and their combined effects on barley tissue culture response are discussed below.

Previous experiments have demonstrated that the composition of culture media has a strong influence on tissue culture response (Dahleen and Bregitzer, 2002; Barro *et al.*, 1999; Nuutila *et al.*, 2000). Numerous reports have investigated the effects of carbon source and plant hormones on barley tissue culture response (Walmsley *et al.*, 1995; Scott and Lyne, 1994; Slesak and Przywara, 2003). The frequency of somatic embryo production and the regeneration of shoots from barley callus cultures could be improved in the current study by testing the influence of different types of culture media on these two scored traits.

Barley plants used as the source material for the isolation of immature embryos are normally grown in a glasshouse or in a controlled environment growth room (Dahleen, 1999; Sharma *et al.*, 2005). The physiological state of the donor plants, which relates to the growth environment, is known to affect the response of explants cultured under *in* vitro culture conditions (Lührs and Lörz, 1987). The most significant environmental differences between these two growth facilities are the quality and intensity of light, the photoperiod and the temperature (Sharma *et al.*, 2005). Dahleen (1999) used immature barley embryos to determine differences in the *in vitro* culture performance of explants taken from donor plants grown in a glasshouse and a growth chamber. Although the growth conditions were relatively constant in the growth chamber in this study, the environmental conditions in the glasshouse were strongly influenced by the season, which accounted for the

observed differences in the frequency of plant regeneration throughout the year. However, growth conditions cannot explain the seasonal variations observed for donor plants completely grown in growth rooms (Dahleen, 1999; Ritala *et al.*, 2001; Sharma *et al.*, 2005). At present, no explanation for this observation can be found.

In planning future *in vitro* culture experiments to study QTL controlling barley tissue culture response, it is recommended that all DH lines are sown in the same location at the same time of year to eliminate variation caused by the time of sowing.

In the current study, a putative QTL with a low LOD score was mapped to chromosome 3H in Chebec x Harrington DH lines (Section 3.2). This chromosomal location was previously shown to control barley tissue culture response in the DH lines generated in the crosses of Morex x Steptoe (Mano *et al.*, 1996), Rolfi x Botnia (Manninen, 2000) and Azumamugi x Kanto Nakate Gold (Mano and Komatsuda, 2002). In order to detect significant differences in the tissue culture response of Chebec and Harrington, the number of replications (donor plants) could be increased and fewer embryos could be isolated per replicate. It is expected that these changes to the experimental design in combination with alterations to carbon source and hormone components of the culture media and timing of donor plant growth in either the glasshouse or controlled environment room could increase the accuracy of the analysis by minimising the variation in the tissue culture response for plants of the same line (Lorimer, pers. comm.) and providing stronger evidence for the existence of a QTL on chromosome 3H and confirming the location identified in other DH populations (Eckerman, pers. comm). However, one of the reasons that the LOD score was low in the current study could relate to the genetic similarity of Chebec and Harrington in terms of tissue culture response (Section 4.2). Therefore, it also could be useful to screen the tissue culture response of other barley cultivars to detect extreme differences in tissue culture performance and produce DH lines from a

suitable parental cross. Alternatively, there currently exists at the Waite Campus eight DH mapping populations, including Alexis x Sloop (Barr *et al.*, 2003a), Clipper x Sahara (Karakousis *et al.*, 2003b) and Galleon x Harura Nijo (Karakousis *et al.*, 2003a) and these could also be useful for this type of analysis. Parental and DH lines could be tested for tissue culture difference and the number of somatic embryos and the number of regenerated shoots could be analysis using QTL software (Manly and Olson, 1999).

In summary, it was demonstrated in the first experiment that Chebec responded well in tissue culture compared with Harrington and further analysis of the Chebec x Harrington DH population seemed to hold promise. However the DH population did not identify statistically significant QTL, although the weak association with a QTL on chromosome 3H is consistent with previously published results. Replicated experiments were performed and these demonstrated that there was no significant difference between the parental lines in term of tissue culture response, which was different to the results of the first experiment. The differing results may be explained by changes in the location used for the growth of donor plants or alterations to the experimental design.

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APPENDICES

Appendix 1: Horsham soil mix composition

Composted pine bark is mixed with the following fertilizers:

Composted pine bark	600 litres
Osmocote (high P)	1.8 kg

(Scotts International BV, Nijverheidsweg 1-5,6422, PD Heerlen, The Netherlands)

Ammonium nitrate	0.135 kg
Micro max (micro-nutrients)	0.135 kg

(Scotts International BV, Nijverheidsweg 1-5,6422, PD Heerlen, The Netherlands)

Iron sulphate	0.27 kg
Agricultural lime	1.33 kg

The mix is not heat-treated and the pH is about 6.0-6.5

Source: SARDI, Plant Growth Service Section, Waite Campus, Adelaide, South Australia, Australia

Appendix 2: MS Culture medium

INGREDIENTS	
MACROSALTS	**mg/L**
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
MICROSALTS	
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
KI	0.8
$Na2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
IRON SOLUTION	
Na2EDTA	37.3
$FeSO_4.7H_2O$	27.8
VITAMINS	
myo-inositol	100
Thiamine-HCl	0.1
SUGAR	
Sucrose	30,000
OTHER	
2,4-D	3
pH	5.7-5.8

Appendix 3: Experimental designs for replicate experiments, showing the location of the tested lines in the controlled environment room. Each large square represents an independent replicated experiment, and the small squares indicate the position of the different lines on the table.

(1)

113	Harrington	27	14
128	35	119	92
58	25	48	Chebec

(4)

Harrington	25	Chebec	113
92	119	128	27
14	48	58	35

(2)

27	48	113	58
25	Harrington	14	Chebec
92	35	128	119

(5)

27	35	58	119
113	92	25	48
14	Harrington	Chebec	128

(3)

92	119	14	58
25	27	Chebec	113
48	128	Harrington	35