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The Production and Utilization of Doubled Haploids in Barley Breeding

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THE PRODUCTION AND UTILIZATION OF DOUBLED HAPLOIDS IN BARLEY BREEDING

A Thesis Submitted for the Doctor of Philosophy in Botany

By

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ABSTRACT

The potential of anther culture response of four commercial barley cultivars: Abbey, Dallas, Bianka and Decado was investigated on maltose and sucrose-based media. Although there was difference in response among the genotypes the substitution of sucrose by maltose led to an improvement in anther response as well as in green plant regeneration. Higher concentrations of maltose (6-9% w/v) favoured regeneration via embryogenetic route.

In the study of transmission of anther culture responsiveness into hybrids produced from crosses between *Hordeum vulgare* cv. Prisma (P) and four *H. spontaneum* lines (HS2, HS10, HS16 and HS20), it was found that, despite the improvement obtained using maltose, anther culture response was largely dependent on the genotype. A heterotic effect was also observed in the F1 hybrids.

Results presented in this study also focused on the development, evaluation and exploitation of genetic markers in barley. Both protein (ß-amylase, water soluble protein, grain esterase, leaf esterase and hordein)

and molecular (RAPD) markers were used as a means of detecting polymorphism in *H. vulgare* cv. Prisma and four *H. spontaneum* lines (HS2, HS10. HS16 and HS20). Except hordein, all other markers detected polymorphism between *H. vulgare* and *H. spontaneum*. These polymorphic markers showed segregation in the five doubled haploids derived from PXHS2 hybrid.

The segregation of alleles at RAPD loci was evaluated in 33 doubled haploids progeny derived from crosses between Blenheim and TS264. A total of 165 primers were used to generate amplification products through PCR and 22 of them detected polymorphism between the parents and were scored for the 33 doubled haploids progeny. MAPMAKER programme was used to estimate linkage relationships between segregating loci and four linkage groups were established.

DEDICATION

I dedicate this thesis to my mother
who encouraged me through the years.

DECLARATION

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. This thesis has not, in whole or part, been previously presented for a higher degree. Work other than my own is clearly indicated in the text by reference to the relevant researchers or the publications.

Lokman Hakim 12.10.96

The work presented in the thesis is the work of the candidate Md. Lokman Hakim. Conditions of the relevant Ordinance and Regulations have been fulfilled.

Professor O. I. Joarder
(Supervisor)

Dr. A. J. Miah
(Co-Supervisor)

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Barley, Barley Breeding and Genetic Markers

Barley (*Hordeum vulgare* L.), one of the oldest cultivated cereals grown widely in many countries of the world, is the fourth leading cereal in acreage planted after wheat, rice and corn (Bengtsson, 1992). It is grown from near the equator in Ethiopia to beyond the Arctic circle at latitude 70°N in Norway and from below sea level in the Netherlands to the limits of cultivation at 5000 m in Tibet. It was one of the basic crops of early irrigated agriculture in Mesopotamia and Egypt (Harlan, 1976).

Barley is a short duration grain crop with more tolerance to salinity and dry conditions than the other temperate cereals. It can give high yields under cool wet conditions (Poehlman, 1985). Its grain is used mainly for animal feed, malt and human consumption. Animal feed is its largest use, supplying primarily carbohydrate and protein. Protein content ranges from 10-15% which is higher than maize or sorghum and is generally comparable to wheat grown in a similar environment (Poehlman, 1985).

Barley is the preferred cereal for the production of malt and this is its second most important use (Poehlman, 1985). Third in importance but by no means insignificant, is the use of barley for human consumption, mainly in areas where the cultivation of other cereals is limited. It is an important food for highland areas (for example, Tibet, Nepal, Ethiopia and in the Andean countries), in semi-arid regions (for example, North Africa, the Near and Middle East, Russia, India and Afghanistan), and in high latitude countries (for example, Norway, Sweden, Finland and the Baltic regions of the former USSR) (Poehlman, 1985). There are 100 million people in the world for whom barley is a staple food (Xu,1990).

There are two types of barley such as six-row and two-row. These barley cultivars are divided into winter and spring referring to particular genotypic characteristics and not necessarily to the time of sowing. Barley ears consist of spikelets in groups of three at each node of the rachis. In two-row types the lateral spikelets are sterile and rudimentary so do not produce any grain whereas they are fertile in six-row types. The six-row types are frost-hardy and more drought tolerant than the two-row types (Briggs, 1978).

The origin and evolution of cultivated barley has gone through a continuous process with various stages. The two-rowed wild barley (*Hordeum spontaneum* C. Koch) is the oldest ancestor and is the first stage of evolution of cultivated barley (Harlan and Zohary, 1966). The six-rowed wild barley (*Hordeum agriocrithon* Aberg.) is the second stage and the cultivated barley (*Hordeum vulgare* L.) is the third and final stage (Qiquan, 1981). The genus *Hordeum* belongs to the family Gramineae and the tribe Triticeae. *Hordeum vulgare* L. is placed in the section *Hordeum*. According to the taxonomic treatment described by Bothmer and Jacobsen (1985), *Hordeum vulgare* L. comprises both cultivated and wild forms that are interfertile and biologically closely related. The subspecies *vulgare* comprises the cultivated forms. They are morphologically very variable as would be expected since they have been cultivated from ancient times over a wide geographical area and range of environmental conditions. The subspecies *spontaneum* (C. Koch) comprises the wild, two-row forms with brittle rachis. The wild forms have commonly been given species status (*Hordeum spontaneum* C. Koch) although they are fully interfertile with cultivated forms (Bothmer *et al.*, 1981; Shao, 1981). They too show considerable morphological

variation. Also included in the section *Hordeum* are *Hordeum bulbosum* L. and *Hordeum murinum* L. *H. bulbosum* is a distinct species of the Mediterranean and occurs in both diploid and tetraploid forms. It is a strict outbreeder and to some degree is also propagated by means of bulbs (Lundqvist, 1962). *H. murinum* is the species of *Hordeum* that has been most successful as a colonizing weed (Bothmer *et al.*, 1981).

Barley is one of the leading experimental organisms in genetic studies of higher plants. The genetics of barley have been studied extensively for several reasons: *Hordeum vulgare* L. is a diploid species with seven pairs of relatively large (6-8 µm) chromosomes two of which are satellited (Ramage, 1985). The five non-satellited pairs are classified according to their relative lengths, arm ratios and Giemsa C-banding patterns. The two satellited chromosomes are distinguished by the length of the satellite. Barley is almost completely autogamous but artificial hybridization is easily accomplished and a high rate of seed set is achievable (Briggs, 1978). Being a naturally self pollinating species the majority of the genetic loci will be in the homozygous condition. A relatively well classified map of barley has been

developed (Sogaard and Wettstein-Knowles, 1987) and the linkage groups have been equated to the seven pairs of homologous barley chromosomes.

The methods used to develop new cultivars of inbreeding species such as barley have been outlined by Poehlman (1979). Essentially, these methods involve the identification of genetic variability for the trait of interest; sexual hybridization to produce a segregating progeny and an efficient means of selection. The result of the crossing phase is a very diverse population which contains many of the possible recombinants for characters that differs in the parents. In the early generation the genetic variation is not fixed, so the progeny of any single plant continues to segregate. However, with generations of selfing, individual genotypes become progressively more inbred. Thus the genetical effect of inbreeding is to lower, generation by generation, the probability of an individual being heterozygous for alleles at any given locus. The breeding of most inbreeding crop plants such as barley is, therefore, the development of homozygous lines generated by automatic self pollination according to the method of pedigree selection. For this breeding method selection begins in

the F_2 generation on individual plants and continues in the F_3 and subsequent generations by evaluating ear rows and family plots using a pedigree based procedure. In other words, the pedigree or ancestry of the selections can be traced back to individual F_2 plants. The pedigree system has been very successful and the majority of new cereal cultivars have resulted from this breeding strategy. Nevertheless, pedigree selection in common with other methods of plant breeding is a long process and is, therefore, less responsive to change in objectives. However, the most recalcitrant problem in the use of the pedigree system is the difficulty of identifying high yielding genotypes in the early generations where only a limited amount of seed is available and this is normally heterozygous. To overcome this problem the breeder may delay intense selection until progenies are approaching homozygosity and sufficient seed is available for evaluation. The technique of single seed descent (SSD) first used in soybean (Brim, 1966) is one method used to achieve rapid generation advancement. Individual families are produced from single unselected seed, retained in each generation from a sample of F_2 individuals. The essential feature of this scheme is that there has been no conscious selection during the production of inbred

lines.

An alternative method for rapid generation advancement is based on the production and exploitation of haploids. Haploids are sporophytes which possess the gametic number of chromosomes (Kimber and Riley, 1963) which in barley is seven (n = x = 7). The haploid plants are usually viable, vigorous, about two-thirds the height of the diploid plants and are sterile (Kasha and Reinbergs, 1981). Chromosome doubled haploids offer the quickest possible approach to homozygosity and in addition, provide the most homozygous genotypes possible for research purposes (Kasha and Reinbergs, 1975). Doubled haploids have been used extensively in barley for cultivar production and genetical studies (Kasha, 1974). In many cereal species, including barley, the microspore is the only single cell capable of reliable regeneration *in vitro* (Dunwell, 1985a), although there has been some success in establishing a system for the efficient regeneration of rice plants from protoplasts (Cocking and Davey, 1987). Microspores, therefore, have considerable potential in the study of plant cell development (Maheshwari *et al.*, 1982). Doubled haploids have been valuable for detecting linkage associated with

quantitative characters and can also be used for calculating recombination values between linked genes (Pickering and Devaux, 1992). The production of haploids from a heterozygous F_1 (assuming random gamete selection), reflects the genotype of the sperm (anther culture) and is equivalent to backcrossing the F_1 to the double recessive. Any significant deviation from the expected ratio is, therefore, a result of linkage. The location of polygene controlling quantitative characters such as yield has not been easily carried out. However, production of doubled haploids from crosses between parents with contrasting marker genes may be of considerable help (Choo *et al.*, 1985).

Polymorphic genetic markers have many applications in plant breeding, e.g. varietal and parental identification and the chromosomal location of traits of economic importance. Studies based on the transmission of Mendelian genetic markers provide a powerful analytical tool, allowing many genetical principles to be tested. The theory exploits the fact that a marker locus identifies a chromosomal segment and enables that segment to be monitored in subsequent generations of selfing or crossing. The potential usefulness of this approach is

limited by the number of markers available in many plant species. Furthermore, many morphological markers have undesirable effects on plant phenotypes and their use in crop improvement have been limited. The subsequent development of isozyme and molecular markers such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) have represented a significant improvement since they offer greater diversity (Tanksley, 1983; Tanksley *et al.*, 1989; Williams *et al.*, 1990).

1.2. Objectives of the Present Study

Followings are the objectives of this study:

(a) Investigate anther culture response in a range of barley cultivars on maltose and sucrose-based medium.
(b) Heritability study of anther culture responsiveness into F_1 hybrids and apply isozyme and PCR-based techniques to monitor the gametic sample of doubled haploid progeny.
(c) Examine the linkage relationships of molecular markers (RAPD) in doubled haploid progeny.

REVIEW OF LITERATURE

1.3. Methods of Producing Doubled Haploids in Barley

Haploids are sporophytes that have the gametophytic chromosome number. Haploids can be produced in barley by a number of ways. The most commonly used method is anther or isolated microspore culture. Other techniques such as the *bulbosum* method, ovule culture and *hap* initiator gene are restricted to the production of one haploid per floret, compared to thousands of microspores available within an anther. However, the *bulbosum* method, resulting from a cross of barley or wheat with pollen of wild species *Hordeum bulbosum*, is the established method for haploid production in barley (Kasha and Kao, 1970) and exploits the phenomenon of chromosome elimination. Fertilization occurs normally but *H. bulbosum* chromosomes are gradually and preferentially eliminated from the zygote. Embryos are rescued by embryo culture and chromosome doubling is used to produce doubled haploids. Barclay (1975) demonstrated that the pollination of Chinese spring wheat with tetraploid *H. bulbosum* also led to chromosome elimination and production of haploids in wheat. The method was,

however, restricted only to a few lines in wheat which carry the recessive alleles of the crossability genes Kr1 and Kr2 (Falk and Kasha, 1983; Snape *et al.*, 1979). Some progress have been made in barley breeding using *H. bulbosum* derived haploids. The cultivar Mingo was produced by this technique (Kasha and Reinbergs, 1980). The highest reported frequency of haploids produced by *bulbosum* method is 23 doubled haploids per 100 florets while the average frequency is in the range of 5-10 (Kasha and Reinbergs, 1981).

Techniques involving the production of haploids from pollen cells have an intrinsic potential advantage over techniques involving egg cells as there is an immense number of pollen cells (approximately 9000 per floret for barley), compared to the one egg cell available per floret, giving an opportunity to regenerate plants more efficiently. The culture of pollen is also more universally applicable than ovule culture, which has been successful in a restricted range of crops, such as barley, rice and maize (Wenzel and Foroughi-Wehr, 1984). Despite the success of the *bulbosum* system, it has disadvantage that it is more labour intensive and, therefore, less cost effective than anther culture (Snape

et al., 1986).

1.4. The *In Vivo* and *In Vitro* Development of Pollen

The embryogenetic potential of the microspore cannot be fully understood without considering the development of the pollen grain (Wulff and Maheshwari, 1938). In normal gametophytic development, barley pollen grains are formed within the four locules of the anther (Figure 1.1) by a meiotic division of the pollen mother cell (PMC) to form a tetrad of thin-walled, non-vacuolated cells from which four immature pollen grains (microspores) develop (Sunderland and Dunwell, 1977). A vacuole develops as the microspore develops rapidly in volume and a pore becomes apparent in the exine wall. Further expansion of the vacuole occurs as the nucleus assumes a position diametrically opposite the pore. The nucleus then undergoes the first, asymmetric, pollen grain mitosis (PGM) which cuts off a small generative cell from the remainder of the grain which comprises the vegetative cell. The vegetative nucleus then migrates to a position adjacent to the pore, the vacuole is resorbed and cytoplasmic constituents are synthesized and accumulated in the plastids. During this phase, in tricellular

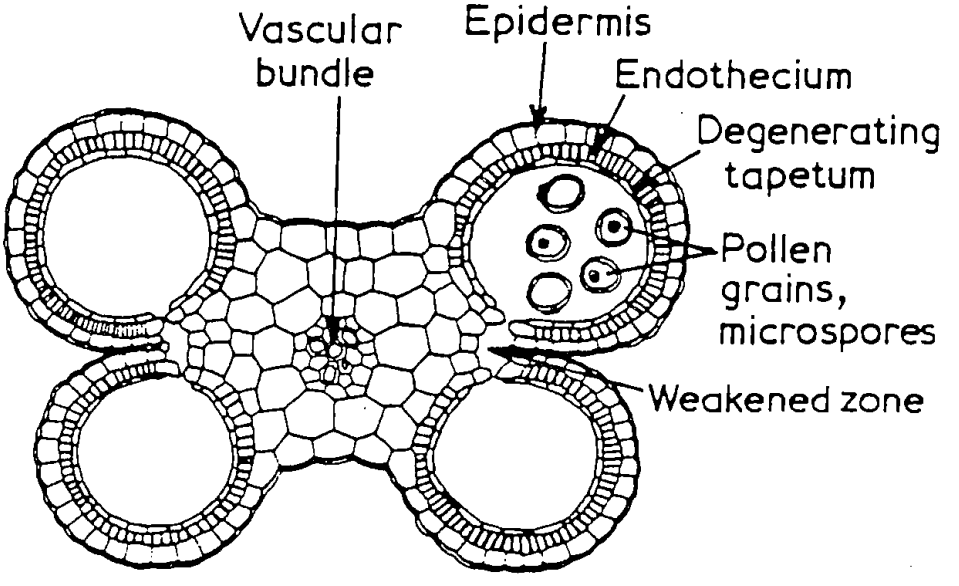

Figure 1.1

The anther of barley stamen (near maturity) in transverse section (after Briggs, 1978).

species, including the Gramineae, the generative cell moves away from the inner surface of the pollen wall and undergoes the second PGM resulting in a mature grain with two sperm cells suspended in the vegetative cell cytoplasm (Dunwell, 1985a). In bicellular species such as the Solanaceae, the second PGM occurs after the generative nucleus has migrated into the pollen tube following germination. Normal developmental sequence of an angiosperm pollen is diagrammatically presented in figure 1.2.

Microspores can be induced on an appropriate culture medium to divert from this gametophytic pathway of development. They develop either by a series of organized cell divisions to form embryoids after extrusion from the exine wall or by the formation of disorganized multicellular callus masses. Further differentiation of root and shoot meristems is followed by development into plants with the gametophytic number of chromosomes. These are then doubled, either spontaneously or through the use of chemical doubling agents, to give doubled haploid lines (Foroughi-Wehr and Friedt, 1984).

The first successful microspore cultures were carried

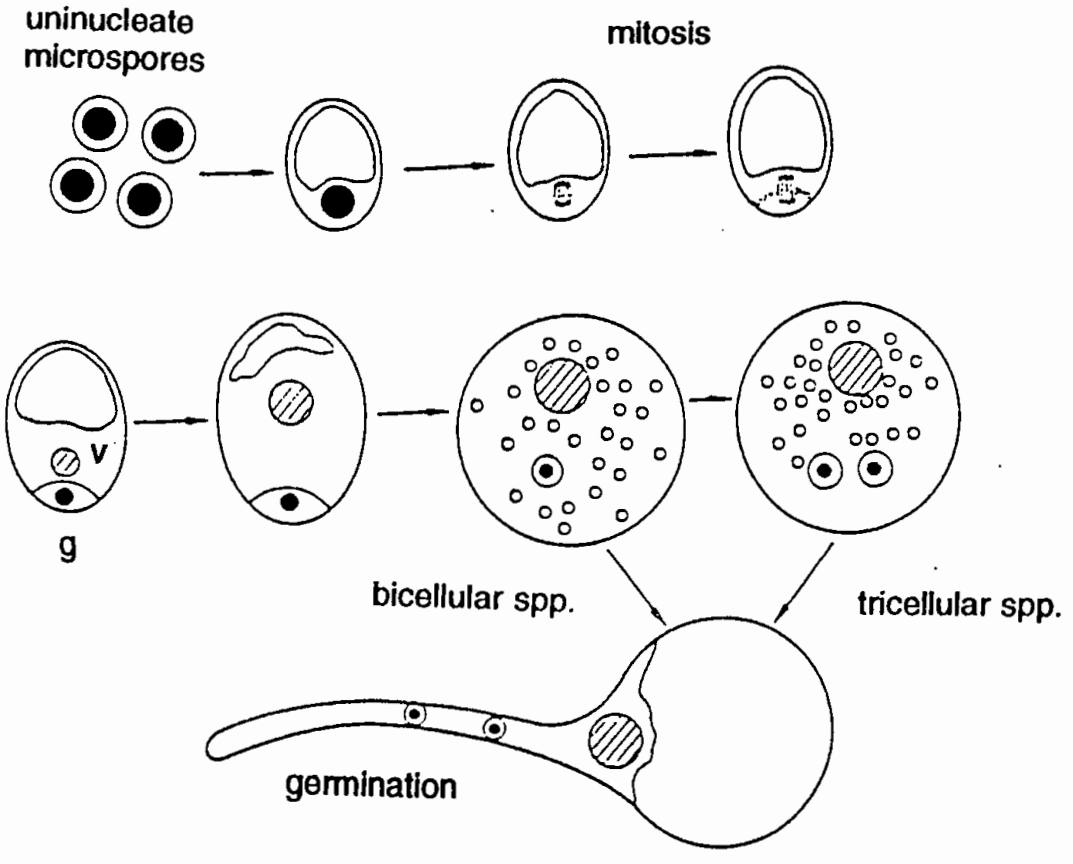

Figure 1.2

Normal developmental sequence of an angiosperm pollen grain following its release from the tetrad as a uninucleate microspore (Sunderland, 1973). The spores enlarge and undergo an unequal division to give rise the generative (g) and vegetative (v) cells. In tricellular species the generative cell undergoes second pollen mitosis resulting in a mature grain with two sperm cells suspended in the vegetative cell cytoplasm while in bicellular species the second pollen mitosis occurs after the generative cell migrates into the pollen tube after germination.

out in the 1950s by Tulecke who demonstrated that mature pollen grains of certain gymnosperms could be switched over from their normal role into callus formation by being cultured in an appropriate medium (Sunderland, 1973). Not all the grains responded in this way. Some showed other forms of behaviour of relatively little significance, while many continued their normal determinate growth pattern and gave rise to pollen tubes and sperms (Sunderland, 1973). Attempts by Tulecke and others to stimulate angiosperm pollen in a similar manner were unsuccessful. Whenever growth occurred it was always in the direction of pollen tube formation. Guha and Maheshwari (1964) discovered that the pollen of an angiosperm, *Datura innoxia*, could be triggered into active growth by culturing anthers (anther culture). This discovery was all the more remarkable because the growth of the pollen was organized and led to the development of haploid plants (Guha and Maheshwari, 1967). Each plant was derived from a single pollen grain. Since then, anther culture has been recognized world-wide as a potentially useful tool in the rapid production of doubled haploids for breeding programmes, and there have been intensive efforts to apply the technique to crops of economic importance on a large

scale (Foroughi-Wehr *et al.*, 1976). Following the success with *Datura innoxia*, response of microspore to culture was soon demonstrated in tobacco (Nitsch and Nitsch, 1969) and rice (Niizeki and Oono, 1968). Barley was first shown to be responsive by Clapham (1971). Whole anthers are usually cultured on a solid medium although liquid medium has been used (Kao, 1981). There has been some success with the culture of isolated microspores (Sunderland, 1978), by the dehiscence of anthers to release microspore into the medium (Sunderland and Xu, 1982; Ziauddin and Kasha, 1990) or by homogenization of anthers and centrifugation to remove the somatic tissue (Cho and Zapata, 1988; 1990). However, the use of these systems has been limited with barley as the viability of the isolated microspores is drastically reduced (Dunwell, 1985b). Another technique is to culture whole inflorescence (Wilson, 1977) but although this system reduces the time needed to process material, the culture yields do not compare with other methods (Wenzel and Foroughi-Wehr, 1984).

1.5. Limitations in the Use of Anther Culture

The utilization of anther culture technology in barley

breeding programmes has so far been restricted because of three major limitations. Firstly, the overall yield of green plants has been very low with large number of albinos being regenerated. Secondly, there are large genotypic differences in anther culture response, as has also been found with the *bulbosum* method and results could not be generalized (Wenzel and Foroughi-Wehr, 1984). Thirdly, there are problems of genetic instability associated with the fact that plant regeneration in barley anther culture has been achieved predominantly via an intermediate callus phase. Regenerated plants show chromosomal disturbances (Powell *et al.*, 1986) and phenotypic variation at the whole plant level (Powell *et al.*, 1984).

1.6. Approaches to the Improvement of Anther Culture Response

Research for the improvement of anther culture response in barley has focused on two main areas. Since the first report of barley anther culture by Clapham in 1971, barley has been the subject of numerous studies in an attempt to clarify the influence of physiological and environmental factors affecting response, and also to find ways of optimizing anther culture protocols so that

they can be applied to any cultivar of interest (Sorvari and Schieder, 1987). The alternative approach has been to identify responsive genotypes and try to incorporate the genes responsible into varieties of interest (Foroughi-Wehr and Friedt, 1984).

1.7. Physiological Factors Affecting Anther Culture Response

1.7.1. Growth Conditions of Donor Plants

Growth conditions of the donor plants have a profound effect on anther culture response. The physiological status of the plants at the time of anther excision also strongly influenced the sporophytic potential of microspores within the anther (Foroughi-Wehr and Mix, 1979). The suggestion that donor plants should be grown at 12^{0}C for the best results has been substantiated by several workers (Foroughi-Wehr and Mix, 1979; Hunter, 1987). Differences in photoperiod and light intensity lead to a high degree of variation in anther productivity. Light intensities as high as 10,000 lux have been recommended for barley (Lyne *et al.*, 1986). Studies have been shown that field-grown materials could be responded better than glasshouse-grown plants

(Clapham, 1973). Growth of the plants in controlled-environment cabinets has been recommended to provide more uniform material for culture (Lyne *et al.*, 1986) and also to avoid problems of plant pathogens since it is thought that chemical disease control measures severely impair the viability of the pollen (Sunderland *et al.*, 1981; Wenzel and Foroughi-Wehr, 1984). It is customary to supply plants with additional nutrients (Lyne *et al.*, 1986) although it has been shown in *Nicotiana* species that nitrogen starvation can induce a higher level of response (Sunderland, 1978). The age of the donor plant also affects anther culture response, with the first-formed spikes being the most productive (Powell, 1988).

1.7.2. Stage of Pollen Development

The importance of sampling anthers at a precisely defined stage of development has been stressed (Dunwell, 1985a). Sunderland (1974) has published a standard method for identifying the optimum stage of development for culture of microspores but the stage can vary between different crop species. In barley, culture of microspores at the mid-uninucleate stage of development is the most

successful, although the precise stage can vary between genotypes and different methods of cultivation of donor plants (Sunderland, 1974). The relative developmental stages of barley pollen are diagrammatically set out in figure 1.3.

1.7.3. Anther Pretreatment

The use of temperature-stress pretreatments has become a routine in anther culture protocols. In barley, pretreatment at 4^0C for a period of 21-28 days is recommended (Huang and Sunderland, 1982) although this optimum contrasts sharply with those used for some other species. The method of pretreatment can also be important (Huang and Sunderland, 1982), with the treatment of deawned whole spikes in Petri dishes being more successful than the treatment of intact tillers in polythene bags, possibly due to the additional effect of stress placed upon excised tillers. Cold pretreatment is known to affect the number of pollen grains diverted from the gametophytic pathway of development (Huang and Sunderland, 1982). When anthers are cultured directly from the plant without an intervening pretreatment, induction frequencies and subsequent callus/embryoid

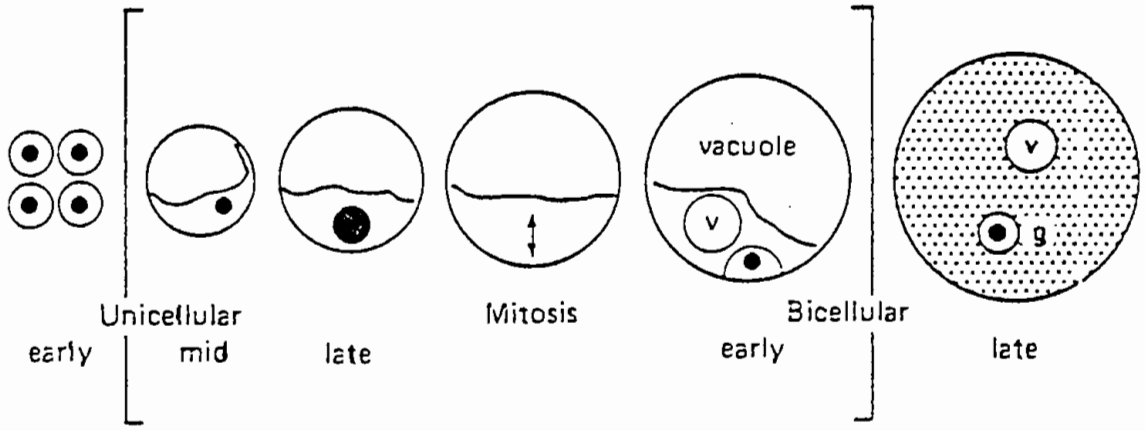

Figure 1.3

Diagram indicating the nomenclature for developmental stages in pollen ontogeny (Sunderland, 1973). The term microspore (or spore) is used to denote the haploid cells formed by meiosis. After release from the pollen meiocytes, the spores enlarge and undergo an unequal division to give rise the generative (g) and vegetative (v) cells. These two cells comprise the male gametophyte or pollen grain.

production are usually low. Pollen that has not been diverted from the gametophytic pathway, and diverted pollen that has not begun to divide, die rapidly (Sunderland, 1982).

1.7.4. Culture Medium

The culture medium has been considered to be one of the most important factors affecting anther culture. However, evidence suggests that the prior variables of donor plant growth, pollen stage and cold pretreatments act to influence the induction of androgenesis while the medium affects the division of the microspores and their subsequent development into multicellular structures followed by plant regeneration (Sunderland, 1982). It is important to adjust the cultural conditions so that the pollen divides but not the somatic anther tissues. However, although divisions in the somatic tissue can occur in rice (Mok and Woo, 1976) and some other species but it has never been observed in barley anther cultures (Wenzel and Foroughi-Wehr, 1984).

Culture media for barley anther culture, as with other tissue culture systems, are usually developed

empirically, often without a great deal of evidence for the actual effects of many of the constituents. Early protocols were based on undefined compounds such as coconut water (Steward and Caplin, 1952). Potato extract media were developed relatively successfully for cereal anther culture (Anonymous, 1976; Chuang *et al.*, 1978) as was the N6 medium of Chu (1978). Culture medium now generally recommended (Lyne *et al.*, 1986) for anther culture of barley is based on that developed by Foroughi-Wehr *et al.* (1976) which is essentially a modification of the Linsmaier and Skoog medium (1965). The advantage of using the defined media has also been stressed when methods of media preparation are considered. Sterilization of the medium by autoclaving can affect its composition not only by affecting the stability of hormones but also by causing breakdown of the carbohydrate to other sugars (Ball, 1953). For this reason filter-sterilization of the medium has been recommended (Lyne *et al.*, 1986).

1.7.4.1. Solidifying agents

It has been found for many years that most types of agar used to solidify culture media contain compounds that are

deleterious to microspore survival (Kohlenbach and Wernicke, 1978). Agarose has been suggested as an alternative to agar and it is now used routinely for several cereal species including barley (Lyne *et al.*, 1986). In barley, the use of alternative and cheaper gelling agents has been investigated by Sorvari (1986a, b) who has shown that plant starches, particularly barley starch, are superior to agar as solidifying agents and can promote the response of microspore in culture. The use of liquid media has also been proposed as an alternative to agar-solidified media (Sunderland, 1978; Kao, 1981). One of the problems with this system has been that although high yields of calli or embryoids may be obtained, plant regeneration is hindered probably due to the anaerobic conditions imposed on the calli which sink below the surface of the liquid (Dunwell, 1985b). The addition of Ficoll to increase the buoyancy of the cultures has been recommended for barley (Kao, 1981) and may give significant improvements in plant yield.

One advantage of using solidified media is that the orientation of the anther can be controlled (Dunwell, 1985a). This has been considered in a number of species including barley (Hunter, 1985; Shannon *et al.*, 1985;

Powell *et al.*, 1988) and anther orientation has been shown to have a considerable effect on response, where anthers cultured on edge with only one locule in contact with medium show much higher levels of response than those cultured flat with both locules in contact, suggesting that some component of the medium is inhibitory to continued development of the microspores (Powell *et al.*, 1988).

1.7.4.2. Mineral salts and organic supplements

There is little information on the effects of major salts on the different stages of microspore development in culture (Dunwell, 1985a), apart from the general observation that they are important in post-induction development (Sunderland, 1974). However, it is known that iron, a minor mineral component in the medium, is involved in the development of pollen embryos from the globular to the heart stage (Heberle-Bors, 1985). There are insufficient information about the specific effects of the organic supplements used in the medium. In some species, such as wheat, maize and triticale, glutamine has been found to partially replace the beneficial effects of potato extract medium (De Buyser and Henry,

1986). Glutamine has also been found to be beneficial in barley anther culture (Olsen, 1987). The addition of inositol is recommended in barley for the growth of multicellular microspores (Wenzel and Foroughi-Wehr, 1984).

Species have been separated into those (Gramineae, Cruciferae) which require the addition of hormones to anther culture media and those (Solanaceae) considered to be hormone independent (Dunwell, 1985b). However, there is some suggestion that cereals may not require exogenous hormones, at least in the early stages of culture (Dunwell, 1985b). This may be due to the type of structures produced from dividing spores. Until recently it was thought that whilst certain species favour regeneration of plants via a direct embryogenic route, cereal always form a disorganized callus from which shoots and roots can be induced to form (Dunwell, 1985a). Auxin is required for callus induction, but it may not be necessary for embryo induction. Embryo formation has now been demonstrated in barley (Dunwell, 1985a; Lyne *et al.*, 1986) and the addition of auxin may be disadvantageous if it diverts the pollen into callus induction instead of embryos as was shown by anther cultures of henbane

(*Hyoscyamus niger*) on medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) (Raghaban, 1978) and also with rice (Lin *et al.*, 1984). These effects might be less pronounced with indole acetic acid (IAA) which is considered to be a less active auxin than 2,4-D (Dunwell, 1985a). Improvements have been demonstrated in barley using a variety of combinations of hormones including kinetin + 2,4-D (Huang and Sunderland, 1982) and IAA + 6-benzylaminopurine (BAP) (Wenzel and Foroughi-Wehr, 1984) but any categorical recommendations about hormone requirements is not practicable (Dunwell, 1985b).

1.7.4.3. Carbohydrate composition

A common requirement for all tissue culture media is a carbon supply and in most anther culture protocols sucrose is recommended as the sole carbon source (Wenzel and Foroughi-Wehr, 1984). However, the research has suggested that sucrose may not be the optimal carbohydrate for all tissue culture systems (Kochba and Spiegel-Roy, 1973 ; Kochba *et al.*, 1982; Hunter, 1987). Furthermore, in barley (Wei *et al.* 1986) and *Nicotiana* species (Aruga *et al.*, 1985; Kyo and Harada, 1985), isolated microspores have been deprived of sucrose for

several days in order to induce embryogenesis or callus formation. Maltose, an α-1,4 linked glucose disaccharide, has been found to be beneficial for embryo induction in anther culture of *Petunia* (Raquin, 1983) and for somatic embryogenesis of *Medicago sativa* (Strickland *et al.*, 1987).There is little standardized information about the optimum concentration at which the carbohydrate should be employed and little information about the extent to which it has an osmotic role (Dunwell, 1985a). Various investigations have separated species into those requiring low sucrose concentrations (2-4% w/v) and those requiring higher concentrations (8-12% w/v). This separation seems to be related to the known distinction between those with bicellular mature pollen (Solanaceae, Liliaceae) and those with tricellular pollen (Gramineae, Cruciferae) with the former group requiring low osmotic conditions and the latter group high osmotic conditions for germination (Dunwell, 1985b). It has been shown in experiments with oil seed rape that media containing high levels of sucrose (16% w/v) support higher level of microspore viability and embryogenic division than the normal concentration of 8% (Dunwell and Thurling, 1985). In barley, low concentrations (2% w/v) of sucrose can inhibit androgenesis (Sorvari and Schieder, 1987) while

high (6-12% w/v) concentrations seem to favour direct embryogenesis. Higher levels of carbohydrate are not, however, conducive to plantlet regeneration and it is usual to transfer callus or embryoids to a medium containing lower carbohydrate (and hormone) concentrations to allow plant formation.

1.8. Incubation Conditions

Temperature is the most critical, and most investigated, of the incubation variables (Dunwell, 1985b). Anthers of most species including barley and the Solanaceous crops, respond well if cultured at 25^{0}C but some, particularly Brassicas, require short periods at 35^{0}C or even higher (Dunwell, 1985b). Little is known about the effect of light quality and quantity on microspore development but the usual recommendation is for incubation in darkness until the time of emergence of the microspore-derived embryos or calli (Dunwell, 1985b, Hunter, 1987), followed by incubation in continuous light.

In barley there are evidences that the density of anthers in culture is important (Sunderland *et al.*, 1981). It is known that cell constituents essential to the development

of multicellular structures leach out of the anthers and so high densities are recommended (Sunderland *et al.*, 1981). The pH of the medium is also important. Huang (1982) showed that anthers easily turned brown and died when cultured on media pH 6 and above.

1.9. The Genetic Approach to Anther Culture Improvement

The effect of genotype is one of the most important factors that affect the efficiency of anther culture (Dunwell *et al.*, 1987). While environmental manipulations have been shown to change the developmental responses of some genotype *in vitro*, it has not so far been possible to produce a uniformly high response among all genotypes. It has been proposed that culture conditions could be optimized for each genotype of interest (Dunwell, 1981). It is, therefore, of practical importance to identify the means by which genetic control of differentiation responses *in vitro* operate (Lyne *et al.*, 1986). Results obtained from a number of studies have shown that anther culture responsiveness is heritable (Foroughi-Wehr *et al.*, 1982). It also appears to be under the genetic

control of at least two separate and independently inherited mechanisms which affect firstly, the ability of microspores to develop into callus and secondly, the ability of these calli to differentiate into plants.

1.10. Polymorphic Assay Procedures

1.10.1. Isozymes

The development of electrophoresis and enzyme specific stains has provided many genetic markers, the variants of which generally have little deleterious effects on phenotype. Isozymes or multiple molecular forms of enzymes, are enzymes that share a common substrate but differ in electrophoretic mobility (Markert and Moller, 1959). The majority of isozymes exhibit codominant expression and relatively high levels of polymorphism (Tanksley, 1983). Isozyme variability has been used extensively for a range of purposes such as, delineating phylogenetic relationships (Ayala, 1975), estimating population genetic parameters (Brown, 1979), producing preliminary linkage maps (Goodman *et al.*, 1980), tagging morphological and physiological characters of interest for convenient screening in breeding programmes (Tanksley

et al., 1982) and characterizing germplasm collections (Brown, 1978). Polymorphic isozyme and storage protein systems have been investigated for use in the classification of a wide range of crops including barley (Neilson and Johansen, 1986). Thompson *et al.* (1990) have used isoelectric focusing in conjunction with seven protein and isozyme marker phenotypes to uniquely characterize 27 out of 29 barley varieties examined. However, many plant species, particularly inbreeds, exhibit low levels of isozyme polymorphism (Nevo *et al.*, 1979). Furthermore, many of these biochemical markers have the disadvantage of being developmentally regulated, with phenotypes expressed only at certain stages of development or only in some specific tissue or organ. Isozymes and molecular markers may provide a convenient means of assessing population structure and diversity. If one assumes that polymorphism for the marker system is indicative of variability for other characters, then this form of screening may ensure that germplasm resources represent a wide spectrum of genetic variability. The first systematic isozyme studies of barley were carried out by Frydenberg and Neilsen (1965), and Nilson and Hermelin (1966). More extensive studies on barley isozymes is limited primarily by their general

lack of polymorphism. The restricted number of isozyme loci and relatively low levels of polymorphism has limited the application of biochemical markers in barley breeding programmes. Associations between isozyme and protein loci with genes controlling spring/winter growth habit in barley cultivars (Forster *et al.*, 1991) and with a range of quantitatively controlled traits in barley doubled haploids (Powell *et al.*, 1990), have been reported.

1.10.2. Polymerase Chain Reaction

Genetic markers are of great value in breeding programmes and in genetic research (Weining and Langridge, 1991). Traditionally, markers based on morphological differences between individuals have been used. Restriction fragment length polymorphisms (RFLPs) have introduced a new dimension to the development of genetic maps and mapping of agronomically and physiologically important traits. The major strength of RFLPs is that they have the potential to reveal an unlimited number of polymorphisms (Weining and Langridge, 1991). Molecular genetic maps are commonly constructed by analyzing the segregation of RFLPs among the progeny of asexual cross (Soller and

Beckmann, 1988). A further recent methodological advance has been the development of the polymerase chain reaction (PCR). This is a very powerful technique, now used in many areas of biology, allowing *in vitro* amplification of specific DNA sequences from undetectable quantities of target DNA (Saiki, 1990). PCR can be used to detect polymorphisms in the length of amplified sequences between the annealing sites of two synthetic DNA primers (Weining and Langridge, 1991). When the distance varies between two individuals the banding pattern generated by the PCR reaction is essentially a genetic polymorphism and can be mapped in the same way as other genetic markers. PCR now makes possible the rapid development of chromosome markers for many organisms by disclosure of useful polymorphisms (Ledbetter *et al.*, 1990; Love *et al.*, 1990). PCR is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers hybridizing to opposite strands of the target sequence with their 3' ends pointing towards each other (Mullis and Faloona, 1987; Saiki *et al.*, 1988). The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotide primers and the four deoxyribonucleotide triphosphates (dNTPs). The reaction mixture is then

cooled to a temperature that allows the oligonucleotide primers to anneal their target sequences, after which the annealed primers are extended with DNA polymerase. Repeated cycles of heat denaturation of the template, annealing of their primers to their complimentary sequences and extension of the annealed primers with a thermostable DNA polymerase (*Taq*) isolated from the thermophilic bacterium *Thermus aquaticus*, result in the amplification of the segment defined by its 5' ends. Since the extension product of each primer can serve as a template for the other primer, each successive cycle essentially doubles the amount of DNA fragment produced in the previous cycle (Figure 1.4). This results in the exponential accumulation of the specific target fragment up to several million-fold in a few hours. The application of PCR was examined by Williams *et al.* (1990) and Welsh and McClelland (1990) who proposed, instead of using a pair of carefully designed and fairly long oligonucleotide primers, the use of a single, short oligonucleotide primer, which binds many different loci, to amplify random sequences from a complex DNA template and the markers generated are called Randomly Amplified Polymorphic DNAs (RAPDs). When genomic DNA is used as a template, it is possible to amplify a specific single

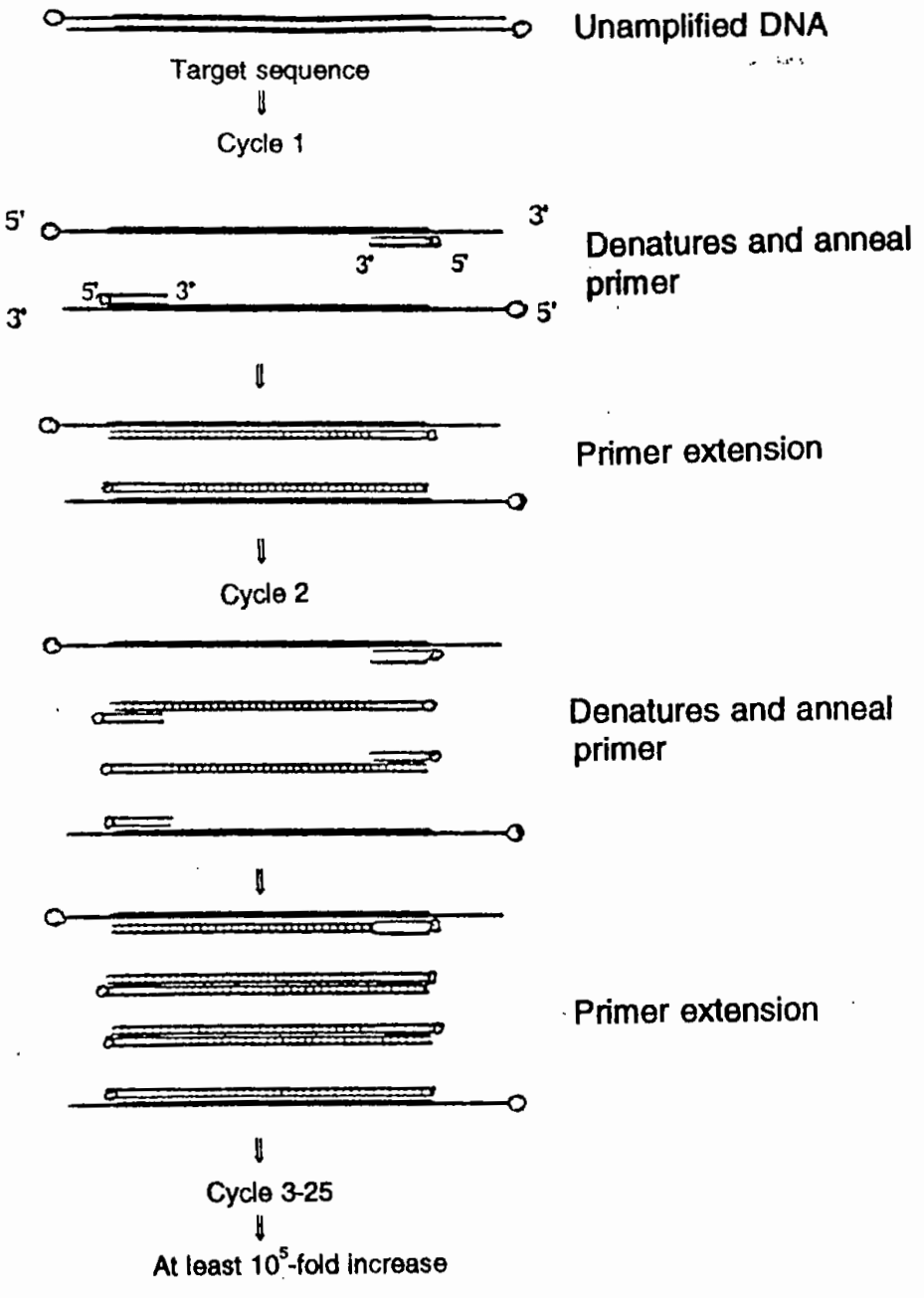

Figure 1.4

PCR based amplification of a DNA fragment.

gene by the selection of the appropriate primers. The length and sequence of the primers were found to exert a major influence on the resulting amplification products. For plants the nucleotide sequences of each primer must be at least 9-10 base pairs long, between 50 and 80% G+C (G=Guanine, C=Cytosine) in composition and contain no palindromic sequences. PCR based markers have been exploited in plant genetic studies for the amplification of wheat genomic sequence (D'Ovidio *et al.*, 1990), detection and mapping of polymorphic DNA markers in tomato (Klein-Lankhorst *et al.*, 1991), development and chromosomal localization of genomic-specific markers in *Brassica* (Quiros *et al.*, 1991), creating linkage maps (Rafalski *et al.*, 1991; Rieseberg *et al.*, 1993), locating disease resistance genes (Martin *et al.*, 1991; Michelmore *et al.*, 1991; Paran and Michelmore, 1993), genetic fingerprinting (Wilde *et al.*, 1992) and characterization of somatic hybrids (Baird *et al.*, 1992).

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant Material

Four two-rowed spring barley cultivars viz. Abbey, Dallas, Bianka and Decado were used to study anther culture response in a range of maltose and sucrose-based media.

In order to study the inheritance of anther culture response, *Hordeum vulgare* cv. Prisma (P), four *Hordeum spontaneum* lines (HS2, HS10, HS16 and HS20), and the four F_1 hybrids (PXHS2, PXHS10, PXHS16 and PXHS20) were studied. Following convention, the first name in the cross corresponds to the maternal parent. *H. spontaneum* lines are spring barley types and carrying resistance genes for *Erysiphe graminis* f. sp. *hordei* (Jahoor and Fischbeck, 1987). The regenerants from the Prisma X HS2 F_1 hybrid were used to monitor the segregation of protein and RAPD marker.

For genetic marker analysis, a commercial spring cultivar Blenheim, an advanced SCRI line TS264 and 33 doubled

haploid lines derived from F_1 hybrid of Blenheim X TS264 were used. Seeds of all cultivars, parental lines, F_1s and doubled haploids were obtained from the Scottish Crop Research Institute (SCRI), Dundee, Scotland.

2.2. Growth of Donor Plants

Anther culture donor plants were grown in pots under glasshouse condition with approximately a 14 h photoperiod at a temperature 16 ± 2^0C. Each genotype was represented by 10 individual plants.

2.3. Anther Culture Procedure

2.3.1. Sampling of Spikes and Staging

The tillers were harvested sequentially from each plant when the distance of emergence of the flag leaf (interligule length) was between 35 to 60 mm. This distance has been shown to be correlated with the developmental stage of anthers within the spike at which the microspores are at the early to mid-uninucleate stage. Tillers were removed and sheath leaves swabbed with 70% ethanol. Spikes were removed aseptically from

the tillers and a test anther dissected from one of the most developmentally advanced central spikelets of each tiller. The test anthers were then cut in half and tapped out in acetocarmine (Darlington and LaCour, 1942) on a glass slide to release and stain the microspores. A coverslip was pressed firmly over the preparation and the microspores viewed under a light microscope (Zeiss photo-microscope). Spikes were retained for culture if microspores were at stages 2-3 according to standard nomenclature of Huang (1982) adopted for barley from Sunderland (1974) (Table 2.1). Microspores at this developmental stage have a fully developed pore, thick exine and an enlarged vacuole. The nucleus is normally positioned at the opposite side of the cell to the pore.

2.3.2. Cold Pretreatment of Spikes

Spikes that were suitable for culture were placed in plastic Petri dishes (90 x 15 mm) containing a second small dish (30 mm) with approximately 3 ml of sterile distilled water to maintain humidity (Figure 2.1). The Petri dishes were sealed with Nescofilm and pretreated for 3-4 weeks in dark at 4^0C (Huang and Sunderland, 1982).

Table 2.1. Standard nomenclature for anther staging of barley after Sunderland (1974) with modification by Huang (1982).

Stage	Pollen characteristics
1	Anthers containing tetrads or young microspores just released from the callose wall. A central vacuole absent. Nucleus in pre-DNA replication phase (GI) of the cell cycle.
2	Anthers containing mid-uninucleate microspores. Exine well developed. Vacuole present but nucleus small and still in the cell cycle.
3	Anthers containing late uninucleate microspores. Nucleus enlarged and in either DNA replication phase (S) or post-DNA replication phase (G2).
4	Anthers containing microspores undergoing the first pollen division (PGM).
5	Anthers containing bicellular pollen grains with a generative cell. Generative nucleus cut off by a wall. Microspore vacuole still present.
6	Mature anthers containing starch-filled pollen grains with two gametes formed after the second PGM. No vacuole.

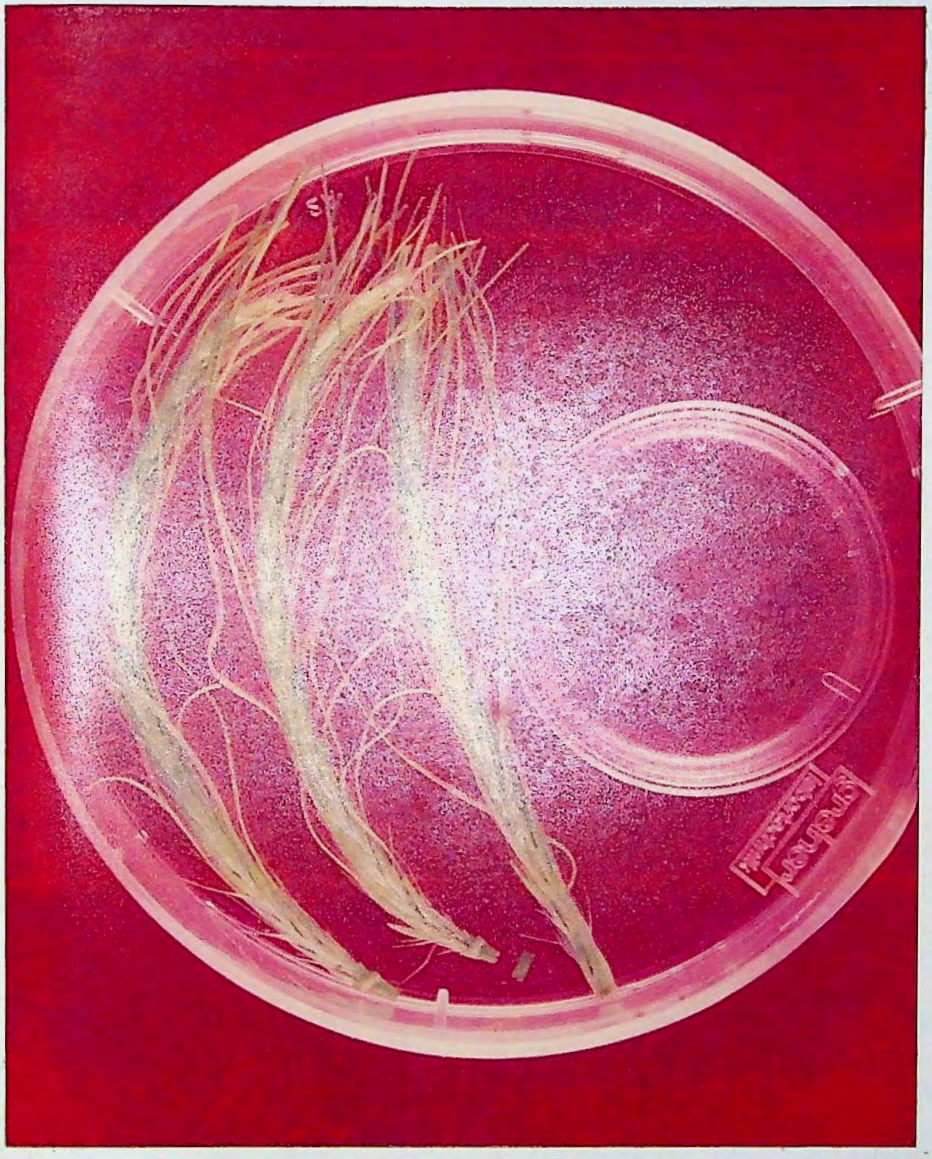

Figure 2.1

Barley spikes ready for cold pretreatment.

2.3.3. Medium Preparation

The barley anther culture medium used was based on that developed by Foroughi-Wehr *et al.*(1976) with little modification. Table 2.2 shows the composition of the medium. The major inorganic nutrients were made up as a x10 stock solution. Each ingredient was dissolved separately in a small volume of distilled water, the solutions mixed and made up to volume. The trace elements were made up as a x100 stock solution, with all the constituents dissolved together. Stock solutions of major and trace elements were then added to the organic supplements and the carbohydrate (maltose 6% w/v) made up to a volume to give a double strength solution. This was sterilized by filtration through a 500 ml Nalgene disposable filter with a 0.2 µm cellulose acetate membrane. The agarose was also made up to double strength in distilled water and sterilized by autoclaving at 121^{0}C for 15 minutes. The agarose was cooled to approximately 50^{0}C and mixed with the filtered medium under sterile conditions before pouring into 55 mm sterile Petri dishes.

The rooting medium was the same as induction medium with

Table 2.2. Composition of barley anther culture medium.

Component	
A. Major Inorganic Salts	(mg/l)
KNO_3	1900.00
NH_4NO_3	165.00
$CaCl_2$, $2H_2O$	400.00
$MgSO_4$, $7H_2O$	370.00
KH_2PO_4	170.00
NaFeEDTA	36.70
B. Trace Elements	(mg/l)
H_3BO_3	6.20
$MnSO_4$, $7H_2O$	22.30
$ZnSO_4$, $7H_2O$	8.60
Na_2MoO_4, $2H_2O$	0.25
KI	0.83
$CuSO_4$, $5H_2O$	0.025
$CoCl_2$, $6H_2O$	0.025
C. Organic Supplements	(mg/l)
Myo-inositol	100.00
Thiamine-HCl	0.40
Glutamine	700.00
*Indole-3-acetic Acid (IAA)	1.00
**Benzylaminopurine (BAP)	1.00
+Maltose	60000.00
Agarose	8000.00

+ Maltose was the sole carbon source unless stated otherwise in the text. * IAA dissolved in a few drops of 1 N NaOH, ** BAP in a few drops of 1N HCl. All media were prepared with doubled distilled water and had a final pH 5.6. This was achieved by adjusting the pH with 0.1 N KOH or 0.1 N HCl.

only exception was that the carbohydrate concentration was reduced to half (3% w/v). The sterilized medium was poured into 30 ml sterilized plastic bottle.

2.4. Culture Methods

Anthers were dissected from the central florets of each pretreated spike avoiding the smaller often sterile florets at either end of each spike and plated onto 55 mm Petri dishes containing the medium (Figure 2.2). Dishes were then incubated in darkness at 24±2^{0}C. After 28 days the anthers producing calluses and/or embryoids (Figure 2.3) was recorded. These calli or embryoids were then subcultured in the medium having the same composition and incubated in continuously illuminated conditions (1500 lux approx.) with cool fluorescent lights at 24±2°C. After 3 weeks regenerating plantlets were transferred to 30 ml plastic bottle containing rooting medium for growth and root initiation (Fig. 2.4). Rooted plantlets were then transferred to smaller pots filled with universal compost for acclimatization and eventually transferred into larger plastic pots (Figure 2.5) for growth and maturity.

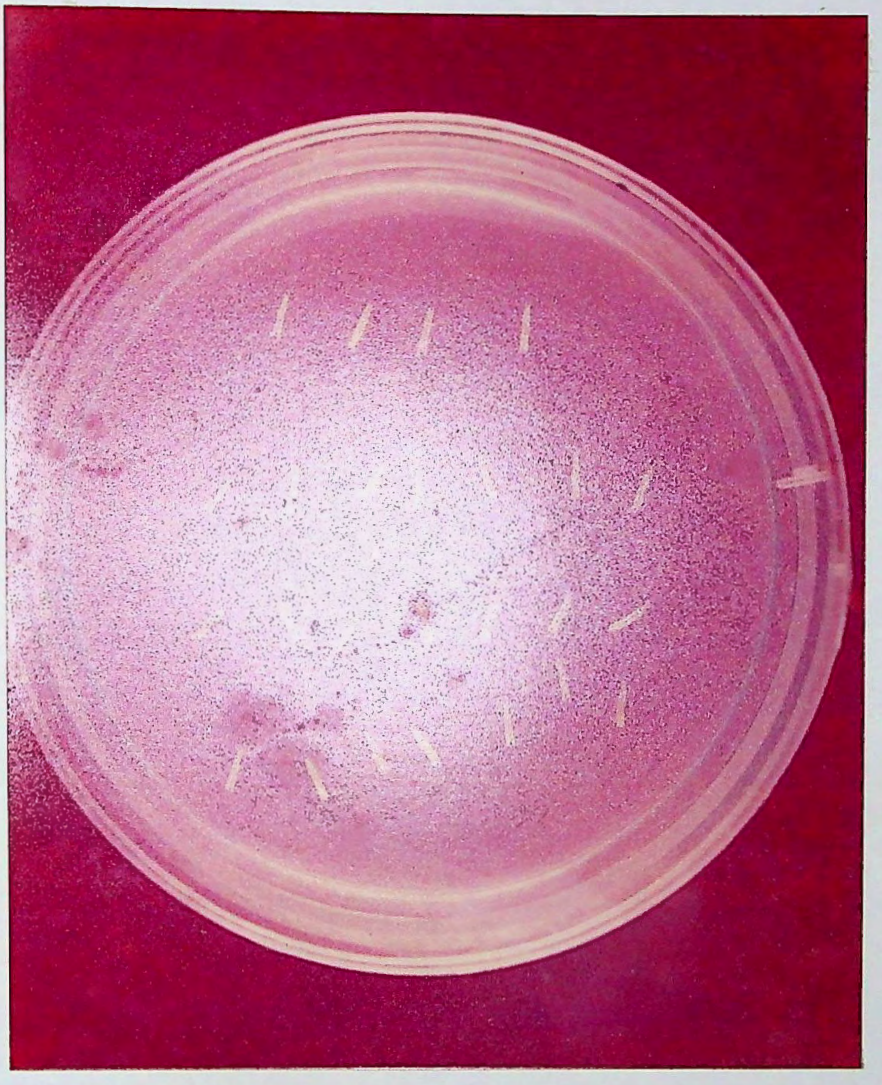

Figure 2.2

Pre-treated barley anthers on semi-solid medium at the start of culture.

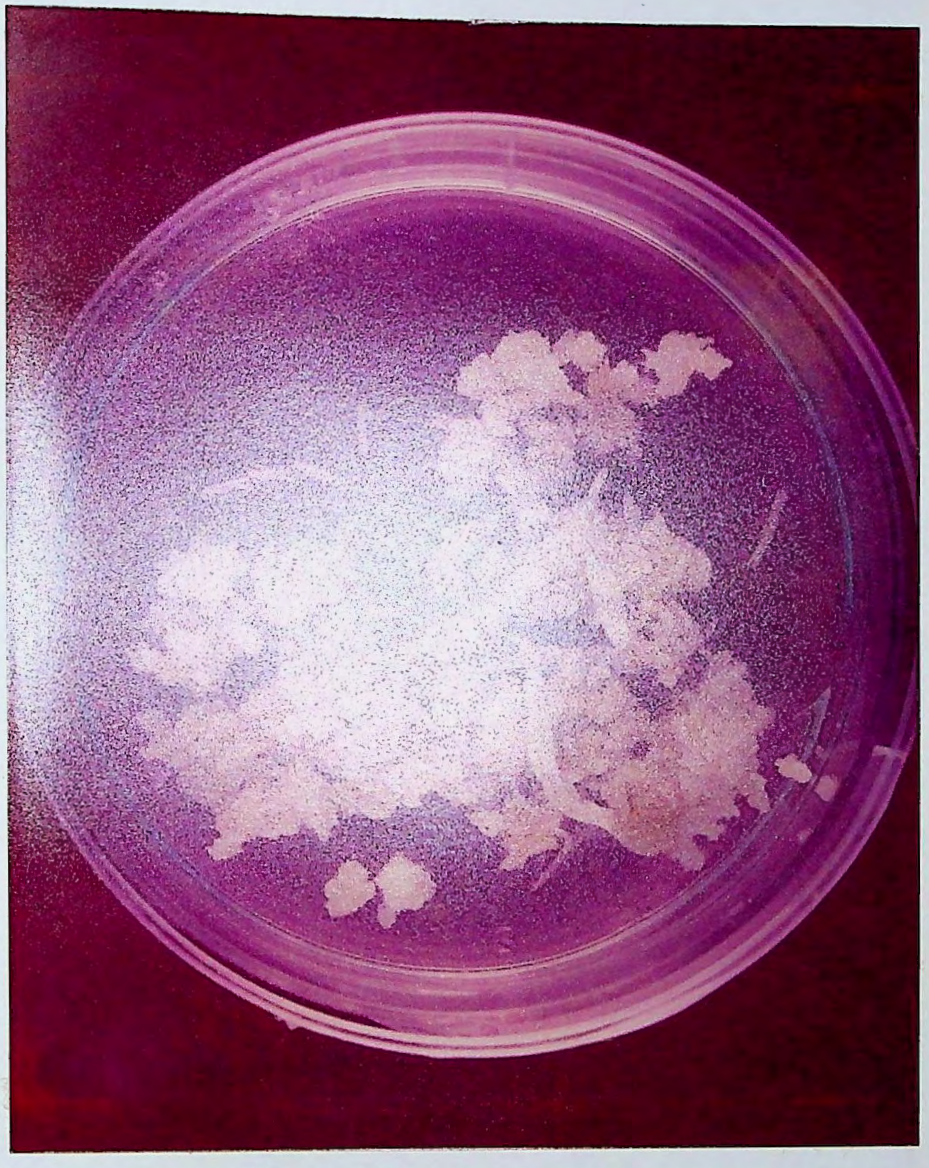

Figure 2.3

Responding anthers after 28 days of incubation in the dark at 24°C.

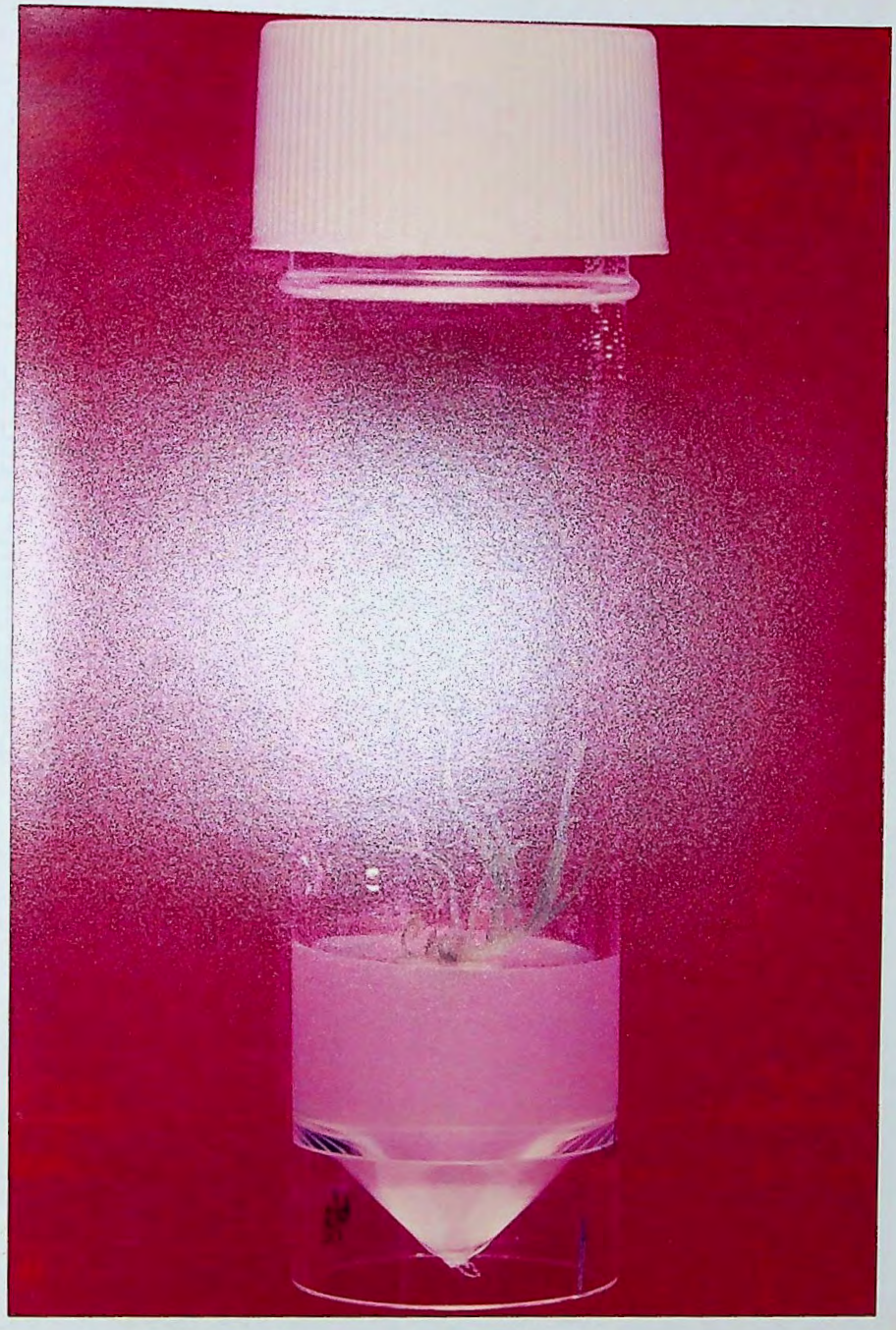

Figure 2.4

Regenerated barley plantlet in the rooting medium.

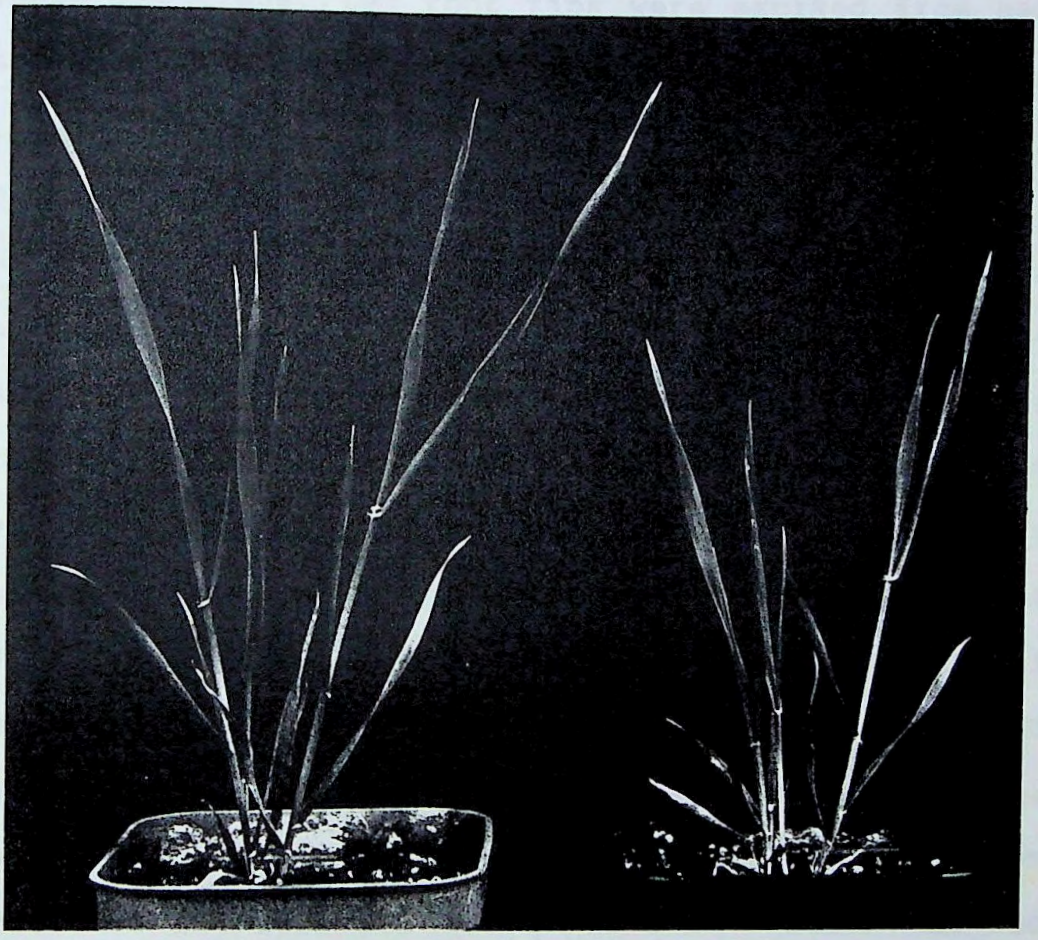

Figure 2.5

Regenerated barley plants established in soil.

2.5. Ploidy Level of Regenerants

The chromosome complement (x=7 or 2x=14) of the regenerants was determined by examining the root tips of young seedlings. Two root tips were excised from each seedling and pretreated by placing in small specimen tubes containing saturated solution of 1-bromonaphthalene. This was prepared freshly by mixing 5 ml bromonaphthalene in 100 ml tap water, stirring and allowing to settle. The tubes were left uncorked for 4-5 hours after which the bromonaphthalene was poured off and replaced with 100% glacial acetic acid. Tubes were corked and left overnight at 4°C to fix the root tips. Root tips were stained by replacing the glacial acetic acid with 1N HCl (previously heated to 60°C) and incubating in uncorked tubes at 60°C for exactly 12 minutes. The HCl acid was then replaced with Leuco-basic fuchsin (Feulgen method; Darlington and La Cour, 1942), tubes were recorked and left for 10-30 minutes to allow the root tips to stain.

The stained meristematic tip of each root was excised and placed in a drop of 45% acetic acid on a glass slide. A coverslip was placed over the preparation and a brass

tapper used to gently break up and spread the material. The slide was then placed between layers of filter paper and pressed firmly, avoiding any lateral movement of the coverslip. Chromosome preparations were examined under a Zeiss photo-microscope.

2.6. Electrophoretic Techniques

2.6.1. Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS-PAGE)

2.6.1.1. Extraction of barley storage protein (Hordeins)

Storage proteins (Hordeins) were extracted from barley endosperm samples as described by Smith and Payne (1984). 4-8 mg of endosperm were removed from each seed and crushed between pieces of filter paper using a hammer. The endosperm was transferred to an Eppendorf tube and ground to fine flour using a micropestle. The flour was mixed with 200 µl Hordein extraction buffer (10% v/v glycerol, 62 mM Tris [2-amino-2(hydroxymethyl)- 1,3-propanediol]-HCl pH 8.6, 2% w/v sodium dodecyl sulfate (SDS), 0.01% w/v Pyronin G dye [BDH Chemicals] and 5% v/v 2-mercaptoethanol), and samples were incubated at room temperature for one hour with occasional shaking and then

for 2 minutes at 100°C in a water bath to dissociate the proteins.

2.6.1.2. Gel preparation and running

Polyacrylamide gel electrophoresis was performed using the system of Laemmli (1970). This method involves the separation of proteins on the basis of molecular weight, using a vertical gel apparatus (20x15 cm gels). Separating gels consisting of 10% w/v acrylamide, 0.13% w/v NN'-methylbisacrylamide, 38 mM Tris-HCl pH 8.8 and 0.1% w/v SDS, were polymerised chemically with 0.08% v/v N,N,N',N'-Tetramethylethylenediamine (TEMED) and 0.025% w/v ammonium persulfate (APS) to give a final volume of 49.3 ml. This was poured between gel plates and overlaid with a stacking gel of 4.4% acrylamide, 0.06% bis-acrylamide, 12 mM Tris-HCl pH 6.8 and 0.1% SDS polymerised in the same way as the separating gel (final volume 21.6 ml). The electrode buffer (pH 8.3) contained 25 mM Tris-HCl, 0.188 M glycine and 0.1% SDS. 40 µl protein extract from each sample was located onto the gel and run at a constant current 20 mA until the Pyronin G dye front had run to the end of the gel.

Following electrophoresis, the gels were stained by immersion for 2 days in a solution of 0.02% w/v Coomassie Brilliant Blue R (in 4.75% v/v ethanol and 6% w/v trichloroacetic acid [TCA]). Gels were destained by immersion in H_2O for 1-2 days.

2.7. Isozyme Analysis

2.7.1. Protein Extraction

a. Grain esterase

Portions of endosperm (20 mg) from mature dry grains were handmilled and mixed with 50 µl of distilled water. Samples were left to extract for 1 hour and then centrifuged briefly prior to loading supernatant onto the gel.

b. ß-amylase

Samples were extracted in 150 µl of an extraction buffer consisting of 0.1% v/v 2-mercaptoethanol in a 20 mM solution of calcium chlorid and left for 1 hour and then centrifuged briefly prior to loading onto the gel.

c. Water-soluble protein

Portions of endosperm from mature dry grains were handmilled and mixed with 70 µl of 10 mM dithiothreitol (DTT) with sucrose added at a concentration of 200 mg/ml. Samples were left to extract for 1 hour and then centrifuged briefly prior to loading onto the gel.

d. Leaf esterase

Grains were germinated in Petri dishes and left until the coleoptiles had reached approximately 2-3 cm in length. The coleoptiles were then detached and ground in a mortar and pestle with 200 µl of 10 mM DTT in 0.05 M Na_2HPO_4 and approximately 14 mg of polyvinyl polypyrrolidone (PVPP). After grinding the samples were transferred to Eppendorf tubes and kept on ice. Prior to loading onto the gel samples were centrifuged briefly.

2.7.2. Preparation of Thin Layer Isoelectric Focusing Gels

Polyacrylamide gels were prepared by mixing 2.0 ml of a solution of Biorad Acrylamide/bis (37:1, 0.7 g/ml) with 8 ml of 16% v/v glycerol, 0.45 ml of various ampholyte

mixes and 0.3 ml TEMED in a 25 ml conical flask. The mix was covered with a small piece of perforated tin foil and degassed in a desiccator jar attached to a vacuum pump. Two glass plates are required: one 10 mm thick x 220 mm x 120 mm with a piece of dymo-tape stuck along each of the longer edges and the other 2 mm thick x 220 mm x 120 mm. Both were cleaned with 70% ethanol and then the thick plate was wiped over with Repel-Silane and the thin plate with Bind-Silane. The thin plate was placed horizontally in a gel-pouring apparatus (which held the plate firmly in position) over a few drops of water so that a thin film of water spread under the whole plate. The thick plate, with dymo-tape face down, was placed over the thin plate overlapping by 200-300 mm. To the degassed gel mixture, 0.06 ml of ammonium persulfate solution was added (10 mg/ml made up freshly each week) mixed gently by swirling and then poured onto the exposed part of the bottom plate while sliding the top plate over it to give a bubble free layer. The gels usually set in 30-60 minutes and were then wrapped in Clingfilm and stored at 5°C until required. The thick plate was separated from the gel fixed to the bottom plate immediately prior to use. The ampholyte mixtures used for each protein marker are given in table 2.3.

2.7.3. Isoelectric Focusing

The gels were placed on a Pharmacia cooling plate. Electrode strips previously soaked in appropriate electrolyte solution (Table 2.3) were placed along each side of the gel approximately 1-2 mm from the edge. Electrodes were then placed over the strips and the gels were prefocused for 500 volthours (Vh) after which sample extracts were placed on the gel surface 1 cm from the cathode using 5 x 10 mm paper wicks and run for further 3000 Vh. The paper wicks were removed after the initial 500 Vh. Power applied to the gel was 1 W/cm (with maximum voltage of 3000 V).

2.7.4. Staining Procedures

a. Esterases (Ainsworth *et al.*, 1984)

Gels were incubated in 50 mg α-napthyl acetate and 100 mg Fast Blue RR salt dissolved in 2 ml dimethyl sulfoxide and made up to 100 ml with 1 M $NaHPO_4$ (pH 7.6) for 30 minutes at 37°C and then destained in 7% acetic acid.

Table 2.3. pH gradients and electrolyte solutions used for isoelectric focusing of different biochemical markers.

Protein	pH gradient	Electrolyte solution*
Grain esterase	1:1:1 pH 4.0-6.5 pH 4.2-4.9 pH 4.5-5.4	1+2
Leaf esterase	1:1:1 pH 4.0-6.5 pH 4.2-4.9 pH 4.5-5.4	1+2
β-amylase	2:1 pH 4.5-5.4 pH 4.0-6.5	1+2
Water soluble protein (WSP)	1:1:1 pH 4.0-6.5 pH 4.2-4.9 pH 4.5-5.4	1+2

* Electrolyte solution: 1. 0.04M Glutamic acid.
2. 0.2M Histidine.

b. Amylase (Gale *et al.*, 1983)

Gels were immersed in 30 g/l soluble starch for 10 minutes, drained and then washed to remove superficial starch and stained with 3% stock iodine (65 g/l I_2, 1.95 g/l KI) and 1% acetic acid. Gels were developed in iodine solution for a further 10 minutes. White bands appeared in the blue background.

c. Water-soluble protein (Forster *et al.*, 1991)

Gels were placed in a solution of 34.6 g sulfosalicylic acid and 115 g trichloroacetic acid made up to 1 l for 15 minutes. Gels were then immersed in Coomassie Brilliant Blue R solution (0.46 g Coomassie Brilliant Blue R dissolved in 400 ml of 25% absolute ethanol and heated to 70°C) for 10 minutes. To clear the background staining gels were placed in 25% absolute ethanol with 7% glacial acetic acid.

Unless otherwise indicated plates of IEF gels are presented with the anodal end (+) uppermost.

2.8. Molecular (Randomly Amplified Polymorphic DNA Marker) Techniques

2.8.1. Growth of Plants

Plants were grown in 5 inch pots containing universal compost under glasshouse condition (Figure 2.6) for 2-3 weeks. The young leaves were harvested in polythene bags and placed immediately in box containing ice.

2.8.2. Plant DNA Isolation

Total cellular DNA was isolated from seedlings by a modified version of the CTAB method (Saghai-Maroof *et al.*, 1984). 3 gm of fresh leaves were rapidly frozen by liquid nitrogen and ground to a fine powder by mortar and pestle. The powder was then dispersed in 6 ml extraction buffer (50 mM Tris pH 7.5, 0.7 M NaCl, 10 mM disodium ethylenediaminetetraacetic acid (EDTA), 1% hexadecyltrimethylammonium bromide (CTAB), 0.1% 2-mercaptoethanol) and incubated at 60^0C for 60 minutes with occasional mixing. 4.5 ml of chloroform/IAA (iso-amyl alcohol), 24:1, was added and the solution was mixed to form an emulsion which was centrifuged at 3500 rpm for 10 minutes at room temperature. The aqueous phase was removed and

Figure 2.6

Barley plants are grown in pots under glasshouse conditions for the extraction of DNA from leaves. Leaves of some plants are harvested.

added to 4.5 ml of phenol, mixed and centrifuged at 3500 rpm for 5 minutes. The top aqueous phase was removed, added to 4.5 ml of chloroform/IAA and centrifuged as above. The top aqueous phase was subsequently pipetted into 0.6 volume of isopropanol to precipitate the DNA. The chloroform acts to remove the lipid, phenolic and aromatic compounds and the phenol to denature and deproteinise the aqueous phase. The precipitated DNA was lifted out with a glass hook and dissolved in 4 ml TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). RNA was removed from the preparation by adding 4 µl of RNAse A and incubating at 60^0C for 15 minutes. The DNA was then precipitated by adding two volumes of 95% v/v ethanol followed by centrifuging at 2000 rpm for 2 minutes. The DNA pellet obtained was vacuum dried for 5 minutes, resuspended in 1 ml TE buffer and stored at 4^0C.

2.8.3. PCR Reaction

The starting mixture for PCR (polymerase chain reaction) was made in a 50 µl volume containing 0.2-0.5 ng of genomic DNA, 0.2 µM of primer, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 units of *Taq* polymerase (Perkin Elmer Cetus), 10 mM Tris-Hcl pH 8.3, 50 µM KCl, 2 µM $MgCl_2$ and

0.001% gelatin. Amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles and each cycle comprised 1 minute at 92^{0}C, 3 minutes at 35^{0}C and 2 minutes at 72^{0}C using the fastest available transitions between temperatures. Amplification products were analyzed by electrophoresis in agarose gels.

2.8.4. Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving 1.5% w/v agarose (Pharmacia) in 1xTBE buffer (89 mM Tris-OH pH 8.3, 89 mM boric acid and 2.5 mM EDTA) in a microwave oven to give a final volume of 300 ml. This was cooled to 60^{0}C and poured into Perspex gel plates (20 x 24 cm gels). The gels were then set up in tanks with 1xTBE used as the running buffer. Then 5 µl of loading buffer (40% v/v glycerol, 0.025% w/v Xylene Cyanol FF and 0.025% v/v Bromophenol Blue) was added to each PCR products. Samples were then loaded and the gel run at 150 V until the bromophenol blue marker had run to the end of the gel. λ (Lambda) DNA, digested with restriction enzymes Hind III/EcoRI, was used to provide size markers. Following elctrophoresis, the gels were stained with 0.5 µg/ml ethidium bromide in 1xTBE buffer for 30 minutes, briefly

destained in water and examined on the transilluminator.

2.9. Linkage Analysis

For each segregating locus, a goodness of fit to a ratio 1:1 was determined by *Chi*-square analysis. Recombination fractions between all pairs of markers, and map distances were all determined by the computer programme MAPMAKER (Lander *et al.*, 1987). The map was developed using a LOD score of 4.0 and a recombination fraction at which linkage can be detected, as the 99 per cent between markers utilizing 56 backcross progeny (Tanksley *et al.*, 1988). The map of each ordered linkage group was then generated by the MAP function of MAPMAKER.

2.10. Statistical Analysis

Statistical analysis of data was performed using the computer package GENSTAT (Alvey *et al.*, 1977). Significance levels are given as:

$P < 0.05$ *

$P < 0.01$ **

CHAPTER 3

INVESTIGATION OF ANTHER CULTURE RESPONSE OF COMMERCIAL BARLEY CULTIVARS ON MALTOSE AND SUCROSE-BASED MEDIUM

3.1. Introduction

Culture medium composition is a critical factor in the successful induction of microspore division. A common requirement for all media is a carbon supply but the carbohydrate component used has been a relatively neglected area of cell and tissue culture research. In most anther culture protocols, as in the majority of cell and tissue culture systems, sucrose, the major translocated carbohydrate in plant tissues, is traditionally recommended as the sole carbon source (Wenzel and Foroughi-Wehr, 1984). However, the yield of green plants obtained using such protocols has been low. Research has suggested that sucrose may not be optimal carbohydrate for all tissue culture systems (Kochba and Spiegel-Roy, 1973; Kochba *et al.*, 1982; Hunter, 1987). Babbar and Gupta (1986) have shown that different carbohydrates can affect different aspects of pollen embryogenesis. Barrow (1986) found that glucose was the best carbon source for long term culture of cotton microspores substantiating earlier work by Davis *et al.*

(1974) and Price *et al.* (1977). In barley (Wei *et al.*, 1986) and *Nicotiana* species (Aruga *et al.*, 1985; Kyo and Harada, 1985) isolated microspores have been deprived of sucrose for several days to induce embryogenesis or callus formation. Sorvari and Schieder (1987) have demonstrated that low concentrations of sucrose (2% w/v) can inhibit barley androgenesis. Raquin (1983) examined the use of a range of sugars as carbohydrate source for anther culture of *Petunia* and found that maltose, an α-1,4-linked glucose disaccharide, was superior to both sucrose and glucose in its ability to improve embryo induction and development. Maltose has also been found to be more beneficial than thirty other carbon sources tested for somatic embryogenesis of *Medicago sativa* (Strickland *et al.*, 1987). The successful utilization of anther culture in crop improvement is dependent upon the ability to produce consistently large number of green regenerants from any cultivar of interest.

The objective of this study is to investigate anther culture response of different cultivars of barley on medium containing maltose or sucrose as carbohydrate source.

3.2. Experimental Procedure

Details of the procedure used in this study are described in Chapter 2.

Four spring barley cultivars namely Abbey, Dallas, Bianka and Decado were used to test their response on anther culture media which have been modified by supplying the carbohydrate as either sucrose or maltose at a concentration of 1, 3, 6 and 9% w/v. Thus for each genotype there were eight different carbohydrate treatments. The experiment was replicated four times and there were 25 anthers/concentration/replication. Florets were removed from tillers and mixed before plating onto 55 mm dishes so that each dish received florets at random. Cultures were scored after 28 days for the number of anthers responding in culture to form callus and / or embryoids and subsequently for the number of green and albino plants regenerated. In addition, cultures were assessed visually for indications of regeneration having proceeded via callus or via an embryogenetic route.

3.3. Results

The analysis of variance for anther response is presented in table 3.1. There was significant effect of treatment i.e. difference in response of anthers to callus/embryoid formation under maltose and sucrose-based medium was highly significant.

The percentages of anthers responding to culture to form callus/embryoids under 1, 3, 6 and 9% w/v maltose or sucrose are presented in figures 3.1, 3.2, 3.3 and 3.4 respectively. It is observed that under 1% w/v, maltose produced 17, 15, 7 and 12 % and sucrose produced 4, 3, 2 and 3 % anther response respectively for Abbey, Dallas, Bianka and Decado (Figure 3.1). Figure 3.2 showed that the percentages of anther response to form callus/embryoids under 3% w/v maltose were 23, 20, 7 and 16 and under 3% w/v of sucrose were 4, 8, 3 and 8 respectively for Abbey, Dallas, Bianka and Decado. In figure 3.3, it is found that Abbey, Dallas, Bianka and Decado responded to produce 36, 33, 12 and 26 % anther response respectively under 6% w/v maltose and 10, 6, 2 and 6 % anther response respectively under the same concentration of sucrose. At the highest concentration

Table 3.1. Analysis of variance[1] for anther response to form callus/embryoid on maltose/sucrose-based medium.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Replication	3	15.09	5.03	0.71
Cultivar (A)	3	478.34	159.45	22.37**
Treatment (B)	1	1875.78	1875.78	263.21**
AB	3	197.84	65.95	9.25**
Error	21	149.66	7.13	
Total	31	2716.72		

** $P<0.01$

[1] Polled over the carbohydrate concentrations.

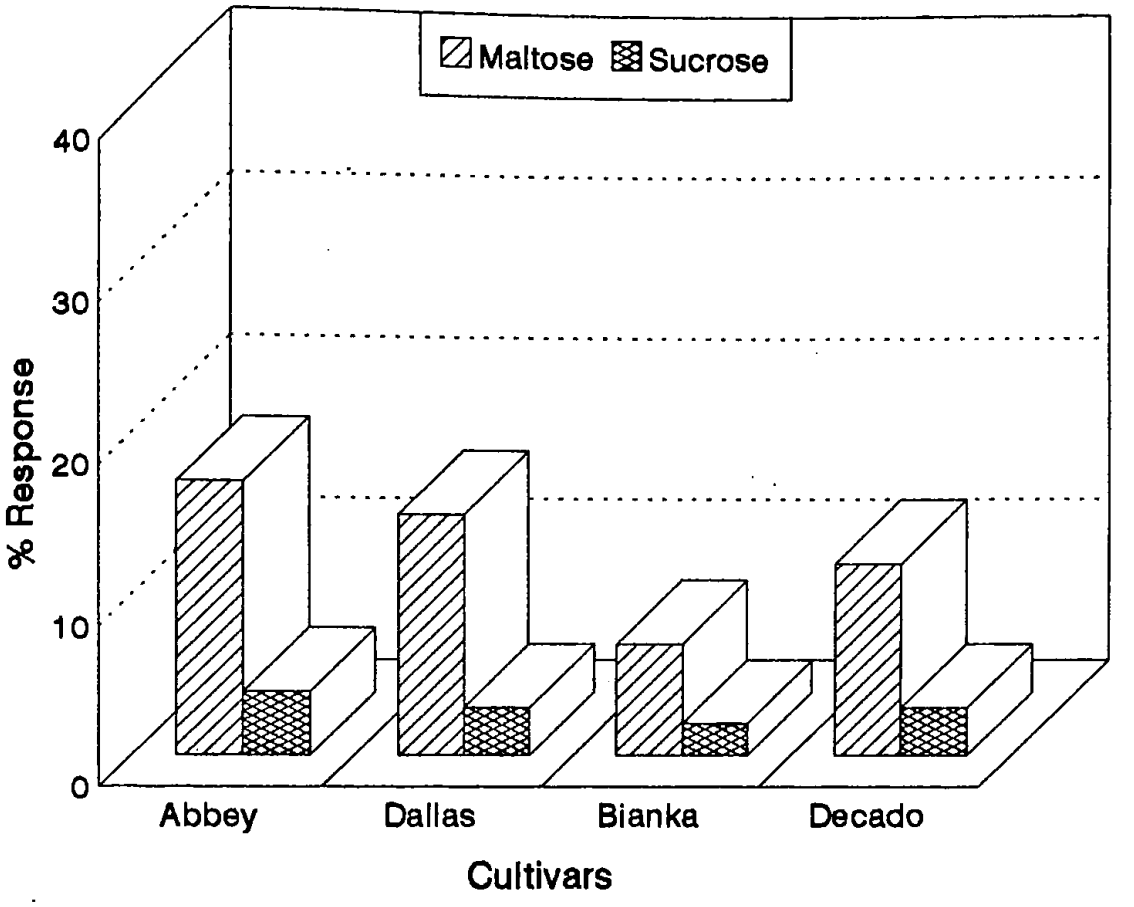

Figure 3.1

Response of anthers to callus/embryoid formation of four barley cultivars cultured on medium containing 1% w/v maltose or sucrose.

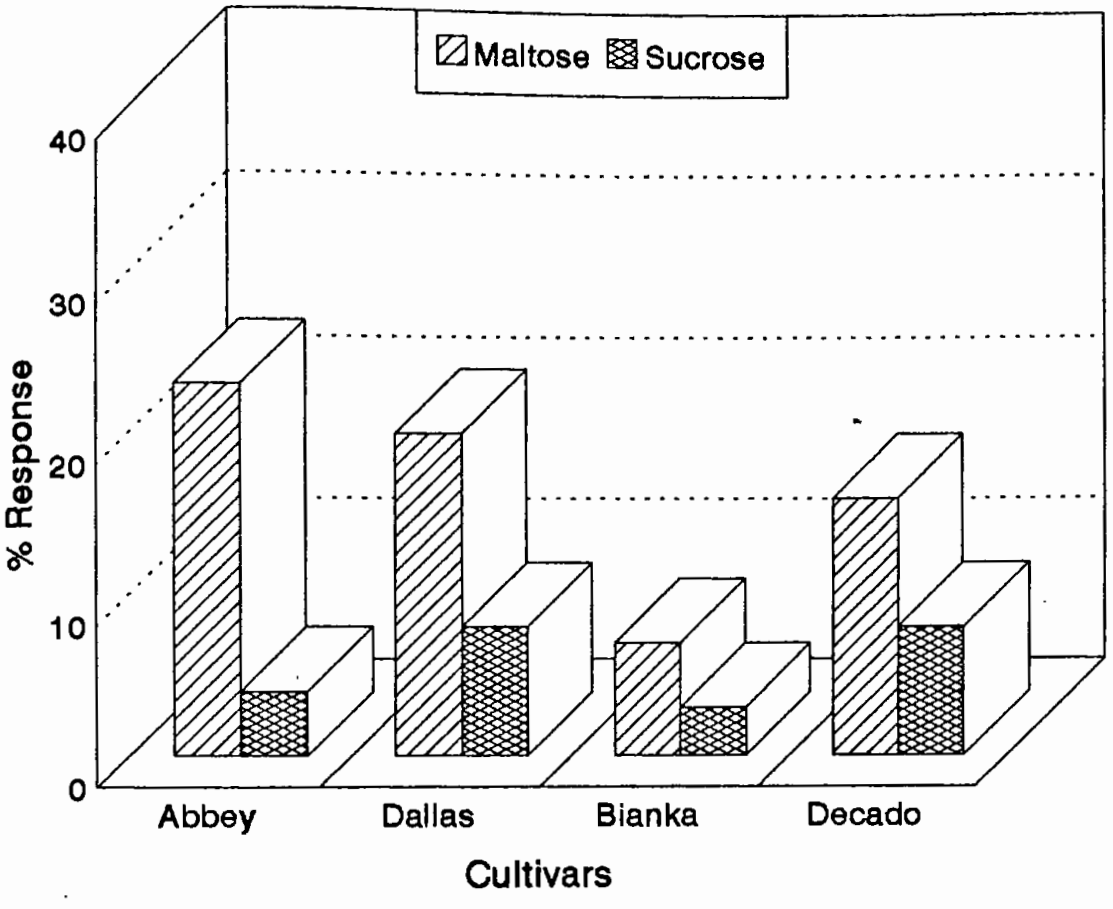

Figure 3.2

Response of anthers to callus/embryoid formation of four barley cultivars cultured on medium containing 3% w/v maltose or sucrose.

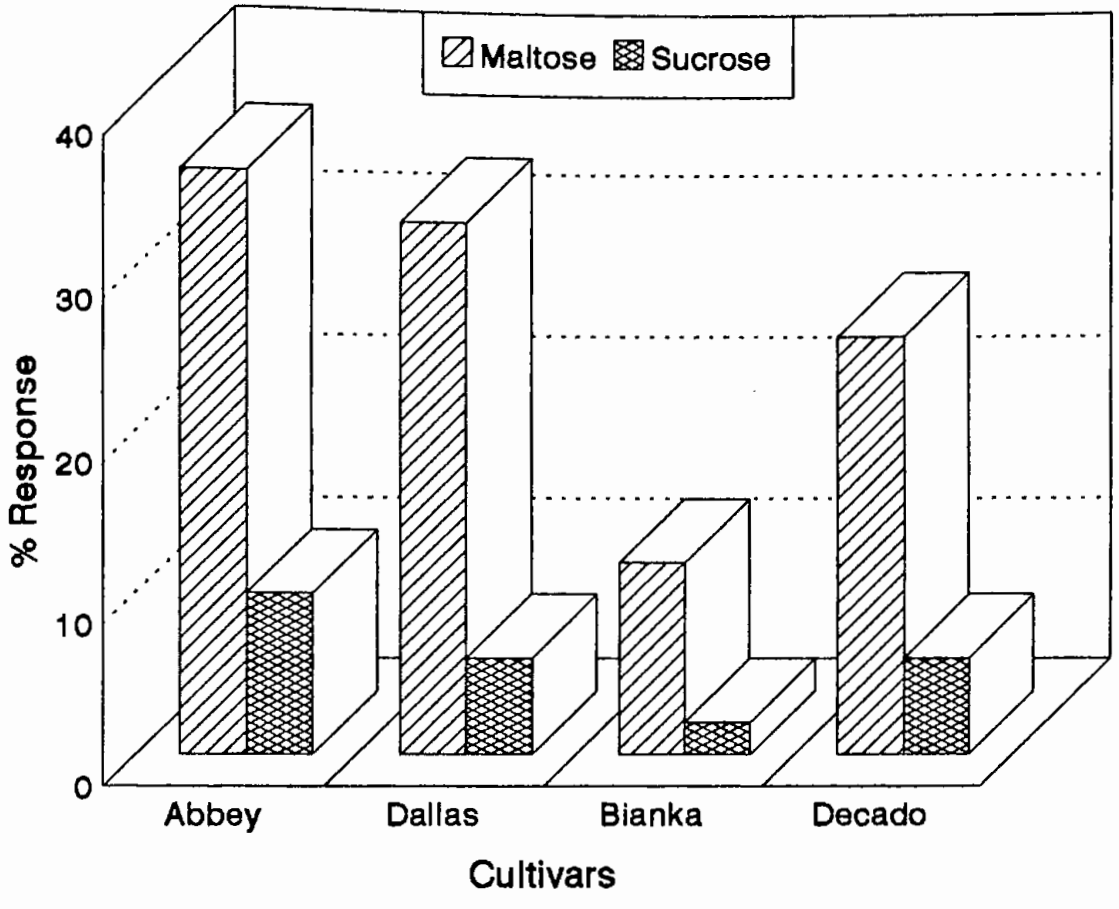

Figure 3.3

Response of anthers to callus/embryoid formation of four barley cultivars cultured on medium containing 6% w/v maltose or sucrose.

i.e. 9% w/v, maltose yielded 30, 34, 16 and 28 % response and sucrose gave 9, 7, 5 and 7 % response respectively for Abbey, Dallas, Bianka and Decado (Figure 3.4). The results clearly indicate that anther culture medium containing maltose is superior to sucrose-based medium in terms of anther response. It also reveals from the figures that there were also genotypic differences in the ability to respond to culture. Abbey, Dallas and Decado responded well but Bianka showed lower level of callus/embryoid formation. It is also evident from the figures that higher concentrations of carbohydrate (6 and 9%) gave better response than the lower concentrations used (1 and 3%). Figure 3.5 illustrates the extent of differential response of anthers of Decado to maltose and sucrose while figure 3.6 demonstrates the response of anthers of Dallas on maltose-based medium.

The analyses of variance for green plant production under the treatment of maltose/sucrose and also on maltose-based media are presented in tables 3.2 and 3.3 respectively. It reveals from table 3.2 that the effect of treatment was highly significant. It also indicates that the differences between genotypes were also significant. Table 3.3 shows that genotypes as well as

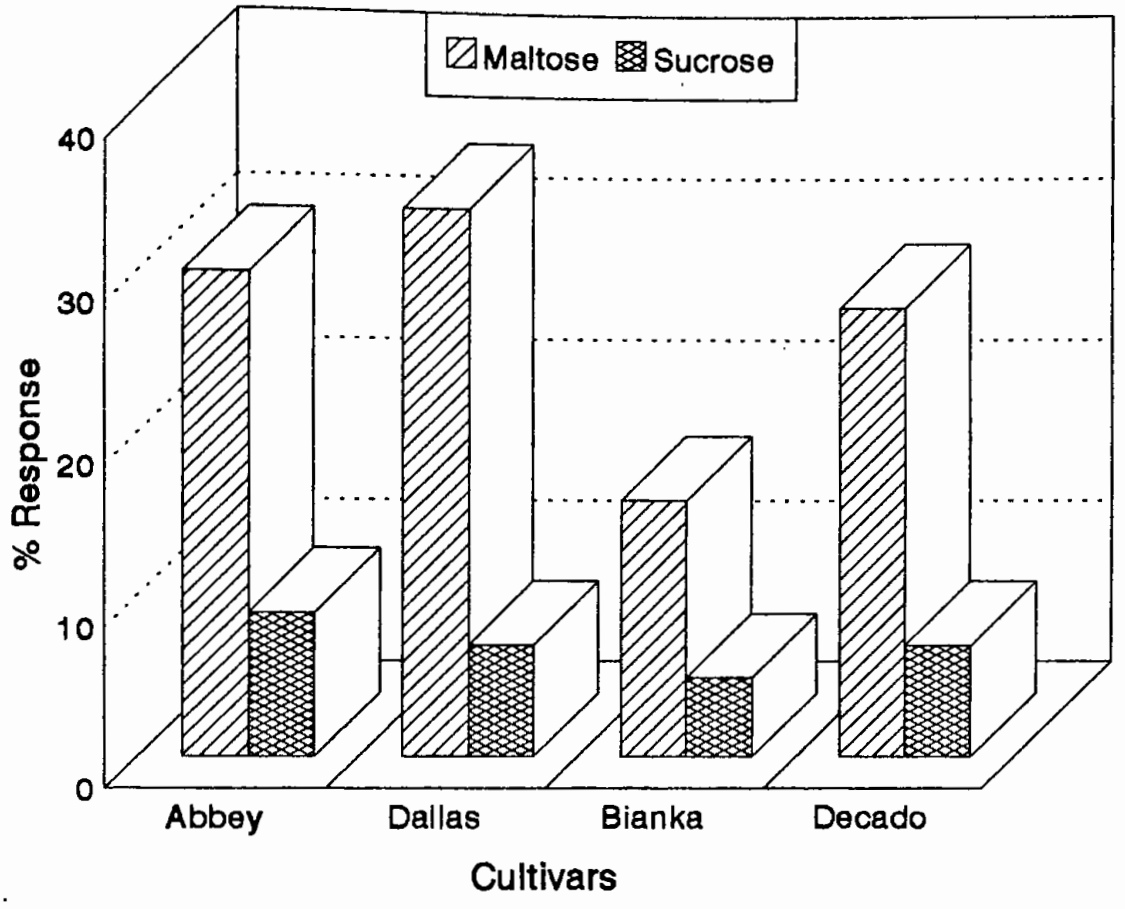

Figure 3.4

Response of anthers to callus/embryoid formation of four barley cultivars cultured on medium containing 9% w/v maltose or sucrose.

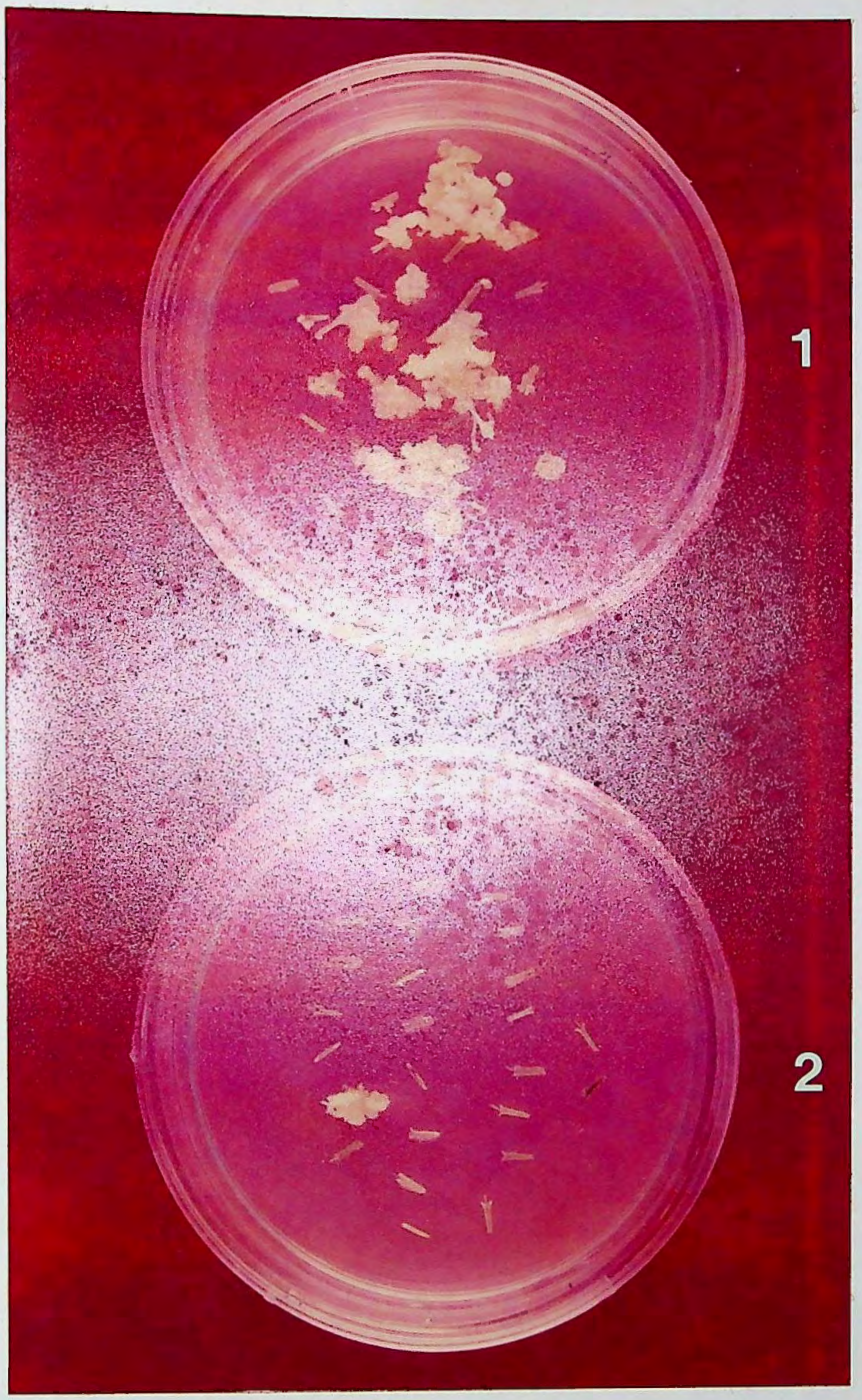

Figure 3.5

Differential response of anthers to form callus/embryoid of cv. Decado on medium containing 6% w/v maltose [1] and sucrose [2] after 28 days of incubation in the dark at 24°C

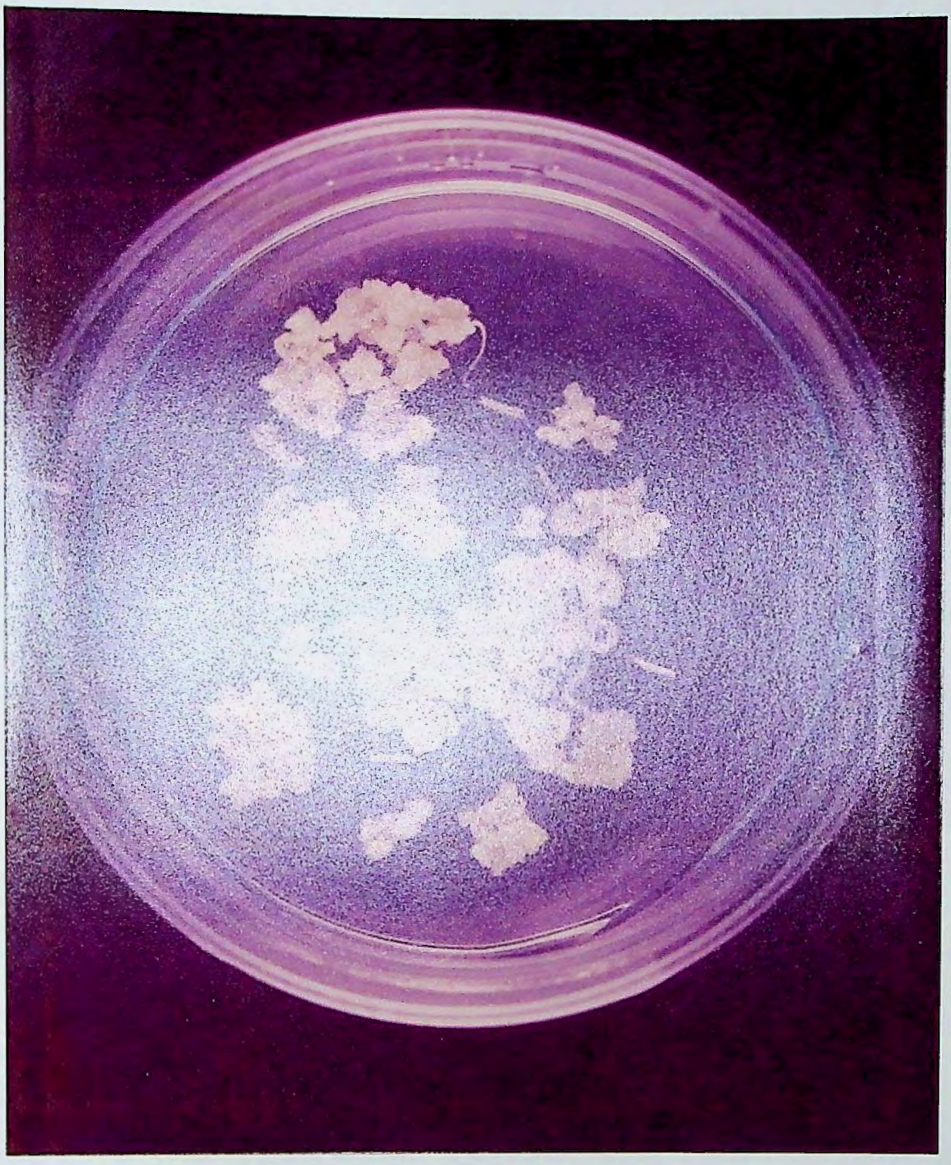

Figure 3.6

Response of anthers to form callus/embryoid of cv. Dallas on maltose-based (6% w/v) medium after 28 days of incubation in the dark at 24°C

maltose concentrations exerted significant influence on green plant production.

The number of green and albino plants regenerated under 1, 3, 6 and 9% w/v maltose or sucrose for each genotype are summarized in figures 3.7, 3.8, 3.9 and 3.10 respectively. The mean number of green plants regenerated under 1% w/v maltose were 5, 7, 2 and 11 and under 1% w/v sucrose were 2, 1, 1 and 2 respectively for Abbey, Dallas, Bianka and Decado (Figure 3.7). At a concentration of 3% w/v, maltose produced 8, 9, 2 and 13 green plants while sucrose produced 4, 4, 2 and 5 green plants respectively for Abbey, Dallas, Bianka and Decado (Figure 3.8). Figure 3.9 revealed that under 6% w/v, maltose yielded 11, 14, 5 and 26 green plants while sucrose yielded 2, 4, 1 and 3 green plants respectively for Abbey, Dallas, Bianka and Decado. In figure 3.10, it is observed that under 9% w/v, maltose produced 12, 16, 4 and 19 green plants and sucrose produced 2, 3, 3 and 5 green plants respectively for Abbey, Dallas, Bianka and Decado. A total of 164 green plants were regenerated on maltose-based media compared to 46 on sucrose-based media. The number of albino plants regenerated on maltose

Table 3.2. Analysis of variance[1] for green plant regeneration on maltose/sucrose-based medium.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Replication	3	3.25	1.08	0.30
Cultivar (A)	3	230.75	76.92	21.32**
Treatment (B)	1	338.00	338.00	93.70**
AB	3	87.75	29.25	8.11**
Error	21	75.75	3.61	
Total	31	735.50		

**P<0.01

[1] Pooled over the carbohydrate concentrations.

Table 3.3. Analysis of variance for green plant regeneration on maltose-based medium under different concentrations.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Replication	3	2.30	0.77	0.32
Cultivar (A)	3	108.92	36.31	15.00**
Concentration (B)	3	40.17	13.39	5.53**
AB	9	15.64	1.74	0.72
Error	45	108.95	2.42	
Total	63	275.98		

** P<0.01

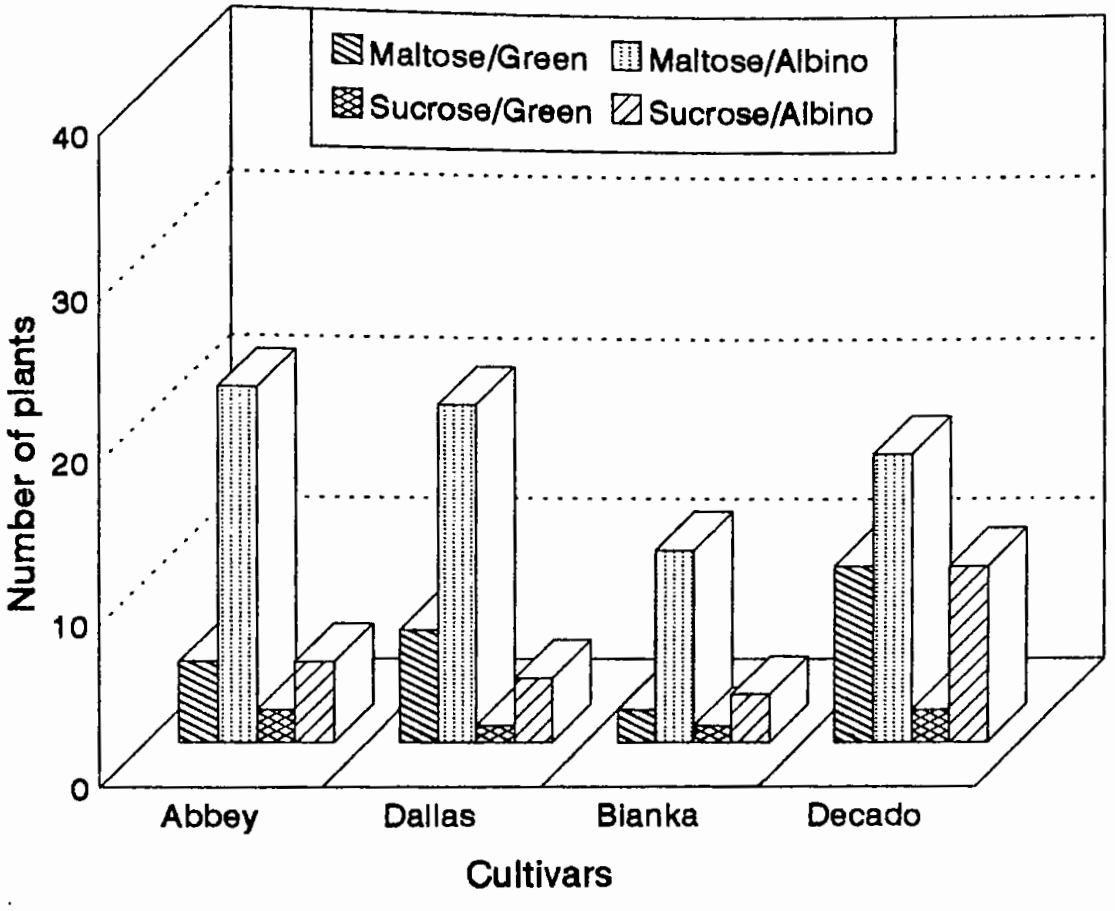

Figure 3.7

Number of green and albino plants regenerated from anthers of four barley cultivars cultured on medium containing 1% w/v maltose or sucrose.

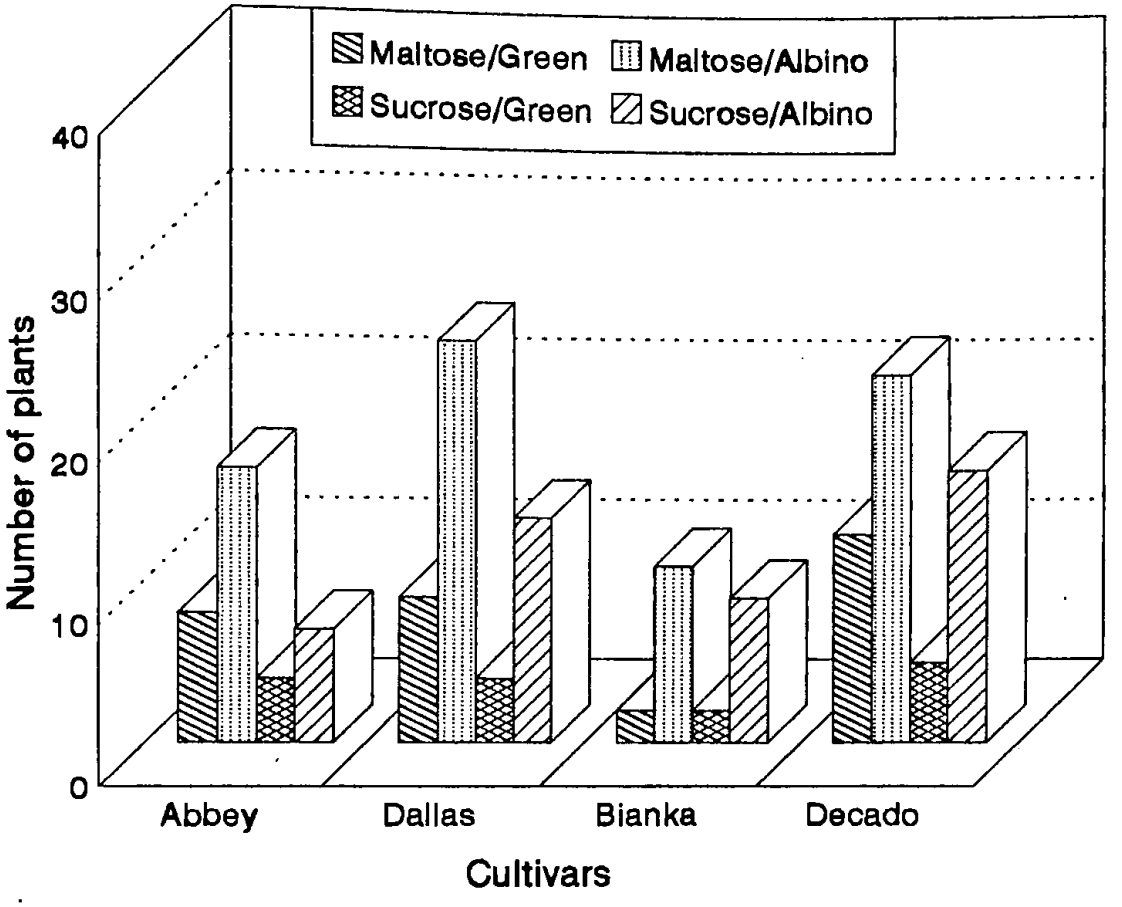

Figure 3.8

Number of green and albino plants regenerated from anthers of four barley cultivars cultured on medium containing 3% w/v maltose or sucrose.

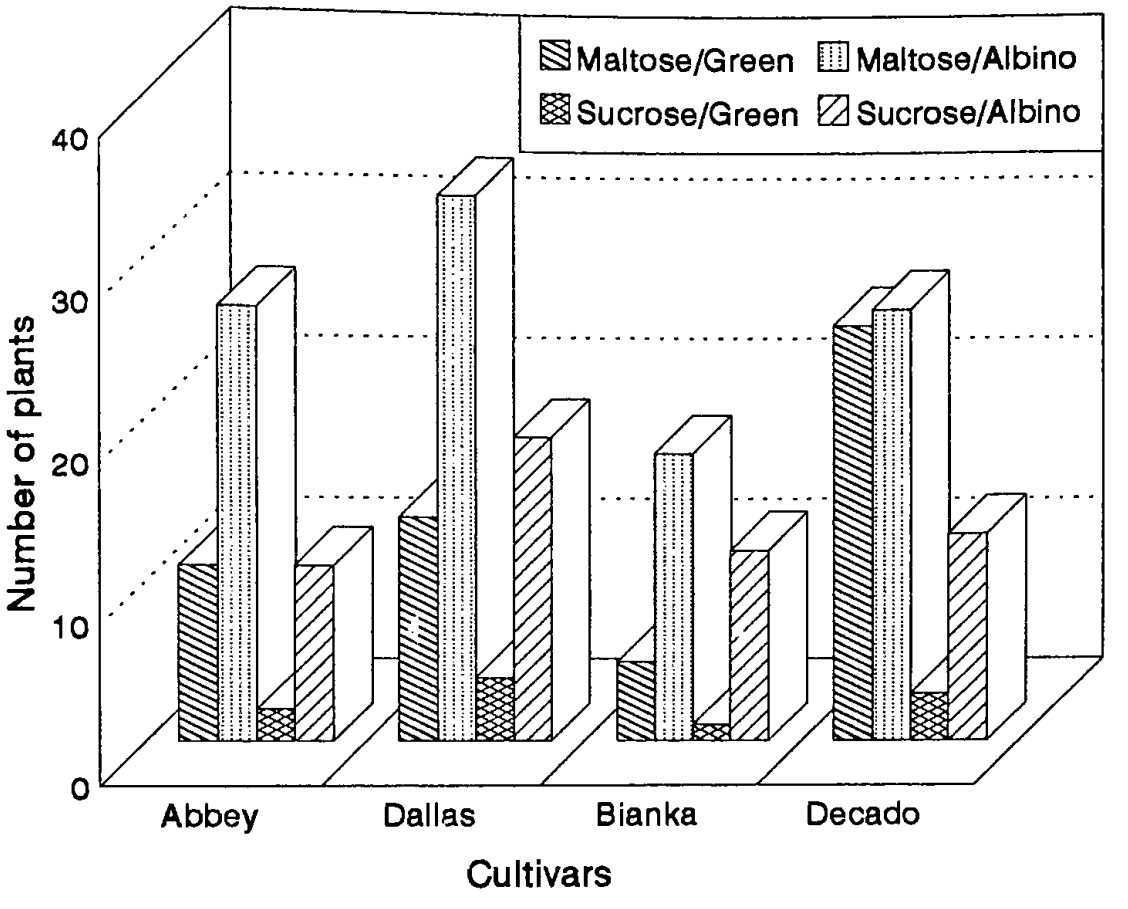

Figure 3.9

Number of green and albino plants regenerated from anthers of four barley cultivars cultured on medium containing 6% w/v maltose or sucrose.

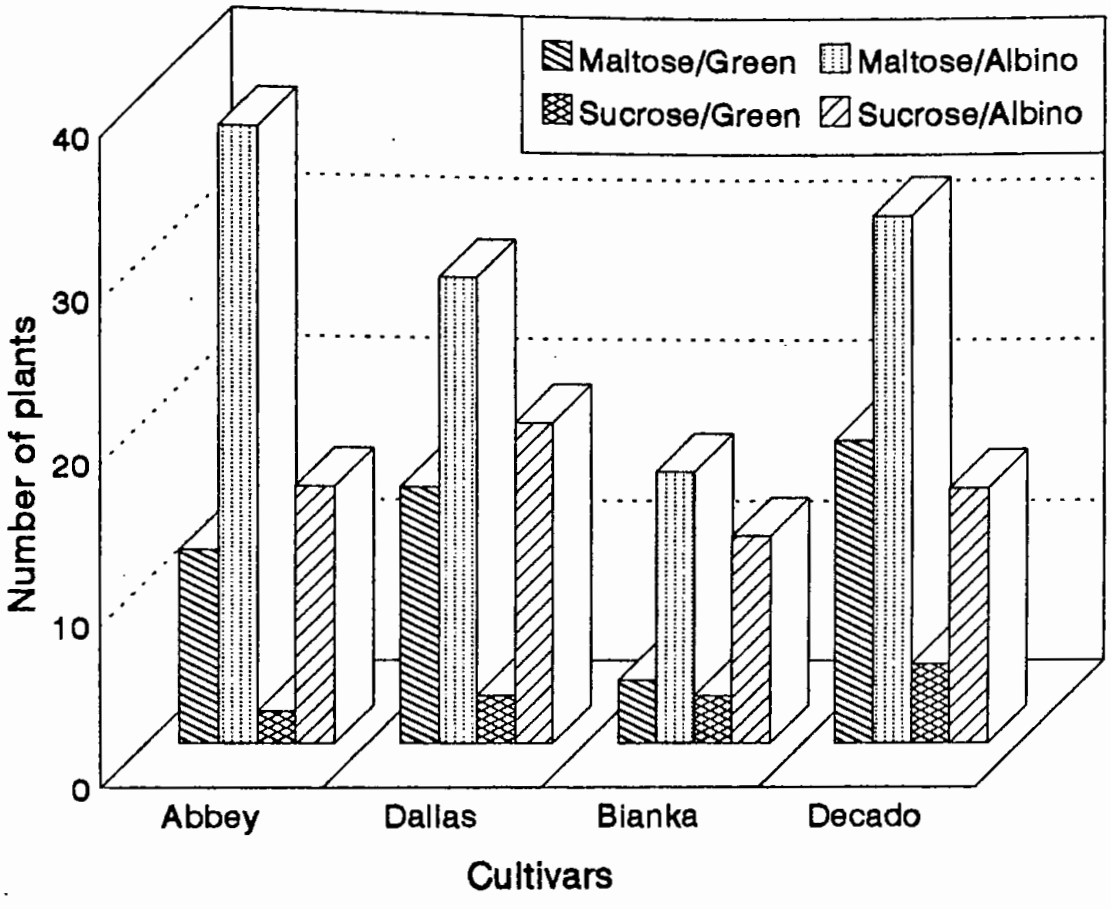

Figure 3.10

Number of green and albino plants regenerated from anthers of four barley cultivars cultured on medium containing 9% w/v maltose or sucrose.

and sucrose-based media were 370 and 190 respectively. In all the treatments maltose produced higher number of green plants than sucrose. There were also genotypic differences in the ability to regenerate plantlets. Decado produced highest number of green plants followed by Dallas and Abbey. Bianka yielded considerably fewer number of plants. Figure 3.11 illustrates the regeneration of green plants in Decado on maltose-based medium. In figure 3.12, the number of green and albino plants regenerated have been averaged over the four carbohydrate concentrations, for each of the four genotypes. It is observed that three times more green plants were obtained from Decado than from Bianka while Dallas and Abbey produced 2 and 1.6 times respectively more green plants than Bianka. The trend of green plants regeneration on sucrose-based medium was more or less similar. However, maltose raised the performance of all four cultivars in comparison with sucrose.

It can be seen from the above figures (3.1-3.4 and 3.7-3.10) that response of anthers to produce callus/ embryoids is not necessarily a reliable indicator of the number of green plants that will subsequently be recovered. Whilst Abbey and Dallas gave higher response

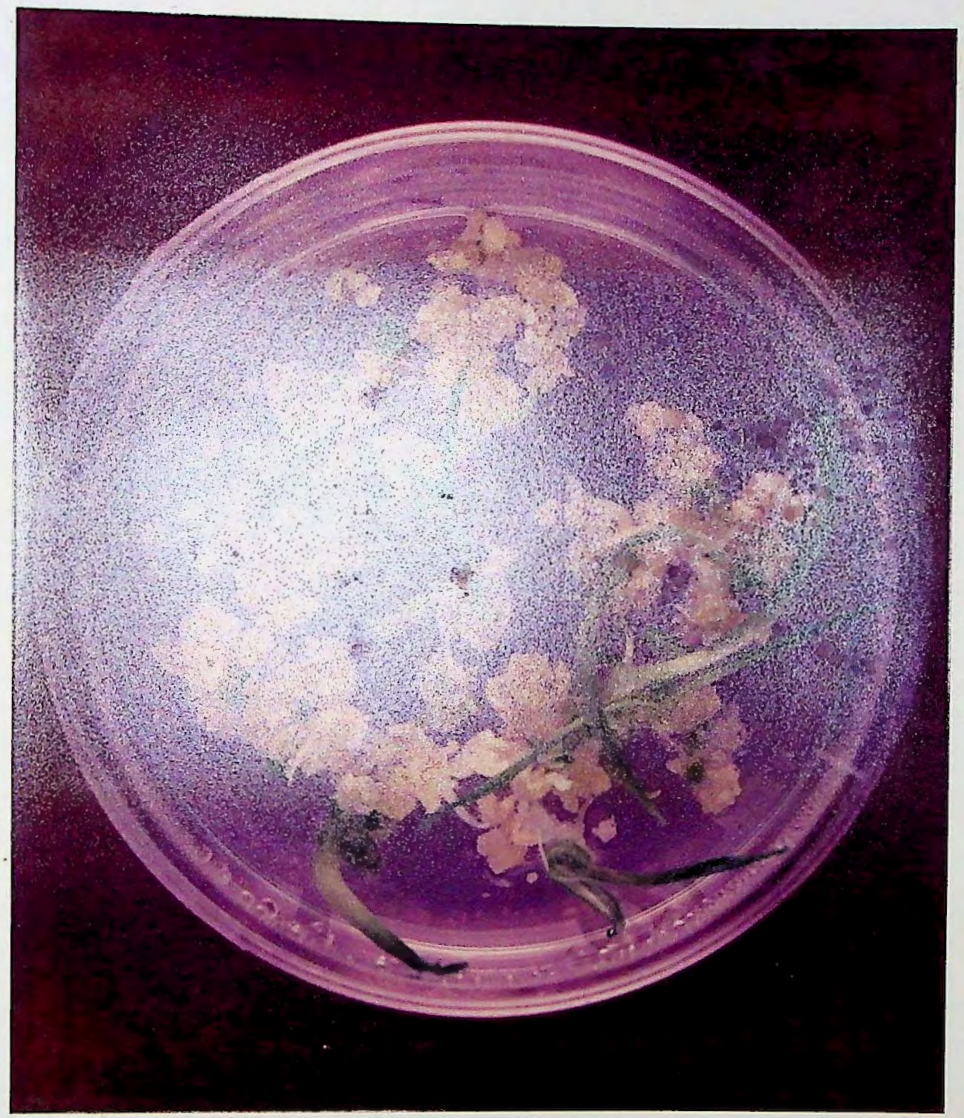

Figure 3.11

Regeneration of plants for cv. Decado on maltose-based (6% w/v) medium during subculture.

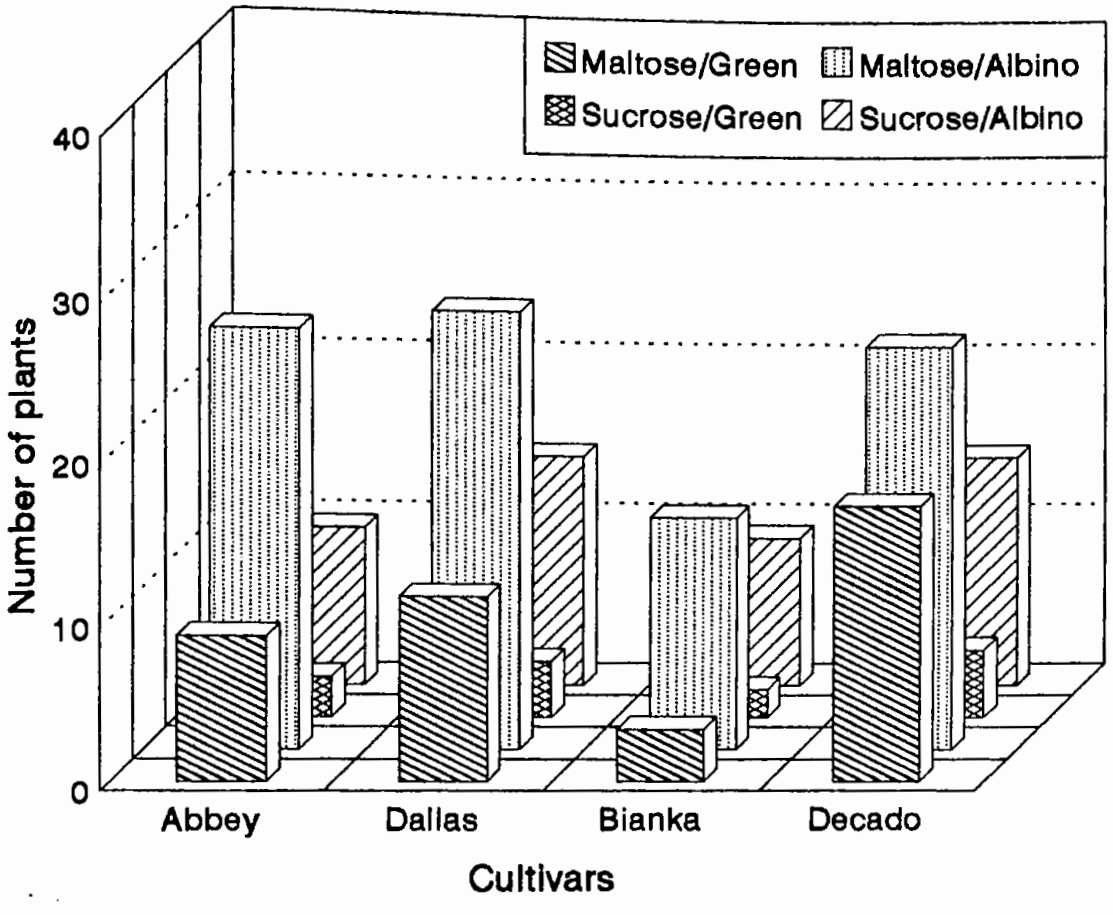

Figure 3.12

Number of green and albino plants regenerated from anthers of four barley cultivars cultured on medium containing maltose or sucrose (averaged over four concentrations).

to culture to form callus/embryoids than Decado but both of them produced lower number of green plants than Decado. Bianka yielded relatively fewer number of callus/embryoids and fewer number of green plants.

Observations of growing culture showed in general that although both callus and embryo-like structures were seen, on maltose a high proportion of embryogenetic structures were developed from microspores, whilst on sucrose more callus was visible. Figure 3.13 illustrates the development of macroscopic structure in Dallas while figure 3.14 shows the formation of embryoids from the same cultivar on maltose-based medium.

3.4. Discussion

3.4.1. The Effect of Maltose as Carbohydrate Source

The results presented in this study demonstrate clearly the superiority of maltose as the carbohydrate source in barley anther culture media.

Using isolated microspore culture system, Hunter (1988) reported that maltose yielded stimulating response in

Figure 3.13

Response of anther of cv. Dallas on maltose-based (6% w/v) medium.

Figure 3.14

Formation of embryoids from anthers of cv. Dallas (arrow marked) on maltose-based (6% w/v) medium.

microspore culture of barley. He suggested that sucrose may inhibit the embryogenetic development of cultured barley microspores and showed that glucose and fructose, added to the medium in conjunction with maltose, can be inhibitory at low concentrations. Raquin (1983) also showed that glucose and fructose are inhibitory to pollen embryogenesis in *Petunia*. Hunter (1988) has put forward a hypothesis to explain the results he obtained. Microspores require a supply of carbon which is converted to hexose phosphates for respiration and biosynthesis. It is hypothesized that above a threshold concentration of intracellular hexose, cell metabolism is inhibited or cells die. Since sucrose is degraded rapidly to its breakdown products, glucose and fructose, by the highly abundant enzyme invertase, which is present in anthers at fairly high activity, there are subsequently very high levels of glucose produced in the vicinity of the microspores causing inhibition. Maltose, on the other hand, is degraded by the much less abundant glucosidase, releasing glucose much more slowly and thereby avoiding the inhibitory glucose threshold concentration. After the first few critical days of culture, the inhibitory effect of glucose is lost. If the hypothesis is correct, then the improvements obtained in anther culture response are

due to the removal of an inhibitor (sucrose) rather than an intrinsic stimulatory effect of maltose.

These results are consistent with the findings of several other workers. Shannon *et al.* (1985) reported an effect of anther orientation on anther culture response. It was found that when anthers were cultured on sucrose-based media, the degree of response was dependent on the way the anthers were placed on the culture medium. When anthers were cultured flat with both lobes contact in the medium, there was little or no response. When anthers were cultured on edge, embryoids developed but only in the upper lobe which was not in contact with the medium. From Hunter's hypothesis, embryoids are only able to develop in those parts of the anther not in direct contact with the medium, since those parts that are in direct contact subject to the inhibitory effects of the glucose being broken from the sucrose. In contrast, when anthers are cultured on maltose-based media, embryogenesis can proceed irrespective of anther orientation. An alternative explanation of the orientation effect is that it is due to an inhibitory effect of the auxin in the medium. Powell *et al.* (1988) found that even on the sucrose containing medium, the

effect of anther orientation was non-significant if 2,4-D (used by Shannon *et al.*, 1985), was replaced by the less active auxin, IAA. Experiments to investigate the potential of various plant starches as alternative to agarose as solidifying agents showed a stimulation of embryoid and callus formation even in the absence of any other carbon source especially with barley starch (Sorvari, 1986a,b; Sorvari and Schieder, 1987). Starch is hydrolyzed by amylases produced by the anther wall or by the microspores themselves (Roberts-Oehlschlager, 1988) to release maltose, glucose, maltotriose, maltotetrose and other oligosaccharides (Briggs, 1978) and presumably, therefore, acts as a slow release source of glucose and provides conditions more conducive to culture.

Roberts-Oehlschlager *et al.* (1990) have shown that barley anther extracts catalyze rapid rates of sucrose hydrolysis but very low rates of maltose hydrolysis and that glucose is probably liberated from maltose at a rate similar to that at which it can be utilized by developing microspores. They have, however, suggested that high levels of glucose are initially advantageous but become inhibitory unless rapidly metabolized. These workers also believe that the poor response of microspores to

culture on a sucrose-based medium might alternatively be due to the inhibitory effect of fructose (a bi-product of sucrose hydrolysis).

Recent works have shown that anther culture response in wheat can also be increased by substantiating sucrose by maltose as the carbohydrate source used in the medium (Last and Brettell, 1990). These workers also found that maltose was hydrolyzed by anther-derived enzymes at a slower rate than sucrose and they suggest that the glucose concentration does not affect the level of response but sensitivity of fructose is important.

3.4.2. The Effect of Carbohydrate Concentration

There has been no general agreement on the optimal concentration of carbohydrate for use in barley anther culture media. For sucrose-based system anything ranging from 2-12% w/v and even higher has been recommended. Foroughi-Wehr *et al.* (1976) recommended 6% w/v of sucrose for barley anther culture. The present study has shown that high carbohydrate concentrations (6 and 9% w/v) are beneficial. This ties in with earlier observations that members of the Gramineae in contrast

with the Solanaceous species favour high osmotic conditions (Dunwell, 1985a). There have been a number of studies involving the use of osmotica. Sorvari and Schieder (1987) demonstrated that the use of the inert osmoticum melibiose in a solid barley anther culture medium stimulated levels of green plant production and at the same time decreased the number of albinos formed in culture. However, this effect has not been seen in liquid culture systems (Kuhlmann and Foroughi-Wehr, 1989). Xu (1990) reported that sucrose is better than a sucrose/mannitol combination in barley anther culture medium and concluded that sucrose has a purely nutritional effect. Mannitol has been used in pre-incubation systems to replace cold pretreatments (Ziauddin *et al.*, 1990). It is thought that mannitol provides an osmotic balance for microspores during this preculture system and also provides a brief period of carbon starvation prior to culture during which time it is postulated that beneficial factors are leached from scenescing anther walls (Ziauddin *et al.*, 1990). However, it appears that mannitol is not inert, but seems to actually have the effect of enriching anther culture medium with glucose either by being converted itself to glucose or by bringing about the release of glucose from

endogenous starch reserves within the anther (Roberts-Oehlschlager *et al.*, 1990). Therefore, the combination of sucrose + mannitol used by Xu (1990) may give a poor response by leading to inhibitory levels of glucose in the medium.

3.4.3. The Formation of Callus or Embryoids

In the study by Sorvari and Schieder (1987) it was shown that there was less callus formation at high sucrose concentrations, with direct embryoid formation favoured. At low sucrose concentrations mainly callus was observed. It had previously been thought that whilst members of the Solanaceae favoured the development of doubled haploid plants via an embryogenetic pathway, callus production was a characteristic of Graminaceous species. Visual assessments of cultures in this study substantiate the effect of maltose in promoting direct embryogenesis. This has further implications for the successful application of anther culture as it may affect the stability of regenerated lines. The formation of disorganized callus tissue has been associated with the phenomenon of somaclonal variation (Larkin and Scowcroft, 1981) and regenerants are thought to be less

likely to undergo genetic change in culture if they are derived from organized embryo structures (Sunderland and Huang, 1985). However, secondary callusing was often observed in cultures and it was not possible to pin point the origin of all regenerated plants. This callusing is probably under the influence of the auxin present in the medium (Wenzel and Foroughi-Wehr, 1984). Auxin is necessary for the induction of callus and has been employed in the anther culture media of cereals which have traditionally been thought to be hormone-requiring species. However, the species that are considered to be hormone-independent (Dunwell, 1985a) are also those that are known to undergo embryogenetic pathway of regeneration. If maltose promotes embryogenesis in barley, then it is possible that auxin will no longer be necessary for induction. It may even be disadvantageous if it promotes callusing at the expense of embryo formation. Another explanation is that callusing is promoted by conditions of low osmotic pressure (Sorvari and Schieder, 1987).

3.4.4. The Production of Albino Plants

One of the major limitations associated with anther

culture has been the large number of albino plants regenerated. Studies have shown that there are protoplastids present in such albinos although they do not contain ribosomes (Wenzel and Foroughi-Wehr, 1984) and an analysis of the chloroplast genome of albino regenerants in barley and wheat has revealed that large regions of the chloroplast DNA have been deleted; in contrast, no alterations have been detected in chloroplast DNA from green pollen plants of barley (Day and Ellis, 1984). It has been suggested that the high rates of albinism are due to the expression of recessive genes (Clapham, 1977). Rates of albino production are much higher in androgenetic haploids than in those formed parthenogenetically and this could be because there are not enough protoplastids present in the microspores (Wenzel and Foroughi-Wehr, 1984). Day and Ellis (1984) have suggested that the deletions observed form a natural part of plant development and act to preclude parental inheritance of chloroplasts by rendering those present in the gametes non-functional. Organelle alteration in the generative cell has been proposed as a possible mechanism for maternal inheritance elsewhere (Vaughn *et al.*, 1980). Studies have shown that culturing microspores at early stages of

development can give higher yields of green plants suggesting that pollen differentiation may involve some irreversible alteration of the plastid genome (Dunwell, 1985c). If this is the case, then genotypic differences in the relative proportions of green and albino plants could be due to genotypic differences in the stage of microspore development at which such an alteration might occur. Perhaps, then, the proportion of green regenerants might be increased by varying the stage of development at which microspores are cultured, depending on genotype. However, if the DNA deletions occur at a very early stage of microspore development, problems are encountered with microspore not responding to culture and it may be necessary possibly through varying the donor plant growth conditions to alter the relative rates of chloroplast and microspore development in order to achieve the desired increase. So far, however, any connection between the occurrence of albino regenerants and a mechanism to preclude paternal inheritance remains speculative. Another answer to the problem of albinism is to develop techniques that are so efficient that large numbers of albinos can be tolerated and simply discarded. There is some evidence with barley that variations in culture media and conditions influence the relative

proportions of green and albino plants, and that the proportion of albinos may decline as efficiency improves (Lyne, 1985).

3.5. Conclusions

The substitution of sucrose by maltose in the anther culture medium results in improvement in the anther culture response and the number of green plants regenerated. The efficiency of green plant production will facilitate the production of improved cultivars, but the embryoid origin of microspore-derived plants may be of greater significance. The use of anther culture technology in a plant breeding context requires that the homozygous lines generated are genetically stable and will breed true in subsequent generations (Powell *et al.*, 1984). A culture process involving a callus phase may not desirable, as was shown by observations of variation in callus-derived anther culture regenerants (Powell *et al.*, 1984). The formation of embryoids directly from microspores reduces the length of the culture process and may reduce the dangers of genetic change as there is strong selection in favour of normal cells during the process of embryogenesis (Datta and Wenzel, 1987).

The results obtained in this study also indicate that, for the four cultivars, Abbey, Dallas, Bianka and Decado, the induction of microspores to form embryoids or callus is not necessarily correlated with the response in terms of green plant production.

CHAPTER 4

STUDY OF THE HERITABILITY OF ANTHER CULTURE RESPONSE OF *HORDEUM VULGARE* X *HORDEUM SPONTANEUM* HYBRIDS

4.1. Introduction

Perhaps the most important influence throughout anther culture is the donor genotype (Sunderland, 1980; Genovesi and Collins, 1982). The genotype of the donor plant is the genetic base line which dictates how the pollen develops initially *in vivo* and subsequently how it will respond *in vitro*. There are numerous reports which suggest that species within the same genus and cultivars within the same species respond differentially in anther culture (Dunwell, 1985b). In the very early experiments with rice (Niizeki and Oono, 1968), only two of the ten varieties cultured produced plants. Similar differences have been reported in other cereals such as maize (Anonymous, 1975), wheat (Picard and De Buyser, 1973), rye (Wenzel *et al*, 1977) and barley (Foroughi-Wehr *et al.*, 1976).

The recombination of traits of high frequency response has been reported for several species (Misoo and

Matsubayashi, 1978; Foroughi-Wehr and Friedt, 1984; Dunwell, 1985b). This suggests that specific genes may be associated with anther culture responsiveness. Miah *et al.* (1985) studied anther culture in rice (*Oryza sativa* sub-sp. *japonica* and *Oryza sativa* sub-sp. *indica*). Using two *japonica* and two *indica* cultivars as parents they carried out a four parent diallel analysis. Anther responsiveness was inherited as a recessive character determined by a single gene. Chowdhury (1985) used six genotypes of *Nicotiana rustica* to study interaction between genotype and environment and their effect on anther response. He found significant parental differences within and between crosses which indicated that anther responsiveness was affected by additive gene action.

For anther culture to be used in breeding industry it is important that a broad range of genotypes can be cultured successfully. It has been proposed that culture conditions could be optimized for each genotype of interest (Dunwell, 1981) but this would be laborious and may not necessarily be effective. An alternative approach is to try to broaden the genetic bases for tissue culture ability by selection and combination breeding (Wenzel and

Foroughi-Wehr, 1984). Genotypic differences in the ability to respond to anther culture imply that anther culture responsiveness is heritable (Wenzel and Foroughi-Wehr, 1984). If anther culture responsiveness can be inherited then it must also be transferable to hybrid progenies (Foroughi-Wehr *et al.*, 1982). Results obtained from a number of studies on wheat (Bullock *et al.*, 1982) and rye (Wenzel *et al.*, 1977) have shown that if crosses are made between a cultivar that is responsive to anther culture and one that is unresponsive, then factors affecting responsiveness are transmitted into the F_1. The hybridization of responsive genotypes with non-responsive genotypes in this way might yield information about the inheritance of the genetic factors controlling the production of microspore-derived plants (Powell, 1988).

Hordeum spontaneum is the recognized progenitor species of cultivated barley, *H. vulgare*, with which it produces fertile hybrids (Nevo, 1987). *H. spontaneum* originated in the area of the Near East known as the Fertile Crescent over 5000 years ago (Harlan, 1976). *H. spontaneum* has adapted to survive in a wide range of hostile habitats and thus contains much genetic diversity for resistance

to diseases, pests and ecological stresses (Poehlman, 1985). Much of this diversity is not present in the cultivated barley as the crop has been extracted from a narrow genetic base. *H. spontaneum*, therefore, represents a valuable source of diverse genes for enhancing the germplasm of cultivated barley by introgression. These include genes carrying resistance to powdery mildew of barley (*Erysiphe graminis* f. sp. *hordei*), one of the most commercially important pathogens of barley (Jahoor and Fischbeck, 1987).

The effectiveness of selection for desired traits could be improved if anthers of the F_1 generation could be cultured to produce haploids. Doubling these would instantly fix the genotype which could then be rapidly assessed for the presence of desirable combinations of characters including genes from *H. spontaneum* lines. There do not appear to be any published reports concerning the ability of *H. spontaneum* to respond to anther culture. However, even if *H. spontaneum* does not respond, provided that anther culture responsiveness is heritable and can be transmitted into the F_1 hybrid after a cross between *H. vulgare* and *H. spontaneum*, then this approach could be exploited.

Critical for such an approach is an effective means of screening for the presence of desirable characters. Biochemical/molecular markers have been widely used to follow the transmission of chromosomes, chromosome segments or even the introgression of specific genes such as disease resistance genes from wild relatives (Nevo *et al.*, 1984). Biochemical/molecular markers are, therefore, an important means of screening hybrid lines that have been genetically fixed by anther culture. If biochemical/molecular markers can be linked to a desired character, they will be valuable in the rapid assessment of lines for presence or absence of the trait. Since *H. vulgare* and *H. spontaneum* represent genetically diverse parents, this maximizes the chances of detecting polymorphism.

The objectives of the present study are:

(a) To investigate the responsiveness of *H. vulgare* and four *Hordeum spontaneum* lines to anther culture.

(b) To follow transmission of anther culture responsiveness into F_1 hybrids following crosses between *H. vulgare* and *H. spontaneum*.

(c) To investigate the potential of anther culture as

a means of rapidly fixing useful genes that have been introgressed from *H. spontaneum* into well adapted *H. vulgare* cultivar, using biochemical and molecular markers to monitor the transmission of parental chromosome segments.

4.2. Experimental Procedure

The experiment was designed to investigate the inheritance of anther culture response of *H. vulgare* cv. Prisma and the four *H. spontaneum* lines (HS2, HS10, HS16 and HS20). Anthers of the above parents and their corresponding hybrids (PXHS2, PXHS10, PXHS16 and PXHS20) were cultured on maltose (6% w/v) based media. Five spikes were used in each genotype. A total of seven florets each containing three anthers from each spike were plated onto 55 mm Petri dishes i.e. there were 21 anthers in each Petri dish and it was replicated 5 times. Cultures were scored after 28 days for the numbers of anthers responding to form callus and/or embryoids, and subsequently for the number of green and albino plants generated. Green plants were further assessed for those with a haploid chromosome complement (n=7) and those which had undergone spontaneous doubling (2n=14) in

culture.

Isoelectric focusing was used to investigate the level of polymorphism present in the isozymes of ß-amylase, water soluble protein (WSP), grain esterase, leaf esterase and hordein (storage protein) in *H. vulgare* cv. Prisma and the four *H. spontaneum* lines. Besides isozymes, molecular (RAPD) marker was also used to detect polymorphism between them. Details of the isozyme systems are presented in table 2.3. The segregation of biochemical and molecular markers in the regenerants of PXHS2 hybrid were also monitored.

4.2.1. DNA Extraction

For extraction of small amounts of DNA from very small portion of leaf tissue (to avoid plant injury), a simple and rapid method (Edwards *et al.*, 1991) was followed. Samples of leaf tissue are collected using the lid of a sterile Eppendorf tube to pinch out a disc of material into the tube. This ensures uniform sample size and also reduces the possibilities of contamination arising from handling the tissue. DNA is extracted as follows: The tissue is macerated in the original Eppendorf tube at

room temperature, without buffer, for 15 seconds. 400 µl of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) is added and the sample vortexed for 5 seconds. The mixture is then left at room temperature until all the samples have been extracted. The extracts are centrifuged at 13000 rpm for 1 minute, and 300 µl of the supernatant transferred to a fresh Eppendorf tube. This supernatant is mixed with 300 µl isopropanol and left at room temperatures for 2 minutes. Following centrifugation at 13000 rpm for 5 minutes, the pellet is vacuum dried and dissolved in 100 µl 1XTE for use in the PCR.

4.2.2. Polymerase Chain Reaction

Details of the PCR are given in chapter 2.

4.3. Results

The percentages of anthers responding to culture to form callus and/or embryoids for each genotype are graphically depicted in figure 4.1. From the figure it is revealed that the *H. spontaneum* lines were poorly responded to culture medium. It is also observed that *H. vulgare* x *H.*

spontaneum F_1 crosses respond to a much greater extent than either *H. vulgare* cv. Prisma or *H. spontaneum* parents in terms of initial anther response. Figure 4.2 illustrates the extent of differential response between anthers of the Prisma, HS10 and the corresponding F_1 hybrid (PXHS10). There are also differences in the level of response within the *H. spontaneum* population and within F_1 hybrid population (Figure 4.1). The number of green and albino plants obtained for each genotype are summarized in figure 4.3. Three (HS2, HS16, HS20) of the four *H. spontaneum* lines did not produce any plant while HS10 produced only one albino. The large number of plants regenerated by Prisma and F_1 hybrids were also albino. Prisma produced five green plants and 27 albino plants. F_1 hybrids togetherly produced 127 albino plants against 14 green plants. Hybrid PXHS16 did not produce any green plant. The analysis of variance is presented in table 4.1 and it indicates that the differences in anther culture response of the nine genotypes considered were significant. There were also significant differences between the *H. spontaneum* parents and the F_1 crosses in the level of response.

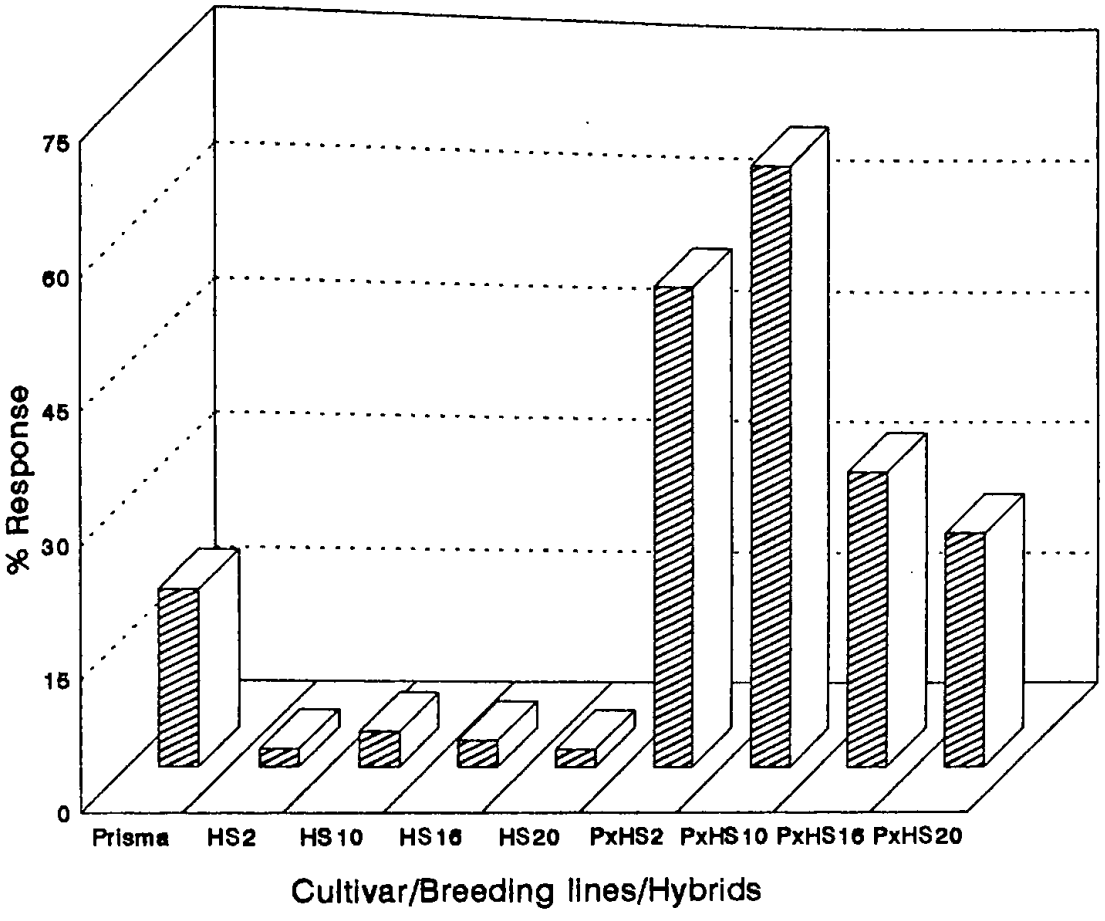

Figure 4.1

Response of anthers to callus/embryoid formation of *H. vulgare* cv. Prisma, four *H. spontaneum* lines (HS2, HS10, HS16 and HS 20) and their corresponding F_1 hybrids.

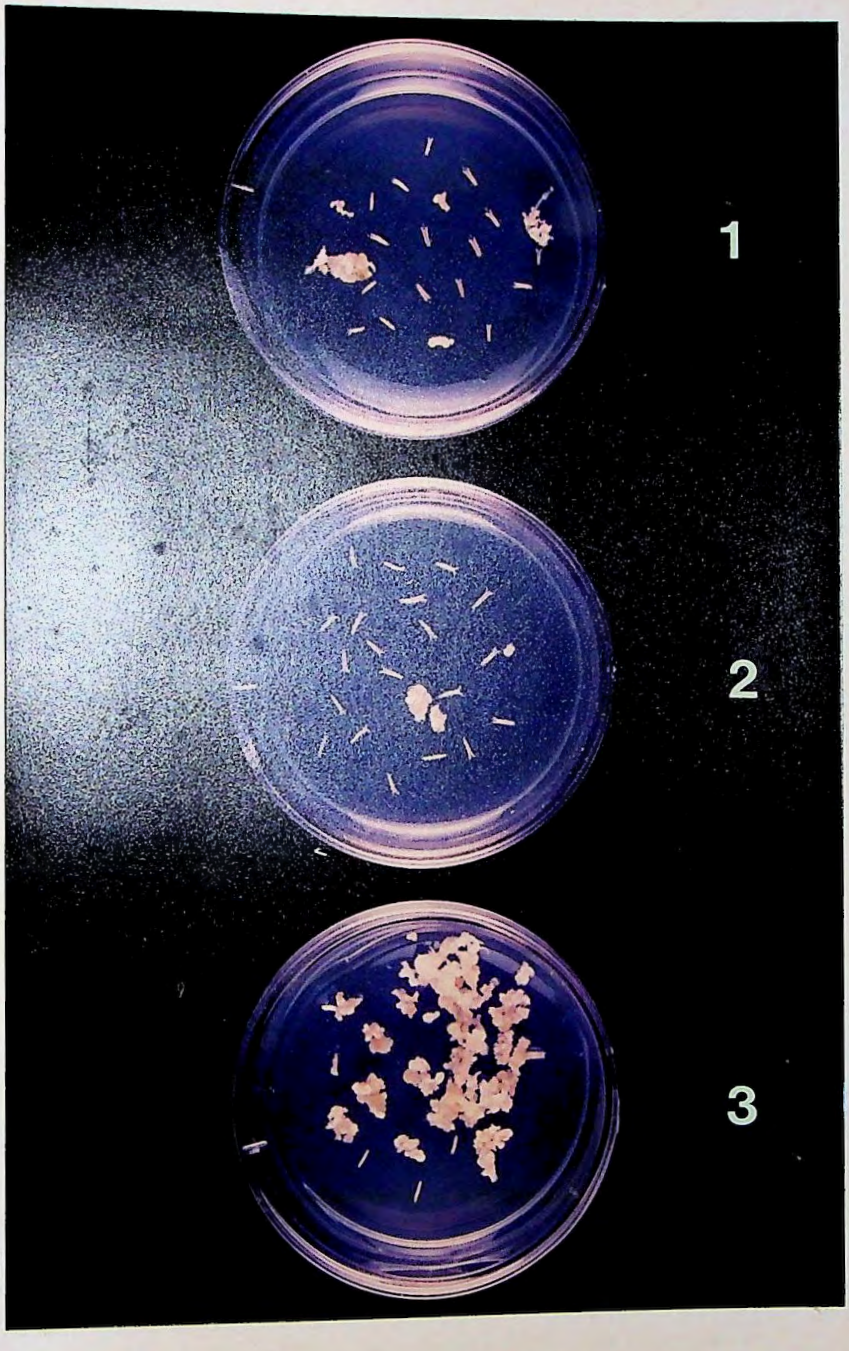

Figure 4.2

Response of anthers to form callus/embryoid of *H. vulgare* cv. Prisma [1], *H. spontaneum* (HS10) [2] and the F_1 hybrid (PXHS10) [3].

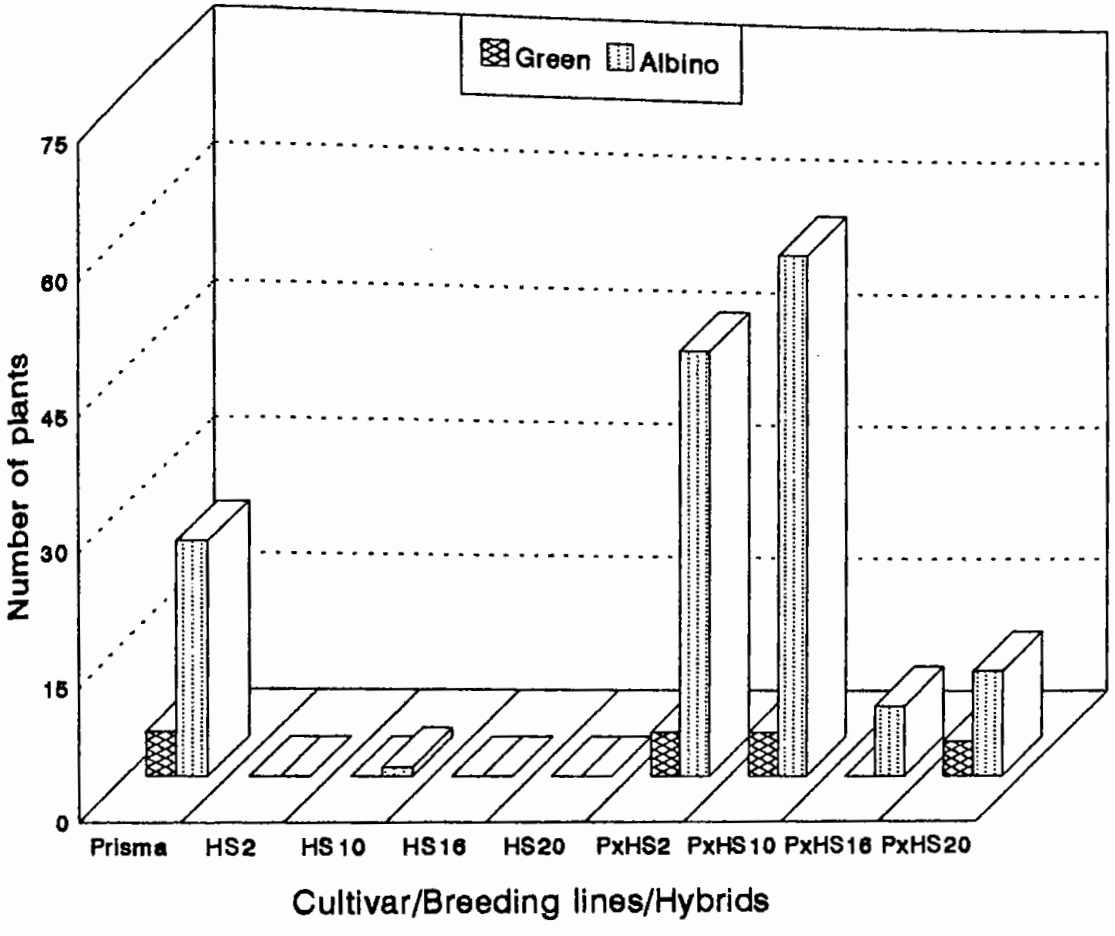

Figure 4.3

Number of green and albino plants regenerated from anthers of *H. vulgare* cv. Prisma, four *H. spontaneum* lines (HS2, HS10, HS16 and HS 20) and their corresponding F_1 hybrids.

Table 4.1. Analysis of variance for anther response of *H. vulgare* cv. Prisma, four *H. spontaneum* lines and their corresponding F1 hybrids.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Replication	4	10.13	2.53	0.71
Genotype	8	1189.60	148.70	41.35**
Error	32	115.07	3.60	
Total	44	1314.80		

** P<0.01

An estimate of the heritability of a trait can be obtained from the regression coefficient of offspring phenotypes on those of their parents. Such a parent offspring regression was used to perform a regression analysis of the mean phenotypes of offspring on the mean phenotypes of both their parents (mid-parent values). In this case the narrow sense heritability h^2_n is:

$$h^2_n = b = \frac{\text{covariance of offspring and parents}}{\text{variance of parents}} = \frac{SP(xy)}{SP(X)}$$

The estimates of the slope "b" provides an estimate of narrow sense heritability (narrow sense heritabilities include the additive genetic components). From figure 4.4 (regression graph) it can be seen that a heritability estimate of 80% was obtained. However, the regression slope is not formally significant.

Despite the high level of initial response to form callus and/or embryoids in anthers of the F_1 lines, there was a low level of green plant regeneration (Figure 4.3). The ratio of the green and albino regenerants for PXHS2, PXHS10, PXHS20 were 1:9.6, 1:11.8 and 1:3 respectively. PXHS16 produced 8 albino plants only. Figures 4.5 and 4.6 illustrate the regeneration of green plants for PXHS2

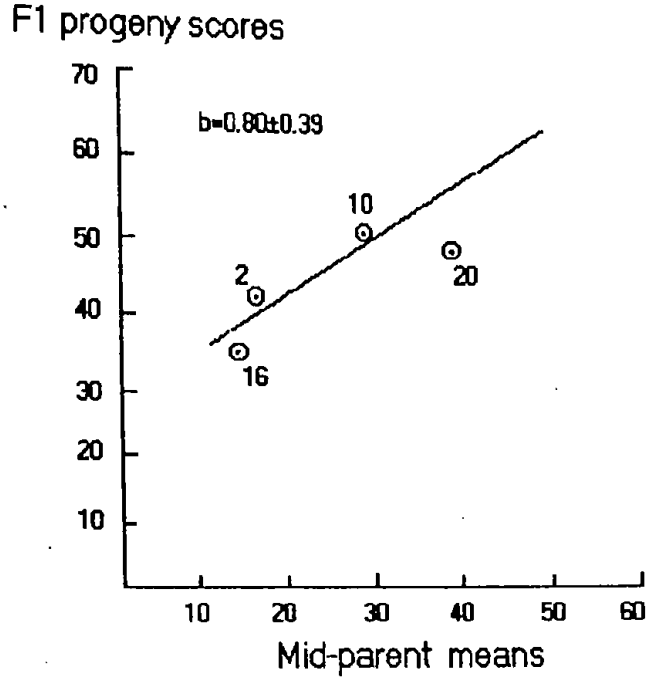

Figure 4.4

The regression of mid-parent means against F_1 hybrid scores of percentage of anther response of cv. Prisma and four *H. spontaneum* lines (2, 10, 16, 20) and the corresponding F_1 hybrids.

Figure 4.5

Regeneration of plantlets in the F1 hybrid of PXHS2 cross.

Figure 4.6

Regeneration of plantlets in the F1 hybrid of PXHS10 cross.

and PXHS10 hybrids respectively. The regenerated green plants were placed in the glasshouse to grow to maturity. The ploidy status of the plants were examined at the tillering stage by root tip study. Two plants (one from Prisma and one from PXHS10) were found haploid while the remaining ones were diploid. Figure 4.7 shows the haploid and doubled haploid plants of cv. Prisma.

The banding patterns obtained for ß-amylase, water soluble protein (WSP), leaf esterase, grain esterase and hordein are presented in figure 4.8. Analysis of banding patterns for ß-amylase (Figure 4.8A) showed extra bands in Prisma when compared to HS2 and HS10. HS16 and HS20 produced identical banding pattern like Prisma. In case of WSP (Figure 4.8 B), the four *H. spontaneum* lines exhibited wide variation in banding patterns among themselves and also with Prisma. Leaf esterase (Figure 4.8 C) and grain esterase (Figure 4.8 D) also displayed polymorphic bands between Prisma and the four *H. spontaneum* lines particularly with HS2. Figure 4.8 E illustrates the banding patterns for hordein which are monomorphic. Besides isozymes, molecular marker (RAPD) detected polymorphism between Prisma and *H. spontaneum* lines (Figure 4.9A). All these polymorphic markers

Figure 4.7

Haploid [1] and doubled haploid [2] plants of cv. Prisma.

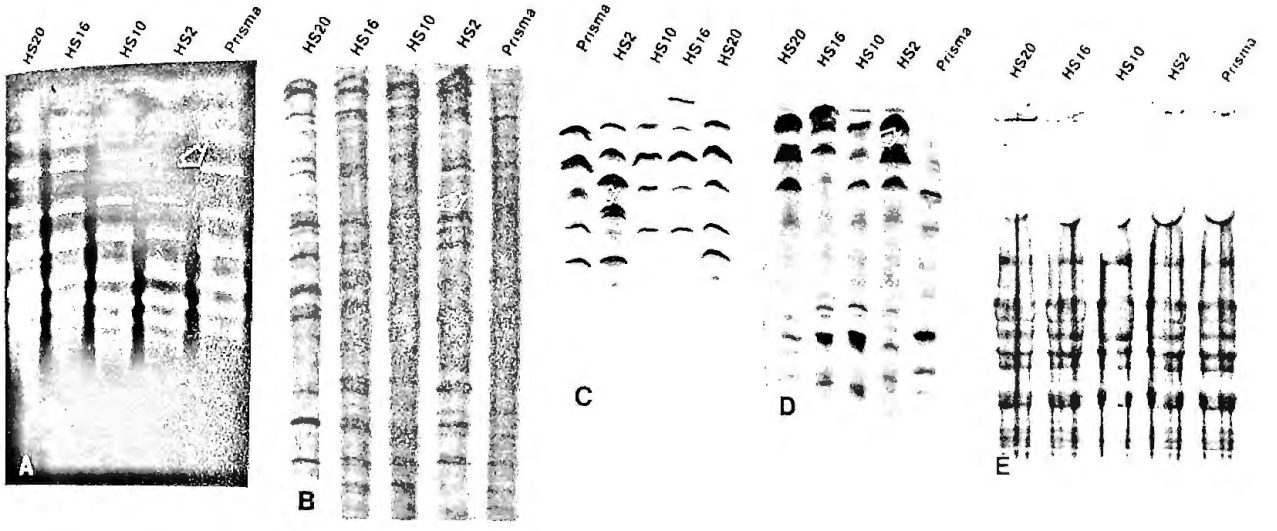

Figure 4.8

Phenotype of cv. Prisma and four *H. spontaneum* lines (HS2, HS10, HS16 and HS20) for ß-amylase [A], water soluble protein [B], leaf esterase [C], grain esterase [D] and hordein [E]. The arrow indicates one of the polymorphic band between Prisma and HS2.

(biochemical and molecular) showed segregation (Figures 4.10 and 4.9 B respectively) in the five doubled haploid plants derived from F_1 anthers of PXHS2. Figure 4.11 shows Prisma (1), HS2 (2) and their corresponding F_1 hybrid (PXHS2) (3) while figure 4.12 shows five doubled haploids obtained from F_1 anthers of PXHS2.

4.4. Discussion

4.4.1. Heritability in Anther Culture

The results obtained in this study demonstrate the ability of *H. spontaneum* to respond to anther culture and there are genotypic differences in the levels of response between the four *H. spontaneum* lines. Genetic differences in anther culture responsiveness imply that the effects must also be heritable (Foroughi-Wehr *et al.*, 1982). A number of studies have been conducted in order to quantify this genetic component. Foroughi-Wehr *et al.* (1982) considered the inheritance of anther culture responsiveness in a study of 55 spring barley F_1 hybrids and 4 cultivars and concluded that culture responsiveness involved at least two independently inherited mechanisms. The first controlled that ability of

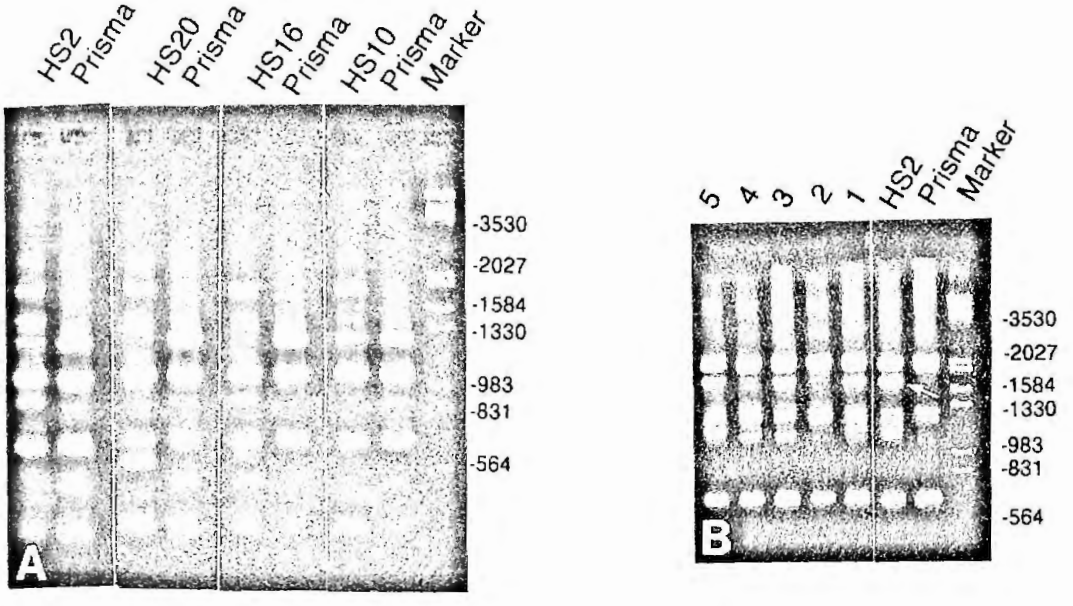

Figure 4.9

Amplification products resulting from PCR showing polymorphicv bands between Prisma and four *H. spontaneum* lines (Primer OPT16) [A]; Segregating markers in the five doubled haploids derived from PXHS2 cross (Primer OPI18) [B]. Marker lane shows molecular size of the product in base ir.

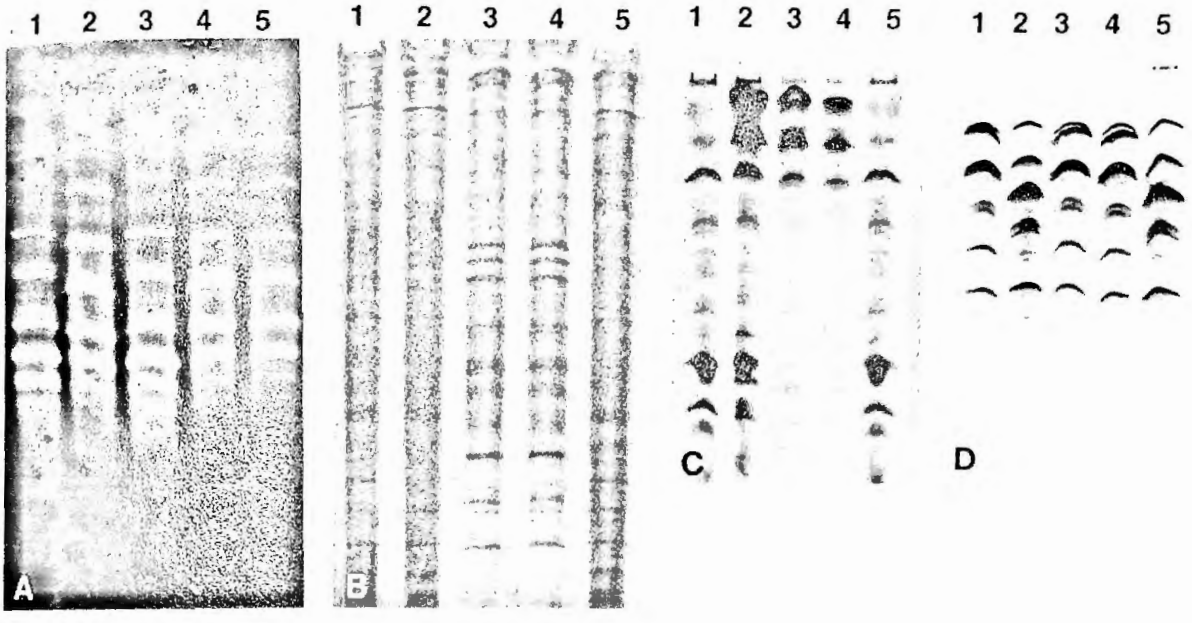

Figure 4.10

Segregation of markers for for ß-amylase [A], water soluble protein [B], grain esterase [C] and leaf esterase [D] in the five doubled haploids derived from F_1 hybrid of PXHS2 cross.

Figure 4.11

Plants of cv. Prisma [1], F1 hybrid (PXHS2) [2] and HS2 [3].

Figure 4.12

Five doubled haploids derived from anthers of F_1 hybrid (PXHS2).

microspore to divide and produce callus and the second influenced the ability of calli to differentiate into plantlets.

These findings have been substantiated by Dunwell *et al.* (1987) in barley and are in agreement with the results obtained in the present study where initial levels of response to culture to form callus and /or embryoids and the subsequent plant formation did not correlate. Foroughi-Wehr and Friedt (1984) suggest that genes determining anther culture responsiveness are dominant or at least partly dominant over non-responsiveness. Lazar *et al.* (1984) investigated anther culture responsiveness in 5 spring wheat varieties and their F_1 crosses. They showed that variation due to genotype was by far the largest component of the total variability between lines. These results provided evidence that both additive and dominance effects contribute to the variation observed among wheat genotypes, but that additive effects predominated. The importance of additive genetic effects in anther culture has also been reported in wheat (Bullock *et al.*, 1982; Deaton *et al.*, 1987), tobacco (Deaton *et al.*, 1982) and rice (Miah *et al.*, 1985). Narrow sense heritabilities have been estimated in a

number of studies of wheat (Lazar *et al.*, 1984; Deaton *et al.*, 1987) and rice (Miah *et al.*, 1985) and found to be quite high (in order of 60-70%). Heritability scores were also calculated in one study (Dunwell *et al.*, 1987) and found to be in the region of 15.5% for green plant production and 28% for initial anther response. A high heritability score for a trait implies that it is controlled by a small number of genes having larger effects. However, heritability is a function of genetic and non-genetic (e.g. environmental) components. Environmental factors can strongly influence anther culture response in barley (Powell, 1988). Lazar *et al.* (1983) have also shown in wheat that there is a significant genotype-environment interaction for anther culturability. It is, therefore, necessary to assess the relative importance of both environmental and genetic factors (Dunwell *et al.*, 1987). Based on the high heritability score obtained in this study, it can be concluded that anther culture response in *H. vulgare* x *H. spontaneum* hybrids has a larger genetic component. This in turn implies that the genetic approach to improving anther culture responsiveness is important, since it has a major effect.

4.4.2. Heterotic Effects

The response of the F_1, obtained from crosses between *H. vulgare* cv. Prisma and *H. spontaneum* lines, was higher than that of any parental lines. This suggests that a heterotic effect is occurring. Heterosis or hybrid vigour for a trait refers to the superiority of the F_1 hybrid over the higher scoring parent. Investigations into wheat anther culture response (Bullock *et al.*, 1982; Ouyang *et al.*, 1983; Lazar *et al.*, 1984) showed that crosses between divergent wheat plants produced a strong expression of hybrid vigour in the F_1 which was not seen in the F_1 between more closely related parents. Heterosis has also been reported for callus growth in alfalfa (Keyes and Bingham, 1979) and tobacco (Keyes *et al.*, 1981). Heterosis can be due to overdominance at one or more loci, or to the dispersion of genes with dominant effects that act predominantly in one direction.

The application of doubled haploids produced by anther culture in breeding programmes has, in general, been most successful in crops with the best anther culture response such as tobacco (Miles *et al.*, 1981), although there has been some success with less responsive crops including

wheat (De Buyser and Henry, 1986). If an F_1 generation displays hybrid vigour, and thus a substantial increase in response to anther culture, then limitations due to low anther culture responsiveness could potentially yield usefully high levels of anther culture regenerants with fixed and stable genotypes. Rapid selections can then be made on these hybrids for desired characters, thus considerably improving the efficiency of selecting desirable gene combinations. Foroughi-Wehr and Friedt (1984) produced F_1 hybrids between winter barley varieties that were either resistant or susceptible to barley yellow mosaic virus (YMV). Anthers were cultured from the hybrids (showing a degree of hybrid vigour) and regenerants were subjected to extensive testing for YMV resistance within one year of hybridization.

4.4.3. The Ploidy of Anther Culture Regenerants

The high proportion of spontaneous doubled haploids obtained here was advantageous, since the inconvenience of using chemicals to induce chromosome doubling was avoided. Spontaneous doubling could be advantageous since it avoids the need for time consuming treatments involving chemicals such as colchicine to induce

chromosome doubling. Chemically induced chromosome doubling would have been time consuming, inefficient, inconsistent, expensive and has been reported to induce mutations in cereal crops (Franke and Rose, 1952; Franchis and Jones, 1989; Hassan *et al.*, 1989). Thus chemically induced chromosome doubling should be avoided if possible. The products of anther culture are not always haploid in constitution (Dunwell, 1985b) and regenerants can be diploid, tetraploid or even higher ploidies in some species. The majority of these non-haploids are derived from single, haploid microspores which have undergone spontaneous chromosome doubling during the first division cycles in culture (Huang and Sunderland, 1982). In many species, plantlets of higher ploidy are produced from pollen at more advanced stages of development than that recommended for anther culture (Sunderland and Dunwell, 1977). In a number of investigations (Huang and Sunderland, 1982; Lyne *et al.*, 1986; Olsen, 1987) up to 90% of the anther culture regenerants obtained have been diploid. It has been suggested that the proportion of haploid to diploid regenerants might be influenced by culture techniques, including the length of the culture phase, as callus cells can undergo spontaneous doubling in culture (Siebel

and Pauls, 1989). It has been suggested that spontaneously doubled regenerants may have a competitive advantage over haploids and the higher number of diploids, therefore, represent a higher survival rate (Jorgensen and Anderson, 1989).

4.4.4. Polymorphism and Segregation of Markers

The results of this study show that there is a high degree of useful polymorphism in *H. spontaneum* lines for four biochemical markers, and that the segregation of these markers can be readily monitored in subsequent generations and in anther culture regenerants. There are several reports of high levels of protein polymorphism in wild barley (Nevo, 1987). An important characteristic of a haploid-producing system is that it should generate lines that represent a random sample of the parental gametes (Powell *et al.*, 1986). However, it has been shown that this may not always be the case. Powell *et al.* (1986) showed non-Mendelian segregation for three morphological markers in anther culture-derived lines and also for one morphological marker in bulbosum-derived lines. In a study by Thompson *et al.* (1990), the segregation of alleles at major gene loci for a number of

biochemical, morphological and molecular markers was followed in anther culture lines derived from four spring barley crosses and their parents. The results showed distorted segregation ratios. The differential transmission of alleles was always in favour of the parent most responsive to anther culture (Powell *et al.*, 1986; Thompson *et al.*, 1990). Biochemical markers represent a useful method for their genetic evaluation if correlations can be established between particular isozyme markers and traits of agronomic importance. Several genotypes of wild barley that are susceptible and resistant to mildew can be associated with single or combinations of isozyme variants (Nevo *et al.*, 1984). Anther culture technology combined with genetic marker technology has, therefore, great potential for the rapid screening of wild barley genotypes and for screening hybrids between *H. vulgare* and *H. spontaneum* for the presence of genes of interest. Besides biochemical markers, RAPD marker also showed high level of polymorphism in *H. spontaneum* lines. Molecular markers also have immense impact for the evaluation of breeding material (Nevo, 1987).

4.5. Conclusions

Anther culture technology can be successfully applied to *H. spontaneum* germplasm. Responsiveness to culture can be transmitted to F_1 hybrids *of H. vulgare x H. spontaneum* and marked heterosis is observed. A high degree of useful polymorphism exists between cultivated and wild barley for biochemical and molecular markers. Doubled haploids can be used to rapidly fix genes from *H. spontaneum* into adapted *H. vulgare* cultivars. The homozygous nature of the anther culture derivatives will allow the progenies to be extensively evaluated.

CHAPTER 5

THE TRANSMISSION OF RAPD ALLELES IN A DOUBLED HAPLOID POPULATION OF BARLEY

5.1. Introduction

The detection and exploitation of naturally occurring DNA sequence polymorphism represents one of the most significant developments in molecular biology (Waugh and Powell, 1992). Restriction endonuclease digestion of total genomic DNA followed by hybridization with a radioactively labelled probe reveals differently sized hybridizing fragments. This form of polymorphism, termed Restriction Fragment Length Polymorphism (RFLP), has been used extensively for genetic studies (Tanksley *et al.*, 1989). However, the technical complexity of performing RFLP analysis, coupled with the widespread use of short-lived radioisotopes in the detection method, has promoted a debate on whether the routine application of RFLPs in large-scale crop improvement programmes is feasible.

An alternative method to RFLP is based on the Polymerase Chain Reaction (PCR). The PCR technique is based on the *in vitro* amplification of specific DNA sequences by the

simultaneous primer extension of complimentary strands of DNA. This method was described and named by Mullis and Faloona (1987). PCR has provided an alternative approach to many procedures in molecular biology and is replacing many standard techniques (Waugh and Powell, 1992). Since its development, PCR has revolutionized many standard molecular techniques, with modifications of the original procedure to suit a range of needs. One such variation generates a specific class of molecular marker termed Randomly Amplified Polymorphic DNAs (RAPDs) developed by Williams *et al.* (1990) and Welsh and McClelland (1990).

The modification of the basic PCR technique which allows RAPDs to be generated is remarkably simple. Instead of using a carefully designed and fairly long oligonucleotide primers to amplify a specific target sequences from a complex DNA template, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template, such as plant genomic DNA. Theoretically, the number of amplified fragments generated by PCR depends on the length of the primer and size of the target genome, and is based on a probability that a given DNA sequence (complimentary to that of the primer) will

occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. For most plants, primers that are 9-10 nucleotides long are predicted to generate, on average, 2-10 amplification products. The primers are generally of random sequence, biased to contain at least 50% Guanines and Cytosines, and to lack of internal inverted repeats. Polymorphisms result from changes in either the sequence of the primer binding site (e.g., point mutation), which prevent stable association with the primer, or from changes which alter the size or prevent the successful amplification of a target DNA (e.g., inversions, deletions, insertions). As a rule, size variants are only rarely detected and individual amplification products are transmitted as dominant markers. Allelic variation detected by RAPDs tend to be inherited in a dominant manner and hence heterozygotes cannot usually be distinguished from homozygous dominant individuals. This problems can be overcome by the use of Recombinant Inbred Lines (RILs) (Burr *et al.*, 1988). In particular doubled haploid families (Powell *et al.*, 1990) are well suited to mapping with RAPDs since dominance-related effects are absent and, hence, linkage can be obtained directly. Such families can be extensively replicated and this

facilitates the partitioning of the total phenotypic variation into heritable and non-heritable components. This approach (called localized mapping) has already been used to map qualitative traits in RILs of *Arabidiopsis thaliana* (Reiter *et al.*, 1992).

The objectives of this phase of experimentations are:

(a) To identify polymorphic RAPD loci between parents.
(b) To monitor segregation of alleles at RAPD loci and establish linkage relationships in a doubled haploid population.

5.2. Experimental Procedure

The experiment was outlined with a view to detect polymorphism between two parents: Blenheim and TS264 by screening with random primers. The polymorphic markers were then evaluated with 33 doubled haploid lines to monitor the segregation of markers. The doubled haploids were derived by anther culture from the F_1 hybrid of Blenheim x TS264.

5.2.1. DNA Extraction and Polymerase Chain Reaction (PCR)

Details of DNA extraction and PCR are outlined in Chapter 2.

5.2.2. Primer and Product Indexing

The following convention was adhered throughout. Consider, for example, the amplification product designated SC10-65-400. It was generated by primer SC10-65 and was synthesized at the Scottish Crop Research Institute, it is 10 nucleotide (nt) in length and numbered 65 in primer collection list. The product of interest 400, generated with this primer is 400 nt in length. Similar is the case for OP (Operon - manufacturer) primer. For example, OPX17-700. OP for Operon and X stands for letter index and under each letter there are 1-20 different primers of 10 nt in length and 700 is the amplification product generated by this primer.

5.2.3. Linkage Mapping

Pairs of linked gene do not segregate according to Mendel,s Law of Independent Assortment. The parental combinations are strongly favoured. Non-parental combinations arise as a result of recombination. The frequency of recombination between two loci is the important index in linkage mapping because it is this frequency that is taken as a measure of their genetic distance apart. The greater the distance between two loci the more likely it is that there will be recombination events in the intervening area and *vice versa*. By comparing the recombination frequencies of numerous loci pairs, a picture can be gradually built up of which loci are linked and, within these linkage groups, estimate made as to the distance separating loci pairs. In linkage mapping the distance between loci is expressed as Centimorgans (cM). 1 cM = the distance that separates two genes between which there is a 1% chance of recombination. MAPMAKER programme (Lander *et al.*, 1987) was used to estimate linkage relationships between segregating loci in 33 doubled haploid populations derived from Blenheim x TS264 cross.

5.3. Results

The two parents Blenheim and TS264 were screened by random primers to detect polymorphisms between them. A total of 165 different primers of arbitrary nucleotide sequence were used to generate amplification products. Figure 5.1 shows that amplification products of different sizes, which are easily resolved by electrophoresis and visualized by staining with ethidium bromide, were generated for each primer. The amplification products were upto 2.5 kb (kilo base) in length. While most of the products were common to both individuals, some products were amplified from one individual but not from the other. For example, a 2.0 kb (kilo base) DNA segment was amplified by primer OPW5 from TS264 but not from Blenheim (Figure 5.1). Of the 165 primers evaluated, 22 detected scorable polymorphism between the parents. The segregation of the polymorphic RAPD loci was also monitored in 33 doubled haploid populations together with their parents. In some cases a primer detected two polymorphic loci between the parents and also segregated in the doubled haploid population (Figures 5.2 and 5.3). A total of 984 amplification products were detected with 165 primers (Table 5.1) and 27 loci were shown to segreg-

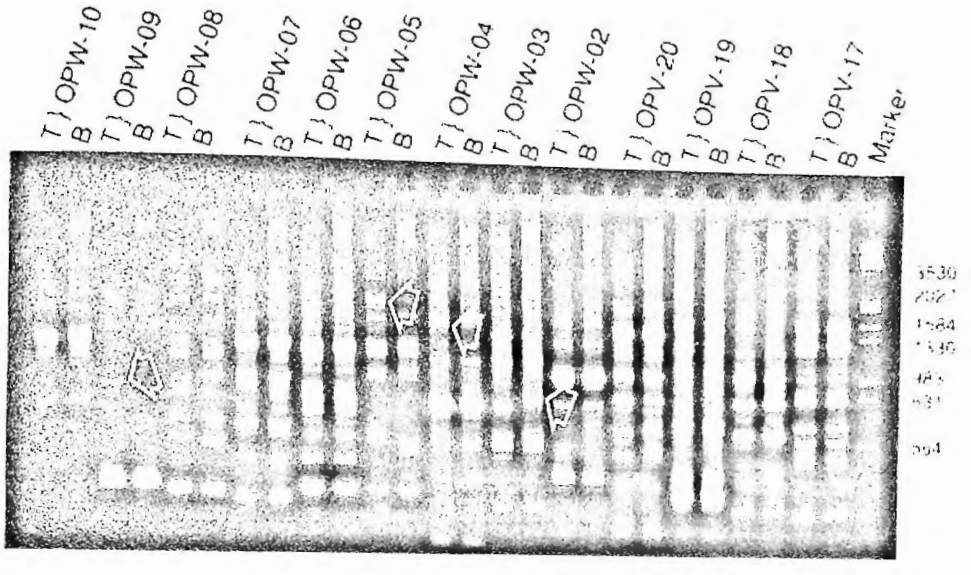

Figure 5.1

Amplification products resulting from PCR carried out on Blenheim (B) and TS264 (T) by different primers. The arrows show polymorphic bands between the parents.

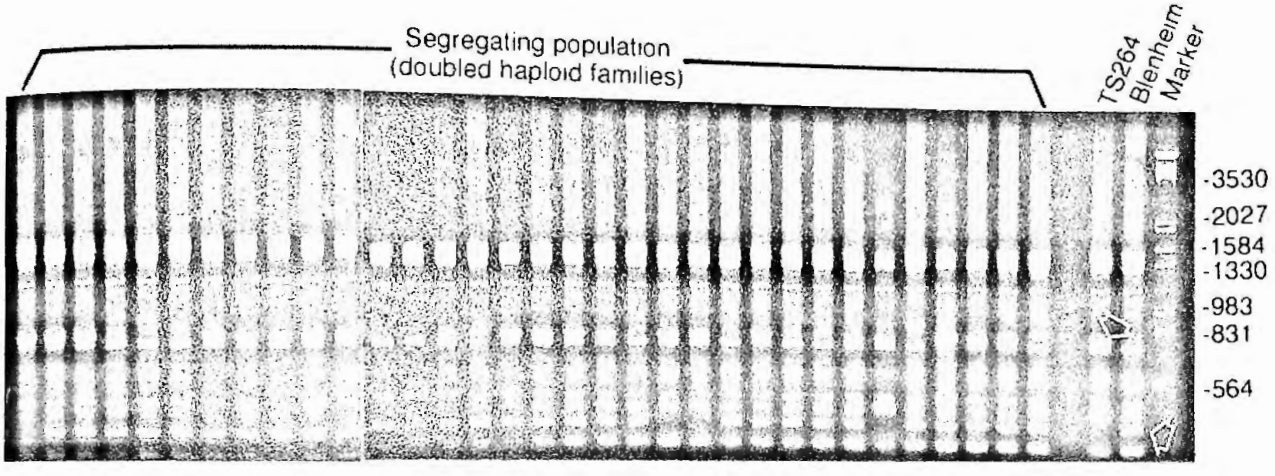

Figure 5.2

Amplification products resulting from PCR by the primer OPQ12 carried out on Blenheim (B), TS264 (T) and their 33 doubled haplois progeny. The arrows show segregating band and lane marker gives molecular size of the product in base pair.

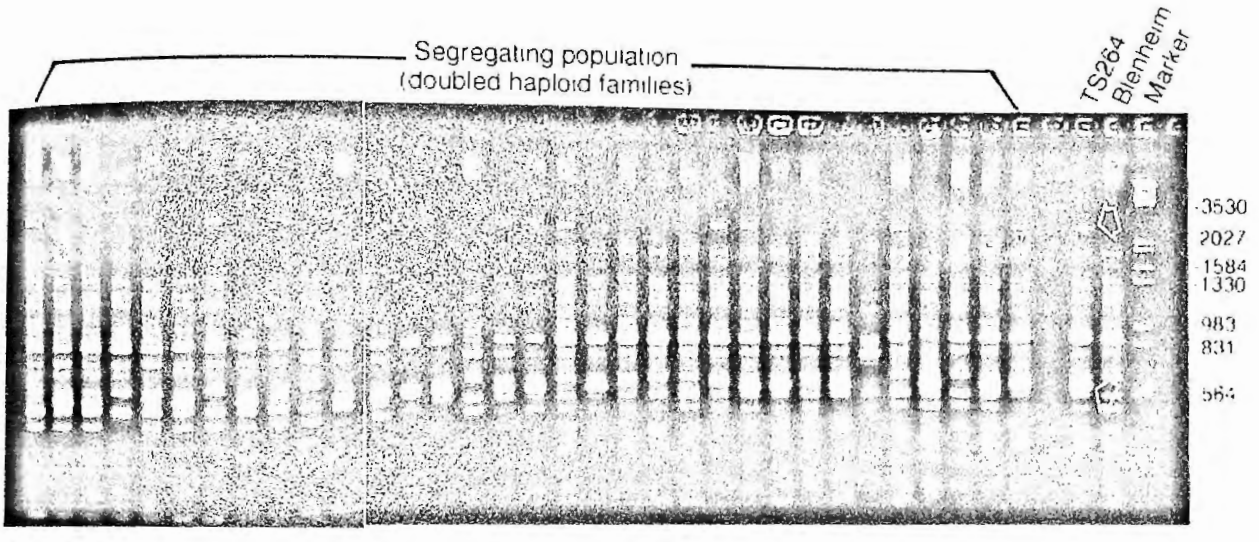

Figure 5.3

Amplification products resulting from PCR by the primer OPT4 carried out on Blenheim (B), TS264 (T) and their 33 doubled haplois progeny. The arrows show segregating band and lane marker gives molecular size of the product in base pair.

Table 5.1. Primers used and amplification products generated in RAPD analysis.

Parameter	Number
Primers screened	165
Polymorphic primers	22
% of polymorphic primers	13
Total amplification products	984
Amplification products per primer	5.96
Segregating loci	27

gate in the doubled haploid population. An average 13% of the primers were found polymorphic and the number of products per primer was 5.96. Data on the segregation of amplification products (loci) are presented in table 5.2. *Chi*-square tests were performed to test for significant ($P < 0.05$) departures from the expected 1:1 ratio. The majority of the loci did not deviate from the expected ratio but seven loci namely, OPX17-700, OPT4-2500, OPQ12-200, OPR9-1400, OPI6-1100, OPW5-2000 and OPB4-700 exhibited segregation distortion (Table 5.2). Of these seven loci, five exhibited an excess of Blenheim alleles while loci OPX17-700 and OPR19-1400 showed an excess of TS264 alleles.

The RAPD loci segregating in this cross were analyzed using MAPMAKER software programme (Lander *et al.*, 1987) for co-segregation of linked markers. Multiple linkage analysis and logarithm of odds ratio (LOD) score of four was established for linkage and Haldane's mapping function was used to convert recombination frequencies to map distances in centimorgans (cM). Eight loci were shown to be genetically linked and four linkage groups were established (Figure 5.4).

Table 5.2. *Chi*-square ($\chi 2$) analysis of segregating RAPD markers in a doubled haploid population derived from the cross between Blenheim (B) and TS264 (T).

Markers identified	Base sequence 5' to 3'	Blenheim (B) Type	TS264 (T) Type	$\chi 2$ (1:1)
OPX17-700	GACACGGACC	11	22	3.667*
OPX3-700	TGGCGCAGTG	18	15	0.273
OPT4-2500	CACAGAGGGA	23	10	5.121*
OPT4-500	CACAGAGGGA	16	17	0.030
OPT7-700	GGCAGGCTGT	17	16	0.030
OPQ12-200	AGTAGGGCAC	28	05	16.030*
OPQ12-700	AGTAGGGCAC	14	19	0.758
OPR19-1400	CCTCCTCATC	06	24	10.800*
OPR15-2000	GGACAACGAG	15	17	0.125
OPR15-700	GGACAACGAG	14	19	0.758
OPR9-200	TGAGCACGAG	17	14	0.290
OPR7-400	ACTGGCCTGA	16	17	0.030
OPI18-980	TGCCCAGCCT	17	14	0.290
OPI6-1100	AAGGCGGCAG	29	04	18.939*
OPH7-830	CTGCATCGTG	21	12	2.455
SC10-65-400	CAGGGGTGAT	21	12	2.455
SC10-65-200	CAGGGGTGAT	20	13	1.485
OPO13-700	GTCAGAGTCC	21	12	2.455
OPO13-400	GTCAGAGTCC	16	17	0.030
OPW4-1400	CAGAAGCGGA	19	14	0.758
OPW5-2000	GGCGGATAAG	22	11	3.667*
OPQ8-2400	CTCCATGGGA	13	20	1.485
OPB4-700	GGACTGGAGT	21	10	3.903*
OPJ5-980	CTCCATGGGG	13	20	1.485
OPP8-700	ACATCGCCCA	18	14	0.500
OPP4-2000	GTGTCTCAGG	18	15	0.273
OPU12-1580	TCACCAGCCA	17	16	0.030

* $P < 0.05$

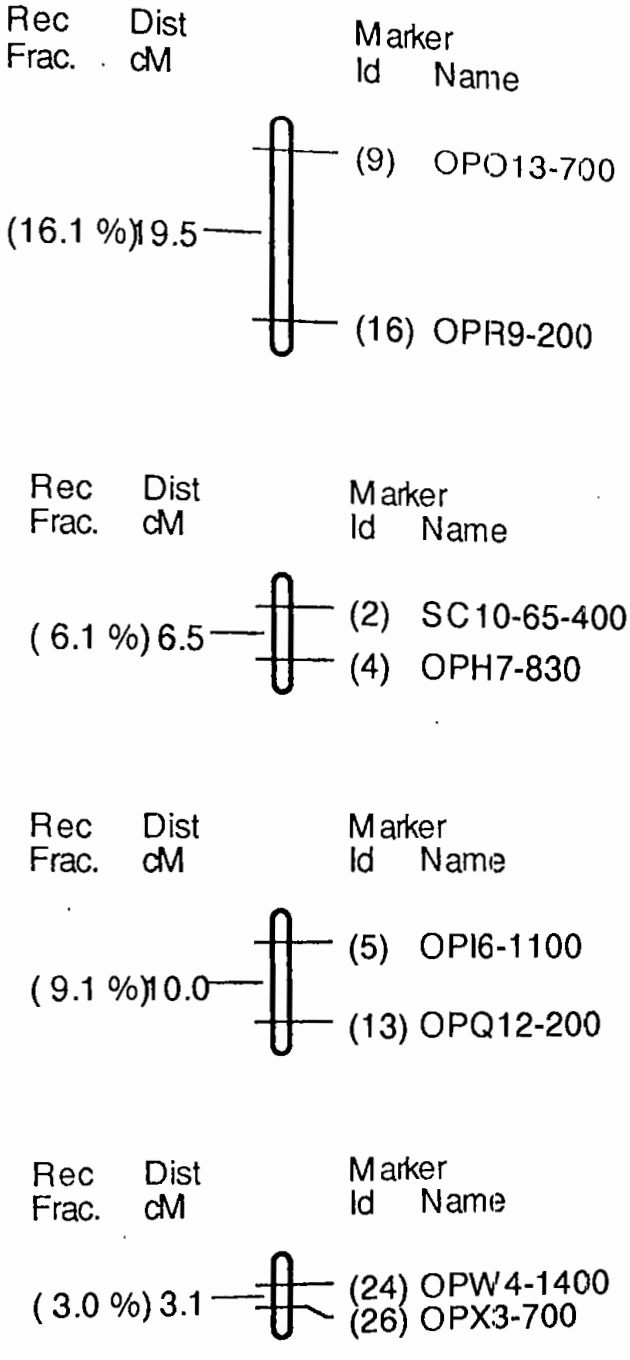

Figure 5.4

Linkage relationships of eight RAPD markers forming four linkage group.

5.4. Discussion

The results obtained in this study demonstrate that RAPDs can reveal polymorphism for genetical and breeding studies. RAPD markers can be used to construct genetic maps. Doubled haploids have been found valuable for calculating recombination values between linked markers. The concept of using linked genetic markers to predict the transmission of another gene complex is not new (Tanksley, 1983). However, this approach has been greatly facilitated by the development of methods to detect polymorphism at the molecular level. The use of RAPD markers provides an efficient, reproducible, and relatively inexpensive approach to comparative genomic mapping studies (Williams *et al.*, 1990). In particular, the level of variation for RAPDs appears to be much higher than most comparable classes of molecular markers (Rieseberg *et al.*, 1993). RAPDs can be exploited as markers that segregate in a Mendelian manner (Rafalski *et al.*, 1991). Doubled haploid families extracted from F_1 hybrids heterozygous for marker genes are expected to segregate for alleles in a 1:1 ratio. Classification of doubled haploid families into two groups carrying the alternative allele allows the transmission of marker

genes to be evaluated and linkage relationships established.

Genetic markers are useful in tagging disease resistance genes, varietal identification and screening germplasm resources (Tanksley *et al.*, 1989). In plant breeding, the usefulness of genetic markers is based on finding tight linkages between the markers and the genes of interest (Tanksley *et al.*, 1989). Such linkage permits one to infer the presence of a desirable gene by assaying for the marker. There are a number of single gene traits that are frequently transferred from one genetic background to another by breeders. One example is genes conferring resistance to pathogens. Traditionally, progenies are screened for the presence of disease resistance genes by inoculations with pathogen. However, simultaneous or even sequential screening of plants with several different pathogens can be difficult or impractical. In contrast, detecting disease resistance genes by their linkage to RAPD markers makes it practical to screen for many different disease resistance genes simultaneously without the need to inoculate the population (Tanksley *et al.*, 1989). With the exception of specific disease resistances, morphological and colour pattern traits

which are often determined by allelic differences at one or two loci, genetic variation in most traits of agricultural importance is attributed to allelic differences at a large but generally unknown number of loci having relatively small individual effects. These loci are termed as quantitative trait loci (QTL) (Soller and Beckmann, 1988). For most quantitative traits little is known about the number, or chromosomal position of individual or interactive effects of genes controlling their expression (Tanksley *et al.*, 1989). Thoday and his colleagues (Spickett and Thoday, 1966) working with *Drosophila* and using special tester strains have shown how polymorphic genetic markers can be used to locate a quantitative trait (bristle number in their case).

Chalmers *et al.* (1992) used RAPD markers to partition the genetic variation in *Gliricidia sepium* and *G. maculata*. Five individuals from 10 geographically and ecologically distinct populations were examined with 11 primers. Overall, 60% of the genetic variation was attributed to between- population and 40% to within- population components. Wilde *et al.* (1992) performed RAPD analysis on a range of cocoa (*Theobroma cacao*) accessions representing a diverse spectrum of available germplasm.

Using only a small selection of 10-mer primers, it was possible to fingerprint all the individual accessions used in this study.

Rieseberg *et al.* (1993) generated a detailed linkage map for *Helianthus anomalus*, a diploid species derived via hybridization between *H. annuus* and *H. petiolaris*. In this study 74 out of 280 (26%) RAPD primers produced polymorphic amplification products between the parents and 162 polymorphic loci were mapped to 18 linkage groups from the 56 *H. anomalus* progeny as well as from the 37 individuals from natural populations of *H. annuus* and *H. petiolaris*.

5.5. Conclusions

Doubled haploids in conjunction with RAPDs provide an efficient way of developing linkage maps. Since doubled haploids represent fixed inbred lines which can be extensively replicated, they provide a means of obtaining estimates of the environmental and genetical factors controlling quantitative traits. Doubled haploid families which have been simultaneously scored for RAPDs and quantitative traits provide a means of locating quantitative traits to specific chromosome.

CHAPTER 6

SUMMARY

The success of the programme on barley breeding has been dependent on the two main strategies. First, a population which exhibits variation for the character on interest is screened using experimental designs allowing accurate partitioning of the total phenotypic variation into heritable and non-heritable components. Second, polymorphic assay procedures are applied which allow allelic variation independent of other genetical and environmental factors to be monitored.

The creation of haploids from heterozygous plants gives individuals with one member of each chromosome pair. Creation of doubled haploids from heterozygous plants give completely homozygous plants in a single generation and this offer the quickest possible approach to homozygosity. The absence of within family segregation, together with the ability of replicate genotypes results in doubled haploids being a valuable source both for cultivar production and the analysis of polygenic sources of variation. Doubled haploids can be derived from plants which have undergone just one round of recombination and therefore, give maximal expression of linkage relationships. These attractive features of doubled

haploids have promoted researchers to develop methods for the efficient production of doubled haploids in barley.

There are a number of ways of producing doubled haploids in barley, but for them to be use in genetic analysis, certain criteria have to be fulfilled, (a) it must be possible to produce large numbers easily and consistently, (b) the lines should be genetically normal and stable, and (c) ideally they should represent a random sample of parental gametes. These constraints have led to the exploitation of anther culture methods for the production of doubled haploid population in barley. This technique involves the use of tissue culture technology to divert normal pollen grain development into the production of haploid embryoids and subsequently doubled haploids.

Four spring barley cultivars viz., Abbey, Dallas, Bianka and Decado were tested for their response to culture media modified by supplying the carbohydrate source as either sucrose or maltose at a concentration of 1, 3, 6 and 9 % w/v. The medium containing maltose was found to be superior to sucrose based medium in terms of anther response as well as green plant regeneration. There were also genotypic differences in the ability to respond to culture. Higher carbohydrate concentration (6 and 9% w/v)

produced better response than the lower ones (1 and 3% w/v). On maltose based media, Abbey showed maximum response to callus/embryoid formation followed by Dallas and Decado while Bianka yielded lower level of callus/embryoid formation. A high proportion of embryogenetic structures were developed in maltose-based media whilst on sucrose more callus was observed. The number of green plants regenerated on maltose-based medium was significantly higher than sucrose. In green plant regeneration, Decado produced highest number of plants (69) followed by Dallas (46) and Abbey (36). Bianka yielded considerably fewer number (13) of green plants. Response of anthers to produce callus/embryoids is not necessarily a reliable indicator of the number of green plants that will subsequently be recovered. Abbey and Dallas gave higher response to callus/embryoid formation than Decado but both of them produced lower number of green plants than Decado.

The substitution of sucrose by maltose as a carbon source in anther culture medium has led to an improvement in the production of doubled haploids in barley. Maltose also induced green plant production by promoting embryogenesis, thus by passing the more conventional and problematic callus phase. Callus formation if often associated with aberrant mitotic activity and

gametoclonal variation resulting in the regeneration of unbalanced and unstable genotypes. In general, maltose raised the performance of all four cultivars with respect to anther response, embryoid formation and green plant regeneration in comparison with sucrose.

In the inheritance study of anther culture response, *H. vulgare* cv. Prisma, four *H. spontaneum* lines (HS2, HS10, HS16 and HS20) and their corresponding F_1s were taken as the experimental material and were cultured on maltose-based (6% w/v) medium. Anther culture response of Prisma was about 19% while the same was very poor (1.90-3.30%) for *H. spontaneum* lines. However, their F_1 crosses (*H. vulgare* x *H.spontaneum*) responded to a much greater extent (26-75%) than either parents. In respect of plant regeneration, three of the four *H. spontaneum* lines (HS2, HS16 and HS20) failed to produce any plant while HS10 produced only one albino plant. The majority of the plants produced by Prisma and F_1 hybrids were albino. Prisma regenerated five green plants and 26 albino plants. F_1 hybrids of PXHS2, PXHS10 and PXHS20 produced 5, 5 and 4 green plants respectively. Hybrid PXHS16 did not produce any green plant. They altogether produced 126 albino plants. Heritability estimate obtained in this study was 80% indicating anther culture response in *H. vulgare* x *H. spontaneum* hybrids has a large genetic

component. A high degree of useful polymorphism was detected between *H. vulgare* cv. Prisma and *H. spontaneum* lines for biochemical (ß-amylase, Water Soluble Protein, Leaf esterase, Grain esterase) and molecular markers (RAPDs). Biochemical marker hordein did not show any variation between *H. vulagre* and *H. spontaneum*. All these polymorphic markers showed segregation in the five doubled haploids derived from F_1 anthers of P x HS2 hybrid.

Two parents Blenheim and TS264 and 33 doubled haploids derived by anther culture from the F_1 hybrid obtained from crosses between the above parents were used in the segregation study of RAPD marker. A total of 165 random primers were used to detect alleles at RAPD loci between the parents. Twenty two primers generated polymorphic amplification products and the segregation of these polymorphic loci were also scored in the 33 doubled haploids progeny. Total number of amplification products generated by 165 primers were 984 and 27 loci were shown to segregate in the doubled haploids progeny. An average 13% primers were found polymorphic and the average number of products per primer was 5.96. Chi-square tests were performed to test the significant departures from expected 1:1 ratio. The majority of the loci did not deviate from the expected ratio but seven loci showed

segregation distortion. Segregation of RAPD loci in this cross was analyzed using MAPMAKER programme. Eight loci were shown to be genetically linked and four linkage groups were established.

The concept of using linked marker gene to predict the transmission of another gene complex is not new. However, this approach has been greatly facilitated by development in methods to detect polymorphism, particularly at the molecular level. Doubled haploid families extracted from F_1 hybrids heterozygous for marker genes will on average segregate in a 1:1 ratio. Classification of doubled haploid families into two groups carrying the alternative allele allows the effect of that particular marker gene on quantitative traits to be assessed. The choice of which marker to use will depend on the precise objective of the study.

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