ANTHER CULTURE OF AUSTRALIAN WHEATS

by

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Contents

Preface

Abstract

The time taken to breed new cultivars of wheat (*Triticum aestivum*) must be reduced if rapidly changing agricultural requirements are to be met. Anther culture provides an excellent way to achieve this reduction but there are currently two major limitations to its practical use in wheat breeding - limited genotype response and low overall success rates. To examine these restrictions, two research priorities were identified, (i) the effect of genotype, the genetic control of response and the culturability of Australian genotypes of current breeding value and (ii) the effect of cultural factors on anther culture response.

An initial investigation was made to determine the applicability of seven media for use for the overall culture of wheat under the local conditions in which experiments were to be conducted. Eight genotypes were used. Only one medium achieved a response from all eight genotypes. This medium, WM2, was liquid rather than solid and was based on that of Datta and Wenzel (1987, Plant Science 48:49).

WM2 was used as the basis for further experiments with 80 000 anthers from twenty two genotypes. Several controllable factors - donor plant environment, microspore stage, cold pretreatment of spikes, incubation temperature and sugar - were varied in seeking an optimum set of conditions. Embryos and green plants were produced. The main conclusions were, (i) genotype is the predominant factor affecting anther culture response, (ii) all the tested factors had significant effects on the culturability of anthers and two-way interactions were also commonly significant, (iii) embryos occurred independently with respect to each other showing no effect of contagion.

The results showed that anthers for culture should (i) be taken from donor plants grown in a glasshouse rather than a growthroom particularly under Spring-time conditions; (ii) contain healthy microspores from early mid uninucleate to binucleate stages; (iii) be from spikes given a cold pretreatment of 4°C for between 3-10 days; (iv) receive an early incubation temperature shock of 30°C for 2-3 days and (v) then be placed at 25°C.

Comparison of four sugars at constant concentration showed that the type of sugar was a major determinant of response, for both embryo and green plant production. More Australian genotypes responded on cellobiose (7 of 9) than on any other sugar tested, while more non-Australian genotypes responded on sucrose (3 of 4). Responses on maltose and gentiobiose were poor. The results with maltose and cellobiose contrast strongly with recent reports on the widespread suitability of maltose and indicate that cellobiose should be the first sugar used when attempting anther culture of Australian wheat genotypes.

Both responsive and non-responsive groups of genotypes were identified. The majority of responsive Australian cultivars were CIMMYT-bred wheats of which most were derived from the Australian breeding line WW15 but not all the CIMMYT-bred wheats were responsive. Similarly, non-responsive genotypes tended to be derived from Insignia, Gabo and Nabawa. Enhanced response was also associated with the translocation chromosome 1B/1R. In spite of the vastly greater culturability of barley, a series of wheat-barley addition lines showed very little response.

The proposed relationship between male sterility and culturability was investigated using cytoplasmic male sterile breeding lines, pentaploid lines and gametocide-treated plants. The main findings were that both cytoplasmic and nuclear genotype affected anther response. Nuclear genotypes of *Triticum aestivum* in the cytoplasms of *Triticum timopheevi* and *Aegilops variabilis* were used. One nuclear genotype responded in alloplasm but not in euplasm, while another responded in euplasm but not in alloplasm. The pentaploid lines and gametocide-treated plants failed altogether to respond. From the results it was not conclusive that male sterility was a consistently promotive factor for anther culture.

This study has demonstrated the value of screening wheats of unknown culturability and local breeding stocks. The genetic commonalities proposed for genotypes of responsive and non-responsive groups can henceforth be used to forecast the aptitude of other wheats to anther culture. The greatest potential value of anther culture is as a tool for breeding wheat. The criteria formulated in this thesis are a step towards applying anther culture technology to current Australian breeding programmes.

The information presented in this thesis is a basis on which anther culture of Australian wheat can be improved. Overall, cultural modifications can greatly improve anther culture response but improvements hinge on the responsiveness of the material used. Where the germplasm is clearly responsive, anther culture has great potential. The implications of these results are considered in the context of the application of anther culture technology to wheat research and breeding.

Declaration

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

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

Deirdre E. Frappell May 1993

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Chapter 1 General Introduction

"Pollen grains are not only the genome carriers, self-reliant, self-supporting, packed with enzymes and substrates for the early progamic phase, instrumental in the recognition of the right way and highly selective in accepting the right partner for a successful fertilization process, but also well preserved in a wrapping which is very tough."

(Linskens 1987)

This inherent high degree of specialization makes pollen one of the most remarkable of plant products. In conventional breeding procedures, the pollen grains endure extreme stress through mechanical handling, desiccation and radiation all of which threaten to damage the enclosed DNA and therefore the genetic information for the next generation. The survival of pollen is primarily due to the extremely resistant nature of its walls, as is shown by the existence of fossilized pollen (Linskens 1987). The realization that pollen could germinate *in vivo*, in the gametophytic state, to produce haploid structures (Gaines & Aase 1926) led, after several decades of research, to the inspiring discovery that haploid embryos could also be generated *in vitro*, by culturing anthers (Guha & Maheshwari 1964, 1966). The manipulation of such specialized and resistant cells in this way heralded the beginning of an exciting field of research for plant breeders, geneticists, biochemists and others; namely that of anther culture.

Pollen embryogenesis is one of the most striking examples of cellular totipotency in plants. This phenomenon is the cornerstone of anther culture. Basically, when anthers containing immature pollen are aseptically cultured on an appropriate medium, some of the pollen may undergo multiple cell divisions resulting in the formation of either unorganized masses of cells (callus) or discrete organized embryo-like structures (somatic embryos). Somatic embryos are formed if microspore development is altered from the normal sporophytic mode to one which is gametophytic. Thus in wheat, embryos are formed if the microspore cell undergoes repeated mitotic divisions instead of giving rise to normal, mature pollen grains after only three divisions. Appropriately handled, these embryos will continue to develop in culture and differentiate to form whole plants which have a haploid number of chromosomes. The formation of plants from male gametes is referred to as "androgenesis".

Somatic embryos formed by culture of a tissue are often called embryoids although they will be referred to as embryos throughout the thesis according to the recommendations presented for haploid terminology at the First International Symposium for Haploids in Higher Plants held at Guelph University, Canada (de Fossard 1974). Similarly, plants formed from the regeneration of embryos will be called plants, not plantlets.

Haploids may be produced in ways other than from anther culture. Firstly, in some species, certain genotypes produce haploids spontaneously *in vivo* (Kimber & Riley 1963) and this phenomenon has been used to produce haploid plants. These haploids originate from either the male (hence androgenic) or female (parthenogenic) gametes.

In vitro, haploids may also be obtained using ovary culture (San Noeum 1976; Dunwell 1985a), which is reviewed in detail by Dunwell (1985b). Anther culture has considerable advantages over ovary culture because male gametes are produced in vastly greater numbers than are female gametes (see Heberle-Bors 1985). Thus, not only are the numbers of haploids greater but, in the case of heterozygous parental plants, their genetic diversity is also greater.

Haploids are also produced by isolated microspore culture (Datta & Wenzel 1987) which is virtually an extension of the principle of anther culture and as such, the factors governing culture success are not dissimilar to those operating in anther culture. The microspores within the anthers are chemically or mechanically released and then cultured in a liquid artificial medium. Furthermore, protoplasts can be isolated from the microspores, making this type of culture suitable for genetic studies where the direct manipulation of the unicellular microspore is required (Lörz *et al.* 1988).

An alternative method for haploid production - other than gametogenesis - is one which uses chromosome elimination from the zygote formed in an interspecific cross. Although fertilization occurs normally, the chromosomes of the male parent are gradually and preferentially eliminated leaving the embryo with only the female chromosome complement. The embryo is rescued and cultured, and germinates into a haploid plant. This technique, now used in several barley breeding programmes, was first developed using *Hordeum bulbosum* as the male parent in interspecific hybrids with cultivated barley (Kasha & Kao 1970) and has since become known as the 'bulbosum' method for producing haploids (Kasha 1974). The bulbosum method of producing barley haploids was the preferred method for breeding purposes over anther culture (Huang *et al.* 1984) until significant increases in yields in barley anther culture (Hunter 1987; Olsen 1987; Sorvari & Schieder 1987) surpassed those possible by chromosome elimination (Picard 1989). Although the 'bulbosum' method can be used with wheat (Barclay 1975; Sitch & Snape 1986; Inagaki 1989), its successful commercial application has been limited to barley (Devaux 1987, 1988). Furthermore, it has the disadvantage of being more labour intensive and less cost effective than anther culture (Snape *et al.* 1986). Wheat haploids have also been successfully produced through interspecific crosses with maize (Comeau *et al.* 1988; Laurie & Bennett 1988; Laurie *et al.* 1990; Laurie & Reymondie 1991) and this procedure is being evaluated in laboratories in several countries.

Haploid breeding procedures offer substantial advantages to the plant breeder. Firstly, haploid breeding has the potential to reduce substantially the time taken to develop new varieties in comparison with traditional methods. Using conventional procedures several generations are needed to produce a set of fixed, homozygous lines from a heterozygous population. Completely homozygous lines can, in contrast, be produced from haploids in a single generation following chromosome doubling. Chromosome doubling occurs spontaneously at a high frequency in culture (Hu 1989) but may be induced chemically with colchicine (Barnabás *et al.* 1991) or less frequently with other agents (Jensen 1974). Secondly, haploid breeding can greatly increase the efficiency of selection for recessive traits in the early generations of a breeding programme as no dominance of characters can occur when plants are either haploid or homozygous. Furthermore, haploid production via anther culture offers an opportunity to study and select mutagen treated plants and to apply selection pressures *in vitro*.

Haploids have resulted in practical breeding advances in wheat with the release of an anther-derived cultivar in China, cv. Jinghua-1 (Hu 1986a) and in France, cv. Florin (de Buyser *et al.* 1987). The genetic modification of crop plants *in vitro* is dependent on the ability to regenerate plants. For haploids to be of use in and incorporated into current breeding programmes they must (i) be vigorous, fertile and hence doubled haploids, (ii) be viable, green regenerants and (iii) have characters of practical breeding importance. The only way in which the third criterion can be satisfied is if haploids are selected from a wide range of genetically useful breeding genotypes. Since the production of wheat regenerants through anther culture is still less than can be obtained with barley (Table 1.1) - the closest related culturable species - the parameters involved in control of wheat anther culture response require further examination.

Table 1.1 Maximum frequencies of green plants reported via anther culture in barley and wheat. (after Kasha *et al.* (1990); References in the table are cited in the source.)

Reference	Medium	Yield per 100 anthers	
		Calluses (embryos)	Green plants
Barley			
Foroughi-Wehr & Friedt 1982	modified MS	7.3	1.3
Kao & Horn 1982	Ficoll	309.0	10.0
Szarejko & Kasha 1989	BAC 3	414.0	14.4
Sorvari & Schieder 1987	EDAM	1730.0	43.3
Olsen 1987	modified MS	-	464.0
Hunter *et al.* 1988	FHG	-	600.0
Wheat			
Picard *et al.* 1987	Miller	28.0	3.8
Jones & Petolino 1987	N_6	8.6	4.2
Szakács *et al.* 1988	Potato	19.6	5.9
Kasha & Oro (unpub)	BAC 1	71.0	46.7
Ouyang *et al.* 1987	Potato II	1983.0	70.6
Liang *et al.* 1987	85D3	-	71.5

Chapter 2 Literature Review

Introduction

The factors affecting androgenesis in wheat are poorly understood relative to other species. Wheat is generally regarded as recalcitrant in culture, a condition which restricts the scope of possible experimentation with this economically important crop plant. Nevertheless, successive improvements in embryo and plant yields with barley have permitted more extensive research into the examination of the mechanisms of androgenesis which, in turn, has helped advance such research in wheat. Since the present studies were initiated in 1988, a representative review of the various factors affecting wheat androgenesis up to 1989 follows.

The genotype of the donor plant from which anthers are taken for culture and the health of this plant, affected by its environment, are critically important in determining culture response as is the stage of development of the microspores within the cultured anthers. Furthermore, shock pretreatments are often used to induce androgenesis which together with various cultural modifications of incubation and medium can have major effects on the success of culture. These factors are discussed below.

Donor plant growth environment

There have been many reports which indicate that a relationship exists between the state of the donor plant, its growth conditions and anther response in wheat and other species (Sunderland 1971, 1978; Picard & de Buyser 1973; Dunwell 1976; Foroughi-Wehr *et al.* 1976, 1982; Keller & Stringam 1978; Heberle-Bors & Reinert 1979, 1981; Wang & Chen 1980; Lazar *et al.* 1984a; Heberle-Bors 1985; Andersen *et al.* 1987; Jones & Petolino 1987;

Simmonds 1989). The physiological state of the donor plant naturally influences the response of microspores in culture since all developmental processes are physiologically regulated. If the donor plant is unhealthy, which may be due to a nutritive deficiency, pest or disease infestation, non-optimal temperature, water or light regimes, then it follows that the anthers of that plant will have been affected in turn and as such, the probability of having poorly responding cultures is more likely. On the other hand, anthers taken from healthy plants grown in more optimum conditions will be more likely to show improved culturability.

The environmental conditions apparent prior to and at the time of flowering are most critical. During this phase of development, alteration of the structures and the mechanisms governing microspore development would be more likely. In wheat, a cool season crop, high temperatures given during sporogenesis have been found to diminish gametic fertility. Subjecting *Triticum aestivum* L. em. Thell. cv. Gabo plants, for example, to 30°C for 3 days at the time of meiosis led to anther indehiscence, pollen abortion and thus male sterility (Saini & Aspinall 1982). Male sterility in wheat also has been environmentally induced through photoperiod reductions, water stress (Kaul 1988) and copper deficiency (Graham 1975). It has been suggested that the light and temperature conditions experienced by tobacco plants affected sexual balance (Heberle-Bors 1982b). Furthermore, both the frequency of anomalous pollen *in vivo* and pollen embryos from anther cultures was increased as a result. Similarly, Dunwell and Perry (1973) concluded that short days were more conducive for embryogenesis than were long days, and the observed frequency of embryogenesis from pollen originating from plants flowering at 18°C was higher than from cultures initiated from pollen of plants flowering at 24°C (Rashid & Reinert 1980). Improved anther responses were obtained when donor plants were grown at low temperatures in wheat (Simmonds 1989) and barley (Foroughi-Wehr & Mix 1979; Lyne *et al.* 1986). Furthermore, photoperiod and seasonal variations have been known to affect embryo yields in barley (Foroughi-Wehr *et al.* 1976), oilseed rape (Keller & Stringam 1978) and tobacco (Dunwell & Perry 1973).

In normal agricultural practices, plants are grown in either the field or in glasshouses and, in experimental research and some more specialized areas of plant production, environmentally controlled growthrooms are also used. Choice of the type of environment in which donor plants are to grow should be taken into consideration as it is now understood that anther response is influenced by the type of environment from which the anthers for culture are taken. Results indicated that glasshouse (Bjørnstad *et al.* 1989) and field grown materials (Ouyang *et al.* 1983, 1987; Lazar *et al.* 1984a; Andersen *et al.* 1987) were superior to those from growthrooms. Although Bjørnstad *et al.* (1989) found field grown plants to be inferior as donors for experiments in wheat anther cultures, they suggested that the observed inferiority may have been related to a prolonged temperature stress prior to heading plus discrepancies in the staging of anthers such that anthers may have been cultured in sub-optimal condition. Such a proviso again points to the importance of considering the environmental conditions influencing donor plant development.

Plant age is also a factor which significantly can affect anther response. Culture of anthers from the earliest developing inflorescences has been correlated with higher levels of embryogenesis. The association between culture response and donor plant age has been ascribed to deterioration in the general condition of the donor plants including a decline in pollen viability (Sunderland 1971). Furthermore, prevention of seed set, by removing older inflorescences, was shown to nullify the effect of plant age on anther response in *Datura innoxia* Mill. (Nitsch 1975). Since temperature, humidity and the gaseous environment are all factors known to affect pollen viability (Stanley & Linskens 1974), the viability of microspores within cultured anthers might similarly depend on these same conditions. Thus culture success would depend on the condition of the donor material. As such, poor performance may be attributable to poor initial pollen viability. Success with haploid induction, therefore, is in part dependent on a knowledge of the physiology of the pollen-yielding plant since the physiological state of the donor plant and thus the microspores affect anther response to culture.

Microspore stage

Microspore stage has also been recognised as a critical factor in the success of anther culture in a wide range of species (Maheshwari *et al.* 1980). This is also the case for wheat. These developmental stages, from pollen meiosis to pollen maturation, have been classified by many researchers. It has been accepted that only microspores with one nucleus (uninucleate microspores) or those on the verge of mitosis, were responsive in culture. However, *Triticum aestivum* is the first species in which calluses and green plants have been induced from anthers at all stages, i.e. from tetrads through to binucleate pollen (He & Ouyang 1984). Considering the recalcitrant nature of wheat anthers in culture (compared to other cereals and dicotyledonous species), this should give wheat an advantage in culture once a suitable induction medium has been defined..

In the uninucleate wheat microspore, both the nucleus and vacuole undergo a visible series of developmental stages. Consequently, in preliminary work with wheat, these categories had been defined as mid and late uninucleate (Ouyang *et al.* 1973; Pan & Kao 1978) but were later broadened to five stages; early uninucleate I and II, mid-uninucleate, late uninucleate and premitosis (He & Ouyang 1984). Furthermore, it was recognized that cells at premitosis had the capacity to produce viable cultures although the frequency of induction was lower than from cultures with anthers staged at mid-late uninucleate (He & Ouyang 1984). Since the responsive range for culture may be broader than first thought, the percentage of microspores capable of embryogenesis would also be improved.

As a physiologically regulated process, microspore development would be affected by the environment. This would include the physiological condition of the plant, which in turn would be affected by the environment in which the plant was growing. Therefore, anthers taken from vigorously growing, healthy plants would tend to contain well formed, healthy microspores and as such, there would be potential success in culture. On the other hand, pollen malformations and sterility caused by various environmental stresses would increase with poorer growth conditions of the donor plant (Kaul 1988), which would result in a reduction in anther culture potential. Consequently, the developmental stage of microspores can be viewed as a genetically based but environmentally influenced character.

Similarly, pretreatments, usually involving temperature stresses, might affect microspore development (Stanley & Linskens 1974). The pretreatment might enhance or detract from culture success depending on the stage when the anthers were collected from the donor plant and also on the efficacy of the pretreatment itself. Consequently, the production of androgenic structures has been shown to vary with the stage anthers are cultured (He & Ouyang 1984). Enhanced embryogenesis has been associated with uninucleate and more specifically, with mid-late uninucleate microspores whereas higher proportions of green plants have been attributed to microspores in the earlier uninucleate stages in wheat (He & Ouyang 1984), barley (Sunderland & Huang 1985) and rice (Genovesi & Magill 1979). Albino plants occur independently of microspore stage suggesting that their formation is under strong genetic control, although a slight increase has been noted from binucleate pollen (He & Ouyang 1984).

Pollen age is also important from quite a different point of view. It has been established that embryos and plants of higher ploidy can be produced along with haploids (see Hu 1986b) and that in many plants this is dependent on the stage of pollen at the time of induction. In the Solanaceae, for example *Datura innoxia* (Engvild *et al.* 1972) and *Nicotiana tabacum* L. (Engvild 1974), plants obtained from microspores at the uninucleate stage were mostly haploids, whereas at later stages, higher ploidy levels were found. In general, the older the stage selected, the higher the ploidy level of the resulting embryos (Hu 1986b).

In order to obtain the best performance from a culture, it is therefore important to select anthers for culture from within the responsive microspore range known for that plant. The variability in microspore development caused by interaction with other donor plant and cultural factors therefore suggests that microspore stage needs to be taken into account when interpreting experimental results. Equally, the stages from which a response could be obtained must be defined in order to maximize culture potential.

Treatments to induce androgenesis

Androgenic capacity can also be improved by subjecting anthers to various treatments, physical and chemical, either prior to culture or afterwards. Such treatments have been used successfully to improve androgenic potential of various species.

Anther or spike pretreatment

Pretreatments have become routine in most anther culture protocols. Of the various pretreatments, that most widely used has been cold shock, of either the entire spike or the anthers when in culture. Other successful pretreatments, usually prior to culture, have included high temperature treatments (Hu *et al.* 1978; Keller & Armstrong 1979; Xu 1985), maintenance of detached inflorescences in water (Wilson *et al.* 1978), osmotic shocks (Chien & Kao 1983), centrifugation (Sangwan-Norreel 1977), irradiation (Yin *et al.* 1984), application of gametocides (Schmid & Keller 1986; Picard *et al.* 1987), use of liquid nitrogen (Coulibaly & Demarly 1979), treatment with elevated carbon dioxide levels (Johansson *et al.* 1982) and anaerobiosis (Imamura & Harada 1981). Commonly in cereals, the whole spike is pretreated, then the anthers are dissected from the florets and plated on to the culture medium (Pelletier & Henry 1974; Wenzel *et al.* 1975; Sunderland & Roberts 1979; Bernard 1980; Dunwell 1981; Huang & Sunderland 1982; Ouyang *et al.* 1983, 1987; Marsolais *et al.* 1984; Heberle-Bors & Odenbach 1985).

At 4°C a shorter pretreatment was required for spikes than for tillers and anthers from pretreated spikes were more androgenic (Huang & Sunderland 1982). Although spike pretreatment appears to be the preferred method, an alternative is first to plate the anthers on the culture medium and then subject the culture to cold (see *post-culture treatment*).

While cold pretreatment may be conducive for some genotypes, equally it may be detrimental to others (Marsolais *et al.* 1984). Genotypic variation in anther response has been the single largest barrier to adopting anther culture as a routine and reliable technique, so it follows that genotypes might reasonably be expected to respond differently to temperature.

Pretreatments can also affect anther response by altering the ratio of regenerants and influencing the rate of chromosome doubling. According to Genovesi and Magill (1979), the ratio of green to albino plants in rice anther culture was much higher following short rather than long pretreatments whereas in barley anther culture, the reverse appeared to be true (Huang & Sunderland 1982). The frequency of chromosome doubling in wheat, hence the fertility of anther derived plants, was found to increase after culture at high temperatures (32-34°C for 8 days) (Hu 1986b). Similarly, the duration of culture can affect the ploidy level of regenerant plants in oat (McCoy *et al.* 1982) and wheat (Liang *et al.* 1987). Among other benefits, spike pretreatment can be used to advantage for storage - a useful option when seasonal flowering means more spikes are produced than can be handled for culture in the available time.

Literature on the mechanism and function of cold pretreatment is sparse, and viewpoints are diverse. Huang and Sunderland (1982) suggested that in barley, virtually all of the pollen grains within an anther theoretically could be switched into morphogenesis because of pretreatment and furthermore, that the number affected was directly related to the duration of the pretreatment. The data indicated a trend in favour of longer pretreatments. According to Nitsch (1975), cold treatment increased the frequency of embryo formation by increasing the number of pollen with similar nuclei, as well as maintaining the pollen in a viable condition. Duncan and Heberle (1976) who gave cold treatment to tobacco anthers after plating, also observed an increase in viable units following treatment but did not find any evidence to support the idea that cold treatment caused symmetrical mitotic division of the pollen grains.

The use of cold pretreatments was found to be beneficial since rapid senescence of somatic tissue was inhibitory to tobacco embryogenesis (Mii 1976) and cold treatment delayed anther senescence. Perhaps, then, one mechanism of low temperature is to slow down sporophytic development, disrupting microspore 'programming' and resulting in a switch to gametophytic development. Alternatively, cold pretreatments might promote senescence of the anther wall which, by damaging the close association of the nutritive tapetal tissue with the germpore, would also disrupt pre-programmed microsporogenesis, perhaps then allowing embryogenesis to be initiated (Foroughi-Wehr *et al.* 1976; Sunderland *et al.* 1984; Wei *et al.* 1986). This switch might occur if the cold shocked microspore development out of phase with that of the tapetum especially if this corresponded to a time in the normal sequence when genes essential to gametophytic development were being activated (Sunderland *et al.* 1984). That the tapetum was dispersed in barley anthers when they were subjected to cold treatment was evidence in support of this theory (Sunderland *et al.* 1984).

Alternatively, the mechanism might be due to variation in the levels of inhibitors associated with pollen development which may be differentially affected by cold treatment at different stages (Sunderland & Wildon 1979). Sugar starvation has been reported to be essential for stabilization of microspores in culture (Wei *et al.* 1986), from which it has been proposed that cold pretreatment is perhaps another form of sugar starvation and as such the mechanisms are similar (Kao 1981). Such starvation eventually compels the surviving microspores into androgenesis as their only alternative means of development.

The actual biochemistry of the effect of chilling is unknown. The dissolution of microtubules at low temperatures (Hepler & Palevitz 1974) suggested that cold treatment may exert an effect through an early interference in establishment of spindle polarity, resulting in a change in spindle organization and thus in abnormal cell division. Microtubule assembly was also disrupted by centrifugation, another occasionally effective culture pretreatment (Hepler & Palevitz 1974) which has been associated with improved anther response in *Datura* (Sangwan-Norreel 1977).

Post-culture treatment

An alternative method to cold pretreatment of spikes, prior to culture, has been first to plate the anthers on to the culture medium and then subject the entire culture to the cold treatment (Duncan & Heberle 1976; Schaeffer *et al.* 1979; Sunderland & Wildon 1979). Investigations into the use of cold pretreatment with anthers began with *Datura innoxia* where it was found to be obligatory (Nitsch & Norreel 1973). In general, such pretreatments involve an initial temperature shock of around 4°C for a specified length of time followed by maintenance at more normal cultural temperatures (usually 25°C) after plating. Some researchers have found this method to be more productive for wheat (Pan & Kao 1978) whereas others using both wheat (Li *et al.* 1988) and barley (Huang & Sunderland 1982) have found pretreatment of individual anthers to be deleterious and less productive.

Culture incubation

Despite strong genotypic control, there has been an increasing acceptance that elevated temperatures are beneficial to androgenesis in cereals (Ouyang *et al.* 1983, 1987; Xu 1985; Li *et al.* 1988; Simmonds 1989). Although cultures were conventionally incubated at a constant temperature, this evidence demonstrates that manipulation of the incubation temperature can affect the productivity or rate of growth of the culture or modify the parameters affecting culture response.

Use of higher temperatures (30-35°C) was instigated following the stimulation of embryogenesis in *Datura innoxia* (Sopory & Maheshwari 1976), after which research into the stimulatory effect of high temperatures for an initial period prior to normal incubation maintenance resulted in some significant advances (Hu *et al.* 1978; Keller & Armstrong 1979; Ouyang *et al.* 1983). The earlier studies with wheat tested temperatures below 28°C (Pan *et al.* 1975; Picard & de Buyser 1975) though subsequent improvements in androgenic performance, which supported the use of high temperatures in culture, were reported after temperatures above 26°C and preferably around 30°C were used (Xu 1985; Ouyang *et al.* 1987; Li *et al.* 1988; Simmonds 1989; McGregor & McHughen 1990). However, the use of higher culture temperatures has been associated with higher proportions of albino plants in wheat (Ouyang *et al.* 1983), triticale (Bernard 1980) and rice (see Chen 1986).

Temperature is therefore important in influencing donor plant growth and pollen development as well as contributing to the induction and maintenance of embryogenic cultures. Therefore, in order to develop a protocol best suited to the experimental material in use, consideration must be given to the use of temperature throughout the culture process.

The culture medium

The culture medium is of critical importance to anther culture. It induces cell division and maintains growth, so without it there is clearly no culture. Thus, the composition of the medium has been considered the most important factor affecting response, although genotype is becoming recognized as having comparable importance.

Many media have been used in tissue culture, each with some modification to suit the research plant and experimental conditions. Typical media consist of macronutrients such as K^+, micronutrients such as Co^{2+}, sugars, hormones and coenzymes (vitamins) together with a supporting substance such as agar. Inducing anthers into androgenesis has required particularly specialized media. The first media to be used for anther cultures were naturally those used for more general tissue culture such as that of Murashige and Skoog (1962), Miller (1963) or Linsmaier and Skoog (1965) and reasonable results were obtained, in part because of the species used. Members of the Solanaceae, for example, were readily responsive. However, as the research field broadened together with the range of species used, it was found that a response could not be obtained in all species and in particular, cereals were recalcitrant. Consequently, more specialized media were developed which included those based upon either the N_6 medium (Chu *et al.* 1975) or another containing at least a 10% aqueous solution of potato extract (Chuang *et al.* 1978). Initially developed for the culture of rice but later used successfully with wheat, rye, triticale, maize (Chu 1978) and potato (Chuang *et al.* 1978; Shimada & Otani 1988), the N_6 medium was more efficient than traditional media and this was in part attributed to the alteration in the nitrogen content and composition.

A notable difference between these generic media - N_6 and potato - was that N_6 types were wholly synthetic while potato media, incorporating ingredients which varied in quality, were not. The production of albino plants has been difficult to avoid but it has been suggested that N_6-type media have favoured better green plant regeneration (Sorvari & Schieder 1987). As a result, wheat anther media have been derived from the N_6 medium (Datta & Wenzel 1987; Sorvari & Schieder 1987).

Improvements in anther culture response have been made by modifying various components of the medium including the basal salts, hormones, organic components and gelling agents (Wernicke & Kohlenbach 1976; Chu 1978; Chuang *et al.* 1978; Maheshwari *et al.* 1980; Kao 1981; Heberle-Bors 1985; Köhler & Wenzel 1985; Xu 1985; Ouyang 1986; Datta & Wenzel 1987; Marburger 1987; Chu & Hill 1988; Feng & Ouyang 1988; Shimada & Otani 1988; Zhou & Konzak 1989). Each improvement has resulted in the advancement and broadening of knowledge about factors affecting the culturability of wheat but, until recently, these improvements have been small compared with those made by modifying the sugar component (Hunter 1987; Sorvari & Schieder 1987).

Several properties of traditional media have been associated with poor anther culture performance such as toxicities attributed to agar (Kohlenbach & Wernicke 1978; Sorvari & Schieder 1987; Jones & Petolino 1988; Simmonds 1989). Autoclaving of media causes some degeneration of sugars (de Lange 1989) with the release of such products as fructose, which inhibits androgenesis (Raquin 1983). These problems have been addressed through the use of media containing alternative gelling agents (Kao 1981; Lyne *et al.* 1986; Sorvari 1986a,b) including liquid media (Wernicke & Kohlenbach 1976; Kao 1981, 1988), filter sterilization (Lyne *et al.* 1986; Chu & Hill 1988) and alternative sugars to sucrose (Wei *et al.* 1986; Hunter 1987; Olsen 1987; Sorvari & Schieder 1987).

Agarose has been used by some researchers for barley (Lyne *et al.* 1986; Hunter 1987) and improved responses have been gained with media gelatinized with other starches (Sorvari 1986a,b). Nevertheless, the compound which has been most widely adopted over agar has been the non-ionic synthetic polymer of sucrose, Ficoll, which first improved anther response in barley (Kao 1981). Increased embryo induction in barley (Kao 1988; Olsen 1987) and wheat (Datta & Wenzel 1987 Chu & Hill 1988; Jones & Petolino 1988; Simmonds 1989; Zhou & Konzak 1989) together with enhanced green plant regeneration and green to albino ratios in wheat (Simmonds 1989) have been attributed to the use of Ficoll.

The primary factors responsible for improved response on Ficoll medium have been suggested to be physical rather than chemical in nature (Lazar *et al.* 1985) and associated with the fact that the anthers readily float on this medium. These factors probably include improved aeration, nutrient exchange and toxic waste dispersion about the developing androgenic structures (Simmonds 1989). In solid media, such substances are thought to accumulate in the substrate surrounding the cultured anthers and contribute to necrosis (Simmonds 1989).

Liquid culture requires more care in handling and sterilization but if culture yields are high enough on solid medium, as has been the case with some cultivars of barley (Olsen 1987), then liquid medium is probably unnecessary. However, species which have been recalcitrant in culture -including wheat - have been shown to perform better on liquid than on solid media (Lazar *et al.* 1985) with differences of 98% to 5% between callus production on liquid and solid medium respectively (Zhou & Konzak 1989). Furthermore, liquid medium can be replenished, without unduly disturbing the culture, by pipetting out stale medium and replacing it with fresh. For these reasons, necrosis can better be avoided through the use of liquid cultures.

The viscosity of liquid media can also be modified by altering the concentration of sugar used. Historically it was recommended that higher concentrations of sugar in the media were needed for culture of Poaceae (6-15%) whereas lower concentrations were used for solanaceous species (2-5%) (Matsubayashi & Kuranuki 1975). Discrete embryo formation in contrast to the formation of callus and also better green plant regeneration have been attributed to higher sugar concentrations in barley anther cultures (Sorvari & Schieder 1987). In a patent on barley anther culture, sugar concentrations no greater than 0.03 mol/l or approximately 10% w/v were defined (Hunter 1987).

Most tissue culture media use sucrose as the carbon source but it has been shown that this sugar was not necessarily the best carbohydrate form for anther cultures (Wei *et al.* 1986). Significant increases in anther response in barley were obtained which showed that not only could other sugars such as cellobiose or gentiobiose replace sucrose in the induction medium but that most out-performed it (Hunter 1987). Furthermore, it was found that the sugar needed to be composed exclusively of glucose molecules. Significant improvements in embryo and plant formation were also obtained with melibiose (Sorvari & Schieder 1987).

Other factors

In barley, the density at which anthers were plated has been found to affect anther response, with high densities (c. 40 anthers/ml medium) being beneficial (Xu & Sunderland 1982; Huang *et al.* 1984; Xu & Huang 1984). There has been comparatively less emphasis on the importance of anther density for wheat cultures but culture vessel size has been implicated (Chuang *et al.* 1978). The effect of anther placement and orientation on the culture medium has not been reported in wheat whereas in barley it has received more attention and has been reported to affect culture response (Hunter 1985; Shannon *et al.* 1985; Powell *et al.* 1988). In liquid culture, anthers do not remain on their side so orientation is irrelevant.

There has also been suggestion that certain chemicals are released by the differentiating microspores and/or the anther tapetum (Sunderland & Roberts 1977; Wernicke & Kohlenbach 1977; Xu *et al.* 1981) which can enhance embryogenesis. Termed "anther factors" (Xu & Huang 1984), these apparently beneficial substances were thought to be by-products of culture and cell proliferation or degeneration products of the nutritive tapetal tissue. Evidence for anther factors has been obtained by preconditioning the induction medium with anthers (Xu *et al.* 1981; Xu & Huang 1984) or ovaries (Xu *et al.* 1981; Köhler & Wenzel 1985; Datta & Wenzel 1987; Ziauddin *et al.* 1990) with the suggestion that ovaries were more effective than anthers (Xu *et al.* 1981). However, much of the research in this area was related to the performance of barley microspores in culture where the intact anther was not directly involved.

Green and albino plant regeneration

An additional problem peculiarly associated with cereal anther culture has been that albino plants often occurred in higher frequencies than green plants. In terms of breeding, albinos are non-viable culture products and as a result, regeneration of these plants in high frequencies has been considered a major limitation. Genotype (Andersen *et al.* 1987; Knudsen *et al.* 1989; Simmonds 1989; Zhou & Konzak 1989), microspore stage (He & Ouyang 1984), the physiological state of the donor plants (Bullock *et al.* 1982) and high culture temperatures (Bernard 1980; Ouyang *et al.* 1983; Chen 1986) have been strongly implicated in causing albinism. Since alterations in and deletions of plastid DNA have been observed in albino plants derived from anther cultures of wheat and barley (Sun *et al.* 1979; Day & Ellis 1985), it seems likely that albinism is, in part, inherited via the cytoplasm, with the chloroplasts (Vaughn *et al.* 1980; Day & Ellis 1984). Consequently, the ratio in which albino and green plants are regenerated has been introduced as yet another important aspect of anther culture response in cereals (Wenzel *et al.* 1977; Foroughi-Wehr *et al.* 1982).

Culture temperature (Wang *et al.* 1977; Bernard 1980; Ouyang *et al.* 1983; Huang *et al.* 1984; Chen 1986; Huang 1987) and cold pretreatment (Genovesi & Magill 1979) have been shown to modify plant regeneration capacity. Components of the induction medium also affected plant production with the use of Ficoll in the induction medium associated with higher green plant regeneration in wheat and, furthermore, with a relative decrease in albino formation (Simmonds 1989; Zhou *et al.* 1991).

Donor plant genotype

Although improvements in anther culture response have been achieved through the manipulation of associated factors such as the growth condition of the donor plants, pretreatments, the media and the culture conditions, the most difficult factors yet to be manipulated successfully are those that are genetically determined, i.e. the plant genotype and associated genetically based factors. Mathias and Simpson (1986) assessed the relative contributions made by media additives and genotype *in vitro* and suggested that the genotype of a plant may be more significant than the medium in affecting culture response. All parameters of culture response are affected by genotype including embryogenesis, growth, differentiation and plant regeneration. Although the genes that control culture characteristics and their location within the genome have not been identified, progress into understanding these systems has been made.

Use of a variety of genotypes will increase the numbers known to be responsive and known to be recalcitrant. This should include a range of cultivars and related species together with genetic stocks such as defined aneuploids and reciprocal crosses prepared for the purpose. With such a wide range of materials it would then be possible to detect and manipulate the genetic factors responsible for the response of anthers to culture.

Genetic determination of anther response

Investigations into the genetics of anther response have included analyses of specific chromosomes or arms, single genes or gene complexes, alien genomes, reciprocal crosses and alien cytoplasms. The research in hexaploid wheat has incorporated conventional information from comparative studies with alien genomes (*Hordeum, Secale, Agropyron, Aegilops, Triticum* spp) and more specific information from studies in tissue culture which have included immature embryo and anther culture.

Culture responsiveness or 'tissue culture ability' (Wenzel 1980), has been divided into three parts: callus or embryogenic induction, total plant regeneration and the proportion of green plants regenerated. Most studies have reported that the control of these three components was from nuclear genes and that, for embryo formation and regeneration, the effects were generally additive and either lacked reciprocal effects (Bullock *et al.* 1982; Foroughi-Wehr & Friedt 1984; Henry & de Buyser 1985; Miah *et al.* 1985; Deaton *et al.* 1987; Agache *et al.* 1989; Tuvesson *et al.* 1989) or had only small reciprocal effects (Foroughi-Wehr *et al.* 1982; Charmet & Bernard 1984; Lazar *et al.* 1984b). Significant maternal or cytoplasmic effects have also been described for several species including wheat, although in most of the studies diverse parents had been used so there has been uncertainty as to whether or not the observed differences could be attributed to maternal or to cytoplasmic effects (Foroughi-Wehr *et al.* 1982; Nesticky *et al.* 1983; Charmet & Bernard 1984; Lazar *et al.* 1984b; Tomes & Smith 1985; Powell 1988; Sági & Barnabás 1989). Moreover, in a number of these studies (Bullock *et al.* 1982; Henry & de Buyser 1985; Dunwell *et al.* 1987), the low levels of response so often encountered in the anther culture of cereals could have obscured reciprocal differences. The only example of a substantial reciprocal effect, suggesting that the direction of a cross should be considered, was for embryo and green plant formation in barley (Powell 1988).

The three components have been reported to be under different genetic regulation, with polygenic determination and independent inheritance (Deaton *et al.* 1987; Lazar *et al.* 1987; Szakács *et al.* 1988; Knudsen *et al.* 1989) allowing a plant to display a high capacity for embryo formation but a low ability to regenerate green plants, or *vice versa*. Callus production was negatively correlated with regeneration capacity in three spring wheat crosses (Deaton *et al.* 1987) and genotype effects have been found to be highly significant for embryo formation but not for regeneration (Tuvesson *et al.* 1989).

Further clarification of the different types of androgenic response shown by anther cultures has been obtained from chromosome homoeologies. Chromosome substitution and addition lines have allowed more specific determination of the genetic control of culture response. Complete ditelosomic (Shimada & Makino 1975), substitution (Szakács *et al.* 1988) and alien addition (Lazar *et al.* 1987) aneuploid analyses have been done. In wheat, several individual chromosomes, chromosome arms or genes have been implicated in the control of anther culturability.

A considerable effect on embryogenic induction of anther cultures has been attributed to chromosomes 1B (Szakács *et al.* 1988) and 1D (Agache *et al.* 1989). According to Kobayashi and Tsunewaki (1980), a main genetic factor for haploid induction in anther cultures of wheat was the presence of the 1B/1R chromosome. Lazar *et al.* (1987) found callus formation to be significantly better from 1R addition lines than 2R, 3R, 6R or 7R. An improved anther culture ability associated with the presence of this chromosome was found in the wheat cultivar Aurora (Henry & de Buyser 1985) from which it was proposed that genetic factors involved in response were located on the short arm of rye chromosome 1 (1RS). Similarly, 1BL/1RS has been related to preferential plant regeneration over embryogenesis with 1BL/1BS (Müller *et al.* 1989).

Chromosome 2D had a strong influence on green plant regeneration ability (Szakács *et al.* 1988) while 2A, 2B and 2D have been linked to the inhibition of embryo formation (Zhang & Li 1984). The control of plant regeneration has also been attributed to chromosome 3A (Szakács *et al.* 1988). Using A genome aneuploids it was found that 3A promoted the formation of filament callus (Shimada & Makino 1975) and as only albino plants were regenerated from all lines, the role of this chromosome for the regeneration of embryos can not be concluded. However, these results lend less support since all callus was from filaments instead of microspores.

From culture of the complete set of wheat-rye addition lines, it was concluded that rye chromosome 4R contained factors which promoted anther culture response (Lazar *et al.*

1987). Major genes affecting anther culture callus initiation were present on chromosome 4A (Shimada & Makino 1975) and according to Zhang and Li (1984), this chromosome also possessed minor genes which inhibited embryo production in wheat anther cultures.

Although chromosome 5BL in anther cultures might improve embryogenesis (Agache *et al.* 1989), minor genes on chromosomes 5A and 5B have been associated with the inhibition of embryo productivity (Zhang & Li 1984).

A considerable positive effect on embryogenic induction of anther cultures has been attributed to chromosome 7A (Szakács *et al.* 1988).

This research has implicated a number of chromosomes in the control of anther response in wheat and has shown that there is no dominance by any one individual chromosome and that governance is complex. Although similar work has been done with embryo culture (Shimada 1978; Galiba *et al.* 1986; Mathias & Fukui 1986; Felsenburg *et al.* 1987; Higgins & Mathias 1987; Lazar *et al.* 1987; Kaleikau *et al.* 1989a,b) no correlation has been found between factors affecting anther and embryo culture responses (Lazar *et al.* 1987). Nevertheless, there have been no studies using the barley genome. Since barley is a much more responsive species, such a study could yield a number of important results.

Wheat-barley chromosome addition lines

Wheat and barley are the two most important small-grained cereals of temperate agriculture. Hopes that a new type of crop plant, which combine the desirable characteristics of both parent species, may result from wheat-barley hybrids has induced scientists for over 80 years to cross these species (Shepherd & Islam 1981).

The first successful combination of cultivars for the hybrids in terms of seed set was obtained with the barley cultivar Betzes as the female and the wheat Chinese Spring as the pollen donor (Kruse 1973; Islam *et al.* 1975). However, these lines were alloplasmic and exhibited various fertility problems. Euplasmic lines with wheat as the female parent have since been developed with normal fertility (Islam *et al.* 1981) and the response of these wheat-barley addition lines in culture may provide useful information on the role of each barley chromosome on culture response.

Male sterility and gametocides

Heberle-Bors (1985) postulated that all conditions which shift the sex balance of a plant, such as male sterility and feminizing substances, were likely to induce a better haploid production from anther culture.

Male sterility can result from a variety of physiological, ecological and chemical causes, singly or in combination. There are also heritable forms of male sterility in which the nucleus, the cytoplasm or both have been recognized for their involvement. There is a continuum of responses from complete sterility to complete fertility and a sterile plant can exhibit pollen which is either not formed or if formed, is either non-viable or viable but incapable of effecting fertilization under normal conditions. The following classification is after Kaul (1988):

i. *Genic male sterility.* Control of sterility is by nuclear genes. There is no influence from the cytoplasm. Expression and inheritance is simple and Mendelian and in the main conditioned by recessive genes. Plants with this form of sterility display minimum reciprocal differences. This class is also known as chromosomal or nuclear male sterility.

ii. *Cytoplasmic male sterility.* 'Sterile' cytoplasm induces male sterility. In a species showing this form of male sterility, there are at least two cytoplasm types; normal fertile (N) and sterile (S). Irrespective of the nuclear genotype, plants with S-cytoplasm are male sterile and those with N-cytoplasm are male fertile, so the sterility is inherited maternally. Reciprocal differences are exhibited in F_1 generations between male steriles and male fertile crosses. Cytoplasmic male sterility is much less common than genic male sterility.

iii. *Gene-cytoplasmic male sterility.* Gene-cytoplasmic male sterility is characterized by both fertility restorer (fr) nuclear genes, where restoration can be dominant or recessive, and N- and S-cytoplasms. The combination of S-cytoplasm with active (Fr if dominant or frfr if recessive) restorer genes results in a fertile plant, while N-cytoplasm remains fertile regardless of the status of the restorer genes. Thus a cross of Sfrfr (sterile) x NFrFr (fertile) would result in SFrfr (fertile) progeny.

The first report of gene-cytoplasmic male sterility followed the transfer of the nucleus of common wheat into the cytoplasm of *Aegilops caudata* L. (Kihara 1951). The interaction was found to be stable and by returning the nucleus back to its own cytoplasm, fertility was restored. Subsequently, different sources of cytoplasm were used to produce cytoplasmic male steriles.

Stable male steriles in hexaploid wheats using the cytoplasm from *Triticum timopheevi* Zhuk. were then derived (Wilson & Ross 1962). These researchers postulated that factors responsible for the restoration of pollen fertility for this sterility system would be found in *T. timopheevi* itself. Subsequently, the transfer of effective pollen fertility-restoring factors from *T. timopheevi* into hexaploid wheats resulted in restored pollen fertility (Schmidt *et al.* 1962 in Kaul 1988). However, restoration was not simply inherited and was considerably affected by environmental factors.

Many alloplasmic wheats are male sterile. The incorporation of the bread wheat (*Triticum aestivum*) genome into the cytoplasms of *Triticum timopheevi*, *Aegilops caudata*, *A. kotschyi* Boiss., *A. ovata* L., *A. speltoides* Tausch and *A. variabilis* Eig. has resulted in male sterility. Indeed, the first haploid plant produced parthenogenically from wheat was the result of a cross between *Triticum compactum humboldtii* Kcke. and *Aegilops cylindrica* Host (Gaines & Aase 1926). This plant could not be distinguished from other normal diploid plants until flowering when the spread glumes (a characteristic display of sterility) prompted cytological examination revealing the haploidy. High frequencies of haploids also have been induced from the cytoplasm of *Aegilops* spp with *T. aestivum* cv. Salmon (Tsunewaki *et al.* 1976).

Male sterile wheats have been implicated in improved anther response *in vitro* (Picard & de Buyser 1973; Ling *et al.* 1978; Foroughi-Wehr *et al.* 1982; Heberle-Bors 1982a; Charmet &

Bernard 1984; Heberle-Bors & Odenbach 1985; Schmid & Keller 1986; Picard *et al.* 1987). The culture of anthers of cytoplasmic male sterile wheats may provide useful information on the role nuclear and cytoplasmic genotypes play in culture response.

Close parallels between cytoplasmic male sterility and the effects of gametocides have been drawn. Since male sterile plants have been reported to produce higher numbers of haploids than non-sterile plants, it has been suggested that through the application of gametocides, male sterility and hence increased anther culturability could be attained across a broad spectrum of genotypes (Schmid & Keller 1986; Picard *et al.* 1987).

Gametocides are chemicals which kill male gametes or cause abnormal pollen development, allowing the induction of male sterility and hence possibly androgenesis, independent of genetic background. Gametocide chemicals, also known as chemical hybridizing agents, have been shown to induce abnormal mitoses (Bennett & Hughes 1972) and it has been hypothesized that treated microspores would be better disposed for androgenesis (Heberle-Bors 1985).

Addition of a gametocide (CGA) directly to the anther culture medium resulted in enhanced induction of embryos, although the results were extremely variable (Schmid & Keller 1986). Similarly, treatment of donor plants with gametocide RH0007 was associated with significant increases in embryo production and green plant regeneration compared to untreated controls (Picard *et al.* 1987). Additional benefits associated with the application of the chemical hybridizing agent were a 5- to 20-fold increase in haploid and homozygous plant production for any genotype, an enlargement of the target period for collection of source material and acceleration of the time for embryogenesis (Picard *et al.* 1987).

Ethrel or Ethephon (2-chloroethylphosphonic acid) has been known to promote male sterility in several crop species including wheat (Rowell & Miller 1971; Bennett & Hughes 1972). Ethrel is an ethylene-generating synthetic compound that acts as a plant growth regulator having a role in abscission, apical dominance, dormancy breaking and fruit maturation. In cucumber, it enhances femaleness (Kaul 1988) and in wheat, abnormal pollen grain development just before second pollen grain mitosis was observed (Bennett & Hughes 1972) which, at the estimated time of maturity, included multi-nucleated cells. Similar abnormal development was seen for wheat pollen treated with the chemical hybridizing agent RH0007 (Picard *et al.* 1987; Mizelle *et al.* 1989) and CGA (Schmid & Keller 1986). The condition of ethrel treated anthers and microspores was not unlike that observed for cytoplasmic male sterile plants (Rowell & Miller 1971).

Ethrel is known to break down in plant cells at a pH below 4.0 releasing ethylene, which has been widely acknowledged as having hormonal properties in plants and has been linked with both stimulated cell division in anther cultures and culture senescence (Kasha *et al.* 1990). Hormones have been thought to act sometimes by directly affecting gene transcription such that a possible explanation for the action of Ethrel would be that it acts through the regulation of differential gene action (Kaul 1988) and alters the production of long-lived messenger RNA transcribed before meiosis but necessary for normal pollen development several days later (Bennett & Hughes 1972).

The herbicide 2,4-D also has some gametocidal properties which may contribute to its hormonal role in anther culture media (Kaul 1988).

The gametocide used by Schmid and Keller (1985) was a product of Ciba-Geigy (suggested to be pyridine derivatives which, as chemosterilants, cause cytogenetic defects) and that of Picard *et al.* (1987) was a product of Rohm and Haas. Despite the complexity of factors affecting gametocide action, a major drawback to the further research use of these gametocides and chemical hybridizing agents was that they were not actually commercially available (pers. comm. Dr J Schmid, Ciba-Geigy, Rohm & Haas; Picard *et al.* 1987). Unfortunately, later attempts by other researchers to both duplicate and further these studies were unsuccessful (Picard *et al.* 1987) which could be attributable to the variability of effectiveness of many gametocides with genotype, concentration and application time, making their use highly sensitive.

Gametocidal treatment has been reported to be not only genotype- but tissue-specific (see Kaul 1988) and hence dose-specific. Effectiveness also depended on the developmental stage of the plant organ receiving treatment. For these reasons, the physiological state of the donor plant and related environmental factors affecting them add to the complexity governing success using gametocides.

Chapter 3 Aims of the thesis

The difficulty in repeating procedures used in other labs and in obtaining similar results to those published suggests strong influences of plant growth and cultural conditions. Therefore, although research has been done on the factors affecting anther culture response, genotypic differences and sub-optimal culture conditions are still a problem even with the best of current procedures. Hence there is still a need to define these influencing factors more precisely, particularly for genotypes previously unused for anther culture.

When this work commenced, there was no evidence that anther culture had been tried with Australian wheat, and unlike barley (Table 1.1), wheat was not only a low yielding or recalcitrant species in anther culture but definitive protocols for it were scarce. For these reasons, a thorough re-examination of the factors affecting culture was needed, to assess genotype performance in culture and to contribute to the resolution of problems associated with wheat anther culture. An evaluation of cultural parameters which may affect the response of the genotypes and which may be affected by genotype was therefore undertaken. The research programme was designed to include an assessment of microspore stage, donor plant environment, length of cold pretreatment and temperature for incubation for the genotypes used and **to determine ranges in these cultural parameters which would promote response in the broadest range of agronomically important genotypes.**

As the project was undertaken with a view to incorporating anther culture into Australian wheat breeding programmes, a major objective was **to establish anther culture using Australian material**. Information gathered about the response of these genotypes in culture could then be used as a reference from which the culturability of other germplasm could be predicted. A suitable protocol could be developed by screening a range of

Australian cultivars used in breeding programmes at the Waite Agricultural Research Institute and by investigating the effect of different conditions on their response. This would necessitate selection of an appropriate medium and refinement of cultural conditions.

This medium would then be the base for the larger scale investigations into genotype, cultural factors and sugar. The significant increases in barley embryo production found by replacing sucrose with other sugars (Hunter 1987) together with the fact that wheat had not been tested on sugars other than sucrose, instigated **a study into the productivity of different sugars in the induction medium**.

Since barley is more responsive to anther culture than wheat (Table 1.1), it was hypothesized that the critical factor in barley could be on a single chromosome. This hypothesis could be tested using **wheat-barley chromosome addition lines**. These genotypes could also be used to assess if any effects from the barley genome could influence culture response in the wheat background. The results might offer insight into the genetic control of both wheat and barley anthers in culture.

Cytoplasmic male sterility is associated with developmental abnormalities which bear some similarity to developmental abnormalities in anther culture. Pentaploids are also male sterile and gametocides offer a further means of obtaining male sterility. These materials were used to **investigate the effect of male sterility on the anther culturability of wheat**.

Chapter 4 Materials and Methods

Introduction

General materials and methods are described in this chapter. Specific alterations are detailed in the respective chapters. Abbreviations are detailed in Appendix 1.

Donor plants

1. Seed stocks

The sources of seed stocks are detailed in relevant chapters.

2. Germination

Seeds were placed in trays containing a sheet of filter paper overlaying a sheet of moist sponge-cloth to prevent dehydration. Trays were covered with transparent lids and seeds germinated at room temperature. Seedlings were transplanted to 20 cm plastic pots containing 'University of California' soil mix (Appendix 1), without added fertilizer. Experimental plants were grown at the Waite Agricultural Research Institute (34° 56'S, 138° 36'E), Glen Osmond, South Australia.

3. Growth environment of donor plants

The term donor plant refers to those wheat plants from which spikes were taken for culture. Plants were grown in either a controlled environment growthroom or a glasshouse cubicle.

The growthroom was a 'walk-in' model with 400W halogen sodium vapour lamps (average emission 1000 lux) set to a 16 hour day at 15°C with a night of 8 hours at 10°C.

The glasshouse environment was more dependent on the external environment, so in summer, ambient temperature was moderated by a ceiling fan and insolation by shade cloth (50%) hung over the outside panels of the glasshouse. During the winter months, the photoperiod received by the experimental plants was supplemented by additional overhead lighting (Philips 'Philux' 100W 80° reflector), which also contributed to heat input during the colder evenings.

4. Plant care

To promote the development of healthy anthers, donor plants were maintained under optimal conditions. These conditions included daily watering and weekly fertilization with a commercial liquid fertilizer (Aquasol). Pest control consisted of manual removal of insects, spraying with a pyrethroid and dusting with sulphur as the use of systemic pesticides (Metasystox, Lebasid and Temed) was associated with deformation of anthers and empty pollen grains.

Culture preparation

5. Media

The compositions of all media are presented and referenced in Appendix 2 and are discussed further in relevant chapters.

For preparation of all the media, stock solutions were prepared at 10x final concentration (macro elements), 100x final concentration (iron compounds) or 1000x final concentration (organic compounds and micro nutrients but not KI). A solution of KI (1000x) was made up separately. Myo-inositol, the sugar and solidifying agent were added as solids to the final, dissolved solution and this was made up to volume with double distilled water. The pH was adjusted (to that specified in the recipe) with NaOH or HCl and then the new medium was sterilized. All stock solutions were maintained in glass vessels, re-made regularly and stored refrigerated. The stocks of the macro- and micro elements were autoclaved to maintain sterility.

All sugars were obtained from the Sigma Chemical Company. Ficoll-400 was obtained from Pharmacia.

6. *Sterilization*

The anther culture induction media were sterilized either by autoclave in a wet cycle of 20 minutes at 120°C or by filtering by one of two methods. In the first, solutions of thermolabile chemicals were pH adjusted then added by filtration to the autoclaved stock medium (under sterile conditions) using individual pipette filter units with a 0.45 μm cellulose acetate membrane (Millipore Millex-HA). In the second and the preferred method, the entire medium was filter sterilized by vacuum filtration through a 0.45 μm cellulose acetate membrane into a 150 ml plastic disposable filtration unit (Millipore Sterifil-D). Since sugar composition can be affected by autoclaving (de Lange 1989), filter sterilization was adopted for all WM2 based media, all Ficoll media and any liquid medium. Autoclaving of the entire medium was restricted to all solid, agar-containing media including regeneration media and some of the initial cultures during the pilot investigations.

7. *Plate preparation*

Under sterile conditions (laminar flow cabinet), medium was pipetted into the culture vessels using an Eppendorf 'multipette' pipette with a 50 ml combitip (polypropylene barrel/polyethylene plunger). Where micro-test plates (Linbro) were used (Chapter 5), 250 μl were pipetted per well. In all other experiments, 1-2 ml of medium were dispensed per 35 mm γ-irradiated plastic petri-dishes. The regeneration medium (190-2; see Appendix 2) was fully autoclaved and poured into sterile 90 mm dishes or 150 ml culture vessels. All vessels were sealed with Nescofilm™ and stored at room temperature in closed containers. A sample of all newly poured plates was routinely incubated for 4 days prior to use in order to check for microbial contamination.

Anther culture

8. Spike collection

Spikes were collected from the donor plants for pretreatment, prior to culture, when the terminal floret of the spike within the sheath leaf could be felt just reaching the point where the adjoining leaf immediately below the flag leaf angled away from the main stem. The entire tiller was taken which enhanced further tillering of the donor plant and ensured that the spike was obtained in its entirety. Spikes were then prepared for a pretreatment at 4°C, or taken directly for culture. Spikes could be collected from the donor plants two to three months after germination. Although maturation time varied among cultivars, the method described for determining the optimum stage of anther development proved reliable and consistent for all cultivars despite the season or prevailing weather conditions. Warm weather necessitated frequent sampling throughout the day in order to find spikes at the correct stage.

9. Spike pretreatment

Spikes were subjected to a cold shock at 4°C for a length of time which depended on the individual experiment but was usually either 3 days (Xu 1985) or 8 days (Datta & Wenzel 1987). The spike was kept enclosed within the ensheathing leaves and the rest of the tiller cut off just above and below the ends of the spike. The exposed surfaces were wiped with 70% ethanol and the spike enclosed in ethanol-cleaned aluminium foil. Batches of spikes were put into plastic bags and refrigerated (4°C) for the appropriate time. Little to no contamination of cultures was attributable to this method of pretreatment handling.

10. Culture methods

In an aseptic laminar flow cabinet (cleaned with ethanol and exposed to UV light), the pretreated spike was removed from its vessel, swabbed with ethanol, removed from within the sheath leaf and placed in one half of a sterile 90 mm plastic petri-dish on which two drops of double distilled sterile water had been placed using a sterile syringe. The glume and lemma were carefully removed to expose the 3 anthers. One of the 3 anthers was removed from a floret in the mid spike region and set aside for later examination of microspore stage. Anthers, from primary florets only, on one side of the spike were dissected and placed together in one of the sterile water drops. Anthers from florets on the other side of the spike were treated in the same manner, ensuring that equal numbers of anthers were dissected per side. Anthers were not taken from immature proximal florets or the basal two florets and any which were obviously immature, mature or deformed were excluded. The anthers were then transferred either together into a culture vessel containing the medium, or on to different dishes. Commonly, 35 anthers were plated per 35 mm dish and 3 anthers plated per 250 μl well unless otherwise stated.

Cultures were labelled, sealed with Nescofilm and incubated in the darkness until visible embryo formation (~4-10 weeks).

11. Microspore staging

The anther that had been set aside was macerated in aceto-carmine and viewed under a compound light microscope. The stage of development of the microspores was recorded. In a pilot study, the microspore stage of every anther from a number of spikes from each cultivar was determined. As a result, the range described above (*culture methods*) was adopted. Intrinsic variation in the spikes was taken into account by adjusting the number of proximal and basal florets excluded. This resulted in a homogeneous population of microspores, containing predominantly uninucleate microspores.

12. Culture incubation

Induction cultures were maintained at various temperatures, depending on the individual experiment. One of two regimes was used; i) continual maintenance at either 18 or 25°C or ii) a split temperature regime with 2-3 days at 30°C prior to maintenance at either 18 or 25°C. The use of the split temperature regime followed reports of enhanced anther response from use of a post-culture temperature shock in wheat (Ouyang *et al.* 1983; Xu 1985; Datta & Wenzel 1987; Li *et al.* 1988). Cultures were maintained at the specified temperature until androgenic structures were visible (and regeneration procedures adopted) or until 6 months had passed when cultures were discarded as it was clear that no response would be obtained.

13. Regeneration cultures

Embryos were transferred, aseptically, onto solid regeneration medium for further differentiation. Regeneration cultures were incubated with continuous illumination (fluorescent 18W/4300K cool white bulbs) at either 25°C or 18°C (25°C being the norm for tissue cultures and 18°C being a closer approximation of the optimal temperature for growth of spring wheat). Where necessary, green regenerant plants were transferred from 90 mm petri-dishes to larger tissue culture vessels until large enough to be transferred to pots of soil. Albino regenerants and undifferentiated embryos were discarded.

Post culture

14. Haploid status

Green plants produced from culture were transplanted to soil and placed in the glasshouse. The survival rate increased when new plants were transferred with some agar medium left attached to the root system. Plants were initially protected by plastic bags supported on a wire frame over their pots. Haploids were recognized as those plants that produced sterile heads and which set no seed (Nitsch & Nitsch 1969). Cytological confirmation was frequently attempted but - as might be expected from the weak growth - mitotic cells were found far less frequently in haploids than in known diploids. Haploid regenerants also tended to be smaller in growth habit than normal wheat plants, with fine leaves and a 'miniature' appearance.

15. Collection of data

Cultures which underwent differentiation were scored for either embryo formation (haploid growth), or for callus, which is derived from filament tissue (i.e. diploid growth). After the embryos were transferred to regeneration medium they were scored according to how they differentiated; as green plants, albino plants, those forming roots only or those remaining as undifferentiated tissue.

Statistical analyses

Throughout the analyses of the data, Genstat 5 (Lawes Agricultural Trust, Rothamsted Experimental Station 1988) was used and a statistically significant difference was defined at $P< 0.05$.

The following sources of variation were considered in the statistical analyses: (i) genotype; (ii) the developmental stage of the microspores of the plated anthers; (iii) the growth environment (growthroom or glasshouse); (iv) date of seed germination of the donor plant; (v) the induction medium; (vi) the sugar used in the induction medium; (vii) the duration (days) of cold shock pretreatment at 4°C prior to culture and (viii) the culture incubation temperature (°C). Factors (iii) and (iv) comprised the 'donor plant environment' and factors (v) to (viii) related directly to the experimental process.

16. Embryo production

Each of these factors was examined independently to see what effect it had upon the number of embryos produced. The probability of an embryo forming was very small and, mostly, zero occurrences were recorded. Since (1) the event was rare, its mean being small relative to the maximum possible number of events per sampling unit, and (2) an occurrence of the event was independent of prior occurrences within the sampling unit, the criteria for a Poisson distribution were fulfilled (David 1971; Sokal & Rohlf 1981; Zar 1984).

Poisson models were fitted to the data, resulting in analyses of deviance (McCullagh & Nelder 1989). It was established that the residual deviance was not significant in any model which meant that the Poisson model was a reasonable fit so relationships between embryo production and factors thought to affect anther response could be determined. Factors were considered one at a time, ignoring the effects of all other factors.

17. Regeneration product

The second area for analysis of the data concerned the product of regeneration as a result of differentiation of the embryo. Embryos tended either to differentiate into plants (green and albino plants) or to remain in various embryo-like forms but without shoots (remaining as embryos, embryos forming roots but not shoots). Regeneration products were analysed as a conditional binomial because the occurrence of plants was both dependent upon and was a fraction of the embryos produced. Because of this dependency, data from the regeneration variables could not be analysed as a Poisson distribution. The interactive factors considered were the same as were those for the analysis of embryo production. Green and albino plants were considered as a count out of the total number of embryos produced, distinguished from the Poisson analyses where embryos were considered per number of anthers per plate. The order in which the factors were considered in the two-way interaction analysis was important and reciprocal combinations could have different deviances.

As stated above, Poisson analyses were executed on the basis of the number of embryos produced per plate of cultured anthers and binomial analyses on the basis of the number of plants or androgenic structures regenerated per total embryos. As an indication of trend, however, results are presented in the conventional way as the number of embryos per 100 anthers plated (see Kasha *et al.* 1990). The regeneration product is presented as the proportion of green or albino plants per total embryos produced, for a given factor. As the number of anthers cultured per plate was constant, the presentation of results per 100 anthers was a direct arithmetic calculation.

Chapter 5 Anther culture response of Australian wheat to different media.

Introduction

To allow a more detailed investigation into the mechanisms operating in anther culture, it was necessary to select an appropriate medium for culture. Since the breeding material chosen for the following studies (Chapters 6-8) was previously unused in anther culture programmes, the ideal medium needed to be one which not only gave high embryo and green plant yields but also one on which a wide variety of genotypes from different parental backgrounds would respond.

Various media were chosen from an array reported to be of use in cereal anther culture at the time of this investigation. Seven media were selected which included those more widely used for tissue culture (MS, Potato-2) plus some recently reported to improve culture response in barley (EDAM I, Ficoll) and wheat (C-17, W5, WM2) (see Appendix 2). Media which were more tailored to cereal anther culture were included on the basis that they contained media components reported to be beneficial, *viz* the use of alternative gelling agents such as starch (EDAM I, Potato-2) or Ficoll-400 (Ficoll, WM2). Improvements in culture response could be obtained using changes to nitrogen concentrations (W5, C-17), alternative sugars to sucrose (EDAM I, WM2) and the inclusion of glutamine and serine (WM2).

MS medium was initially developed for tissue culture of tobacco and represents one of the most widely used and standard tissue culture media to date. Moreover, it was the medium for pioneering work in wheat anther culture (Ouyang *et al.* 1973; Picard & de Buyser 1973). The N_6 medium (Chu *et al.* 1975) subsequently became the preferred base for anther culture media because of the greater success obtained initially with rice and later because it seemed well suited for the culture of most cereal anthers (Chu 1978; Sorvari & Schieder 1987).

N_6 media are characterized by having a reduced total nitrogen content and an increased proportion of it in nitrate. This nitrogen balance was found to be superior to that of MS for cereal anther culture (Chu 1978) and followed a report that reductions in ammonium nitrate led to significant increases in callus production from barley anthers (Clapham 1973). A detailed study of the role of potassium nitrate for wheat anthers in culture resulted in the development of the W5 medium (Feng & Ouyang 1988). Six genotypes including the highly responsive anther-derived cultivar Jinghua-1 were cultured on media with a range of potassium and nitrate concentrations. As the KNO_3 content increased so did the frequency of callus induction but there was an optimum, past which induction significantly decreased for all genotypes. The decrease past the optimum was considered to be due to the NO_3^- ion alone whereas the promoting effect of the KNO_3 was due to both the K^+ and NO_3^- ions. Similar responses were obtained for green plant production. Nitrate and ammonium ions also affected embryogenesis and plant regeneration independently such that the two nitrogen sources are probably used to satisfy different demands in culture. Since the nitrogen content in the induction medium is important in anther culture requirements of Poaceae, and this medium was used for wheat genotypes, it was included as a medium for this study.

Another main type of medium used frequently for wheat anther culture was based on the use of potato (Chuang *et al.* 1978) where the minor inorganic and organic elements were replaced by a 10% aqueous potato extract. Thus the potato medium of Shimada and Otani (1988) was used in this study as a representative of those media.

Culture media need not contain agar. The decision not to use agar in anther culture media was instigated by reports that it had inhibitory qualities (Kohlenbach & Wernicke 1978). Thus, the medium developed for barley by Sorvari and Schieder (1987) not only did not contain agar but replaced it with barley starch which effected the replacement of agar completely (Sorvari 1986a,b). When this starch was used in conjunction with an alternative sugar to sucrose, melibiose, a notable increase in plant yield was obtained with the resultant medium (EDAM I). These modifications were instrumental in bringing yields up to levels acceptable for implementation into barley breeding programmes and also offered promise for studies in wheat anther culture (see Chapter 7).

C-17 medium was more effective than either N_6 or potato-2 media in terms of callus induction and number of genotypes responding (Xu 1985). This medium had been developed for wheat from the N_6 medium with further reductions in nitrate and ammonium ions to which the improved responses had been attributed (Prof. H Xu pers. comm.). For these reasons, the C-17 medium was tested with Australian wheat genotypes.

Both WM2 (Datta & Wenzel 1987) and the Ficoll medium (Kao 1988) were representative of liquid media. Initially, the obstacle to using liquid medium was that the embryos or calluses tended to sink and die (Kao & Horn 1982). This was readily overcome by addition of Ficoll-400 to the liquid medium which by increasing the density, allowed the anthers and the androgenic structures produced to float, promoting better aeration and growth (Kao 1988; Simmonds 1989).

A sample of genotypes was cultured on these media in order to discern which would be most suitable for further anther culture use.

Finally, the effect of medium conditioned with anthers (Xu *et al.* 1981; Xu & Huang 1984) was also investigated using two of these media.

Materials and Methods

Seed of the Australian spring wheat cultivars (Aroona, Halberd, Kite, Machete, Schomburgk, Spear, Warigal) and one non-Australian cultivar (Chinese Spring) was supplied by the Australian Winter Cereals Collection, Tamworth, NSW.

Seed was germinated from June - October 1988 and in March 1989. Plant care, spike collection, pretreatment preparation, culture and regeneration procedures were as detailed in Chapter 4. Spikes were given an average of 3 days cold (4°C) pretreatment.

Seven media were used (Table 5.1). The prepared medium was pipetted (250 µl) into each well of a sterile, plastic, 96-well micro-test plate. Anthers from each genotype were plated according to the protocol described in Chapter 4, where three anthers were placed in each well. Cultures were sealed and incubated in darkness at 25°C or at 30°C for 2-3 days then transferred to 25°C. Embryos and calluses were transferred for differentiation to regeneration medium 190-2 (Zhuang & Xu 1983; Appendix 2) and incubated at 25°C with continuous illumination.

Two media (C-17 & W5) were used to test the effect of conditioning media with anthers. Maltose was substituted for sucrose and to condition the media, fresh anthers were placed in the prepared medium at a rate of 1000 anthers/l. The medium was autoclaved and plates prepared as described above. Anthers of the cv. Warigal containing microspores around mid-uninucleate stage were used for conditioning. Anthers were plated on the media as described above.

Table 5.1 The media used and their properties.

Medium*	Sugar	Solidification	Sterilization
C-17	sucrose	agar	autoclaved
EDAM I	melibiose	barley starch	autoclaved
Ficoll	sucrose	Ficoll-400	filter sterilized
MS	sucrose	agar	autoclaved
Potato-2	sucrose	agar, potato	autoclaved
W5	sucrose	agar	autoclaved
WM2	maltose	Ficoll-400	filter sterilized

* a full comparison is given in Appendix 2.

Results

A total of 16858 anthers were plated over all genotypes and across all media (Table 5.2). Anthers responded in culture on five of the seven media, of which WM2 had a response from all eight genotypes (Table 5.3). No response was obtained from any genotype on W5 or EDAM I. While the production of androgenic structures was greater per genotype on Potato-2 and C-17, only half the genotypes cultured on Potato-2 medium responded and 6 responded on C-17.

A culture response was not obtained for either medium containing maltose in place of sucrose (C-17 & W5) regardless of preconditioning with anthers (Table 5.4). Since no response was obtained on medium conditioned with anthers, a small sample of cultures was made up in which a fresh wheat ovary was placed amongst the plated anthers. No embryos were obtained from these cultures either, although there was a tendency for the anther filaments to etiolate and the cut end to swell.

Table 5.2 The number of anthers plated per genotype per medium.

Genotype	Medium							
	C-17	EDAM I	Ficoll	MS	Potato	WM2	W5	Total anthers plated per genotype
Aroona	120	27	90	18	42	1482	30	1809
Chinese Spring	234	24	90	36	33	1154	147	1718
Halberd	101	78	90	72	24	1441	20	1826
Kite	155					2339	92	2586
Machete	246	183	198	114		1212	60	2013
Schomburgk	213	54	66	42	9	1962	30	2376
Spear	394	169	192	126	75	729	70	1755
Warigal	756	162	267	78	96	1017	399	2775
Total anthers plated per medium	2219	697	993	486	279	11336	848	16858

Table 5.3 The number of androgenic structures produced per 100 anthers plated for each genotype on each medium and the number of genotypes responding on each medium.

Genotype	Medium						
	C-17	EDAM I	Ficoll	MS	Potato	WM2	W5
Aroona	0	0	0	0	4.76	0.20	0
Chinese Spring	2.14	0	1.11	0	3.03	0.61	0
Halberd	3.96	0	0	0	0	0.69	0
Kite	1.29					0.17	0
Machete	0.81	0	0	0		0.08	0
Schomburgk	7.04	0	1.52	2.38	0	0.10	0
Spear	0.76	0	0	0	0	0.82	0
Warigal	0	0	0	0	8.33	1.67	0
Genotypes responding	6/8	0/7	2/7	1/7	3/6	8/8	0/8

Table 5.4 The production of androgenic structures on unconditioned medium or medium conditioned with anthers. The number of anthers plated is shown in brackets.

Medium	unconditioned	conditioned
C-17	0 (924)	0 (420)
W5	0 (516)	0 (300)

Discussion

Embryo and plant production are the forms of yield usually sought from culture because they are requirements for plant breeding. However, an increase in overall yield from anther cultures does not necessarily mean that there has been an associated increase in yield from each genotype or that more genotypes responded.

The most important first step in establishing a workable culture protocol for experimentation and incorporating this technique into breeding programmes is to obtain an increase in the diversity and number of genotypes which will respond. Therefore, efficacy of the media in this study required the largest possible genotype response range. Luckett *et al.* (1991) have since stated that for a protocol to be fully functional, it should give a workable response in all genotypes since it is unacceptably restrictive if only a few genotypes are capable of being cultured successfully.

A response was defined as the formation of any structure additional to the anther. As a result, this sometimes included callus (from the diploid filament tissue) where the difference between callus and embryo was not clear. Although not strictly the desired product of anther cultures, such growths did imply that the medium was capable of inducing and supporting culture growth. The formation of calluses suggested that there was potential to improve this response with further cultural modifications. Embryos were easier to distinguish from callus on the liquid than on the solid media as they often detached from the anther whereas on solid media if the filament was not etiolated, callus which formed could not be discerned as separate to the anther and thus was scored as a response to culture. Although the production of androgenic structures was greater per genotype on Potato-2 and C-17, the lower means obtained on the liquid media, particularly WM2 might be attributable to such scoring, and as such WM2 was considered the most suitable medium for further investigation, particularly of Australian material. The negative correlation between range of genotypes responding to culture and overall anther response has also been found by other researchers (Andersen *et al.* 1987; Foroughi-Wehr & Zeller 1990).

Media conditioned with anthers (Xu *et al.* 1981; Xu & Huang 1984) and ovaries (Xu *et al.* 1981; Köhler & Wenzel 1985; Datta & Wenzel 1987; Ziauddin *et al.* 1990) have been shown to improve androgenic response in microspore cultures. If the promotive factor of conditioned medium was inorganic then autoclaving should not have interfered with its action. However, if the factor responsible was organic, then such preparation would have deactivated it and could be associated with the lack of response obtained. Since previous success with conditioned medium has involved live anthers which were removed prior to plating (Xu *et al.* 1981) and co-culture with live ovaries (Datta & Wenzel 1987), the results obtained in this study suggest that if "anther factors" (Xu & Huang 1984) exist at all, they would be labile compounds.

The response obtained with C-17 was promising but the medium was not effective over enough genotypes to continue its use. Variations of this medium were used recently with Australian wheat (Luckett *et al.* 1991) with embryos produced over a range of genotypes. However, the responses Luckett *et al.* (1991) obtained were significantly influenced by the use of membrane rafts which supported the anthers above a liquid form of the medium and which most likely influenced responses by improving nutrient supply, waste removal and prevention of anaerobiosis. Given variation in genotype response, it was concluded that this system required further development for it to be of use over a wider range of genotypes (Luckett *et al.* 1991).

Potato media were popular in the early 1980's for wheat anther culture (Schaeffer *et al.* 1979; Henry & de Buyser 1981; Bullock *et al.* 1982; Ouyang *et al.* 1983; He & Ouyang 1984; Lazar *et al.* 1984a,b, 1985; Marsolais *et al.* 1984) and are still in use by some research groups (Bjørnstad *et al.* 1989; Zhou & Konzak 1989; Yuan *et al.* 1990; Ekiz & Konzak 1991a,b,c; Zhou *et al.* 1991) but present the problems of insufficient standardization and hence repeatability.

Shimada and Otani (1988) found that their potato medium was significantly better than N_6 for the performance of 31 wheat genotypes. Since only filament callus was obtained from anthers on the N_6 medium (Shimada & Otani 1988) the use of a potato medium for embryogenesis is strengthened. Another attraction to using this medium was that when potato starch was used in place of the boiled potato extract, equally promising results were obtained. This suggested that the medium could be developed away from being chemically undefined towards being more synthetic. The results obtained in this study were supportive of the potato medium in terms of embryo response but were poor in terms of the number of genotypes which were responsive.

The total lack of response by the wheat cultivars on EDAM I was highly disappointing in light of the significant improvements attributed to this medium with barley (Sorvari & Schieder 1987). In part this might have been because EDAM I was so specifically developed, with both the starch and sugar chosen to obtain the best response from barley. Equally, the barley study used only three highly responsive genotypes and so the utility of this medium over a wider genetic background was not demonstrated. Apart from starch and sugar incompatibilities, there might have been inhibitory substances present since the medium was autoclaved rather than filter sterilized (Chu & Hill 1988; Schenk *et al.* 1991).

Despite the reported beneficial qualities of the W5 medium (Feng & Ouyang 1988), no response was obtained from any of the wheat genotypes tested in this study. The outcome on MS was the same, which could be due to its less specialized composition that did not meet the culture requirements of the anthers.

Liquid media have gained increasing popularity for the culture of wheat anthers (Zhou & Konzak 1989; Chu *et al.* 1990; Last & Brettell 1990). The substantial increases in induction and significantly better growth of embryos on media containing Ficoll than without has made the use of Ficoll almost a precondition for liquid anther culture media. Ficoll not only acts as a buoyancy increasing agent which can successfully replace agar but also makes the medium liquid or semi-solid - solid media being conventional. Since better results were obtained with low sugar, high Ficoll concentrations than with high sugar, low Ficoll concentrations (Kao & Horn 1982), the use of sugar in induction media is again questioned. The contrast in response of the Australian wheats to WM2 and this Ficoll medium was striking and possibly attributable to the inclusion of many potentially detrimental organic components in the medium (see Appendix 2). Nevertheless, the benefits derived from the liquid nature of the medium, its filter-sterilization and the use of Ficoll were common to the Ficoll medium and to WM2, on which every genotype responded.

Therefore, WM2 brought together many attributes which had been reported to be beneficial for cereal anther culture. It was based on the N_6 medium (Sorvari & Schieder 1987; Kasha *et al.* 1990) and was liquid, using Ficoll for physical support (Kao 1981; Olsen 1987; Chu & Hill 1988; Jones & Petolino 1988). Furthermore, it was filter sterilized rather than autoclaved (Chu & Hill 1988), contained glutamine (Henry & de Buyser 1981; Olsen 1987) and had a reduction in sugar concentration (Hunter 1987). The micronutrient cobalt was subsequently added as it is essential for wheat development. Therefore, this medium incorporated aspects previously related to good anther culture performance and, in addition, was a wholly synthetic medium which not only reduced preparation time but also ensured consistency of composition which can help experimental reproducibility.

In summary, a suitable induction medium is the key prerequisite for not only raising the frequency of green plants from wheat culture but also for obtaining useful results from an economically important range of breeding material. Clearly, the results showed that the WM2 medium was superior in offering the best potential for further investigations into the differential response of genotype and other factors affecting androgenesis.

Chapter 6 Anther culture of Australian wheats and a study of the factors affecting androgenesis

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Introduction

The response of anthers to culture in most species is complex. Several factors, including genotype and health of the parent or donor plant together with several aspects of cultural conditions such as microspore stage, pretreatments, incubation temperature and sugar, have been shown to be influential (see Chapter 2) but responses are not consistent among research laboratories even when the same protocols are used.

An assessment of the relationship between these factors and anther culture response is imperative when using previously unresearched donor material since many of the factors affecting androgenesis are still ill-defined and, furthermore, vary so strongly with genotype and cultural conditions. Most experiments on improving the method of *in vitro* anther culture of non-Australian wheat have included carefully selected, suitable genotypes. There is now a need to broaden the range of responsive lines.

When this project commenced, no research had been reported on the anther culture of Australian wheat cultivars.

For these reasons and for anther culture to be applicable to current breeding and research material, it is necessary to identify and to investigate those factors which may have a bearing on culture response.

A protocol better suited to the research material at hand would result from such investigations. This will allow an evaluation of the factors that affect culture response and will allow anther culture to be more practically employed in other fields of research.

The objective of this study was to investigate the extent to which certain variables affected anther response of Australian cultivars from which a protocol could be designed which maximized the response in those cultivars. The WM2 induction medium (Appendix 2) was used as the basis for these experiments following the results of Chapter 5 which showed its general applicability. The variables investigated were, the i) genotype of the parent material, ii) conditions under which donor plants for anthers were grown, iii) developmental stage of the microspores within the cultured anthers, iv) duration given for cold pretreatment of spikes, v) choice of sugar in the induction medium and vi) temperature at which cultures were incubated.

Materials and Methods

Seed of the Australian spring wheat cultivars and breeding lines (Table 6.1), Chinese Spring and Pavon-76 were supplied by the Australian Winter Cereals Collection, Tamworth, NSW. Seed of Atys, Orofen and an Orofen F_1 plant derived from anther culture (anther Orofen) were obtained from Dr Richard Brettell, CSIRO Division of Plant Industry, ACT.

Seed germination, plant care, spike collection, pretreatment preparation, culture and regeneration procedures were as described in Chapter 4. The basal medium was WM2 (Datta & Wenzel 1987), which contained $CoCl_2$ (0.025 mg/l), kinetin (0.5 mg/l) (Appendix 2) and a sugar at 6%. The prepared medium was filter sterilized and 2 ml pipetted into each 35 mm sterile plastic petri-dish. Anthers were cultured on the liquid medium according to the protocol described in Chapter 4, where 35 anthers were placed in each plate, with anthers from one side of the spike assigned to a petri-dish of medium containing one sugar and those from the other side to another sugar. Cultures were sealed and incubated in darkness. The regeneration medium used was 190-2 (Zhuang & Xu 1983; Appendix 2).

The six factors assessed in these experiments were genotype, donor plant environment, microspore stage, length of cold pretreatment of spikes, choice of sugar in the medium and the temperature at which cultures were incubated.

Twenty two genotypes were assessed, as were 24 donor plant environments, which were defined by the date of seed germination and the use of a glasshouse (GH) or a controlled-environment growthroom (GR). Twelve microspore stages were defined and used. The cold pretreatment of 4°C was generally applied for either 3 or 8 days. The sugar in the medium was chosen from maltose, cellobiose, sucrose and gentiobiose, all of which are glucose disaccharides. Four incubation regimes were established, based on either 18°C or 25°C and with or without a preliminary incubation for 2-3 days at 30°C (30/18, 30/25).

A full factorial combination of all levels of all treatments represented over a million possibilities, so some rationalisation was necessary. Twelve genotypes (Aroona, Atys, Chinese Spring, Halberd, Kite, Machete, Orofen, anther Orofen, Oxley, Schomburgk, Spear and Warigal) (Group 1) were grown in both the GH and GR, received both lengths of cold pretreatment and all incubation regimes. The remaining 10 genotypes (Bencubbin, Bindawarra, Dagger, Egret, Federation, Gabo, Hartog, Insignia, Pavon-76 and WW15) (Group 2) were grown only in the GH, given 8 days cold pretreatment and the 30/18°C incubation.

Statistical analyses were as outlined in Chapter 4. In brief, the data for genotype and each of the six variables for these two groups were analysed separately. Pair-wise combinations of the factors were analysed to determine the first-order interactions but higher order analyses were not feasible. In the tables of embryo means/100 anthers, a blank indicates that the combination of factors was not tested. In the tables showing the regeneration of plants from embryos, a blank similarly indicates that the combination of factors was not tested or if tested, that no embryos were produced from which plants could be regenerated. In all tables, data are presented with ± standard error. Where no standard error is present, only one dish of anthers was tested and where the standard error is equal to the mean, only one dish responded.

An additional set of Australian cultivars and their F_1 hybrids were cultured using WM2 medium containing maltose. Spikes were collected from donor plants growing in the glasshouse in July and August and were given cold pretreatment for 3 days. Cultures were incubated at 30/25°C.

Results

The Australian wheat cultivars, breeding lines and non-Australian wheats exhibited a range of responses to anther culture (Table 6.1). Androgenesis resulted in the formation of embryos which regenerated green (Figure 6.1) and/or albino plants, or remained undifferentiated. Somatic callus from the filaments (Figure 6.2 A) was commonly observed but since it was derived from diploid cells it was not considered of further interest. Only the formation of embryogenic structures (Figure 6.2 B) from haploid tissue was analysed. As detached calluses can be confused with embryogenic structures, great care was taken throughout all experiments not to include such calluses in the results.

Table 6.1 Anther response for the wheat cultivars and breeding lines used, with the total number of anthers plated for each genotype and the number of embryos and plants produced. Group 1 and group 2, and Australian and non-Australian genotypes are shown.

	Genotype	Total number of anthers plated	Embryos	Plants Green	Plants Albino
	Australian				
Group 1	Aroona	4422	2		
	Halberd	4127	0		
	Kite	5031	8	1	1
	Machete	5223	6	2	2
	Oxley	4827	39	2	11
	Schomburgk	4089	13	5	1
	Spear	4111	0		
	Warigal	4688	49	3	19
Group 2	Bencubbin	2610	0		
	Bindawarra	3384	0		
	Dagger	3398	0		
	Egret	3150	8		
	Federation	3692	1	1	
	Gabo	1873	0		
	Hartog	2160	0		
	Insignia	2375	0		
	WW15	2249	2	1	1
	Non-Australian				
Group 1	Chinese Spring	5519	7	2	2
	Orofen	3258	20	1	2
	anther Orofen	2420	12	1	1
	Atys	4049	5		
Group 2	Pavon-76	2538	0		
	Total	79193	172	19	40

Figure 6.1 Green plants regenerated from anther-derived wheat embryos.

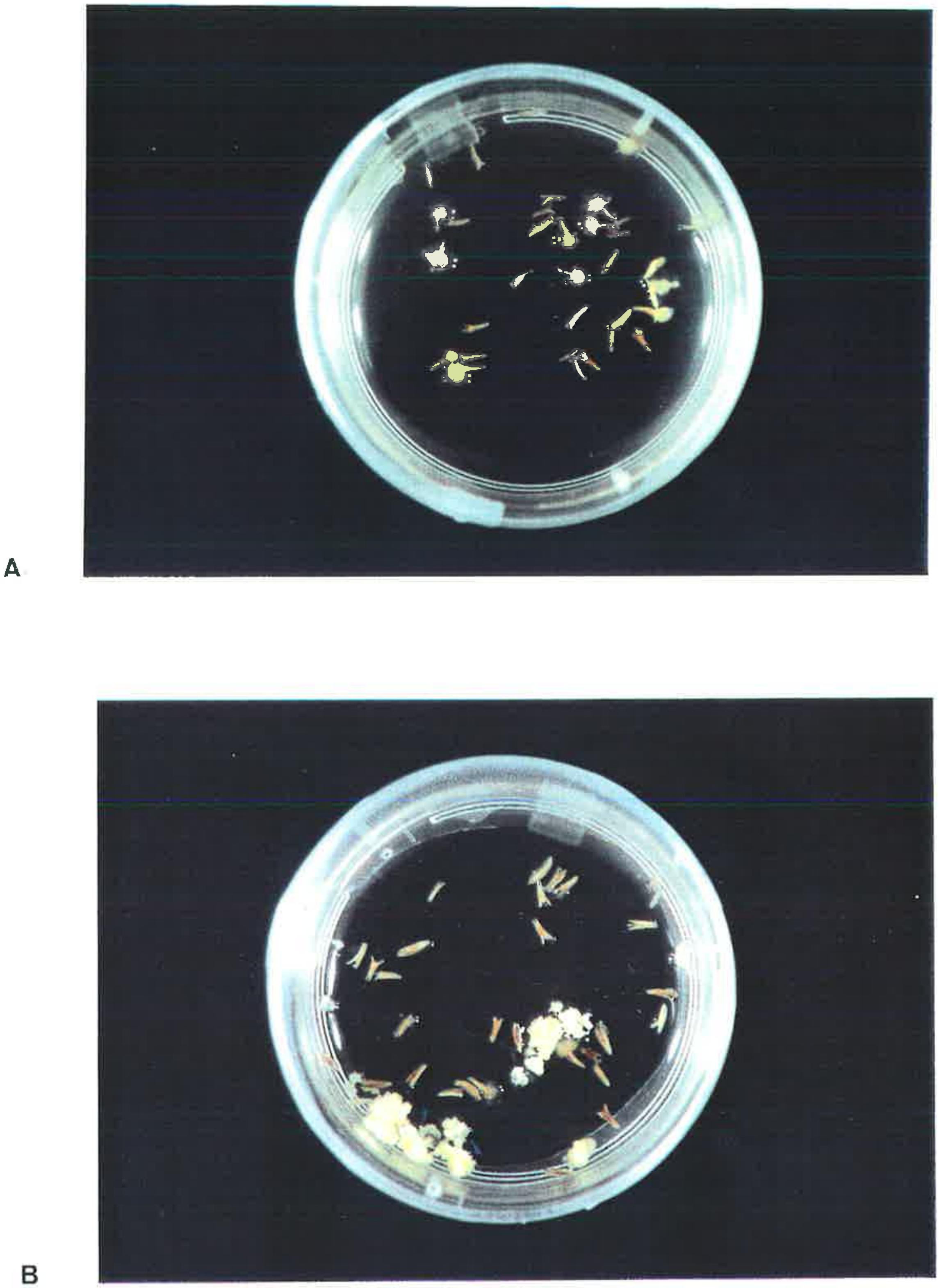

Figure 6.2 Wheat anther cultures with somatic, filament callus (A) and embryos (B). Embryogenic structures (B) are the organised growth and development of haploid microspores whereas filament callus (A) is formed from maternal, diploid tissue and considered an artefact of anther cultures.

Statistical analysis revealed that each factor strongly influenced embryo production for group 1 and group 2 genotypes (Table 6.2). The length of cold pretreatment had no effect for group 2 because variability within this factor was limited. Twelve of the 15 interactions for treatments applied to group 1 genotypes were also significant (Table 6.3) whereas all interactions among treatments applied to group 2 genotypes were not significant. There was no interaction among the donor plant environments, sugars and incubation temperatures which influenced the production of embryos (Table 6.3). Since all the genotype x treatment interactions were significant, the main effects of each treatment will be described together with its interaction with genotype.

Table 6.2 Results for the one-way analysis between factors tested for their contribution to anther response and embryo production for group 1 and group 2 genotypes cultured. Where df = degrees of freedom, χ^2 = chi square statistic, ns = not significant.

Source of deviation	Group 1			Group 2		
	df	χ^2	Probability	df	χ^2	Probability
Genotype	11	196.78	P<0.001	9	32.26	P<0.001
Residual	1455	1121.54		784	86.83	
Microspore stage	11	159.37	P<0.001	11	38.82	P<0.001
Residual	1455	1158.95		782	80.27	
Donor plant environment	20	343.42	P<0.001	10	38.68	P<0.001
Residual	1446	974.91		781	80.36	
Cold pretreatment length	11	119.26	P<0.001	5	8.09	ns
Residual	1455	1199.06		788	111.01	
Sugar	3	37.29	P<0.001	3	13.41	P<0.01
Residual	1463	1281.03		790	105.69	
Incubation temperature	3	77.69	P<0.001			
Residual	1463	1240.63				

Table 6.3 Results of the two-way interactions between factors tested for their contribution to embryo production for genotypes of group 1. Where df = degrees of freedom, χ^2 = chi square statistic, ns = not significant, [a] main effects $P<0.001$.

Source of deviation	df	χ^2	Probability	Source of deviation	df	χ^2	Probability
Genotype	11	196.78		Incubation temperature	3	77.70	
Sugar	3	49.71		Sugar	3	37.87	
Interaction	33	296.25	P<0.001	Interaction	3	3.83	ns[a]
Residual	1419	775.58		Residual	1457	1198.94	
Genotype	11	196.78		Microspore stage	11	159.37	
Microspore stage	11	140.49		Donor plant environment	20	348.21	
Interaction	77	315.16	P<0.001	Interaction	99	251.90	P<0.001
Residual	1367	665.89		Residual	1336	558.84	
Genotype	11	196.78		Microspore stage	11	159.37	
Donor plant environment	20	358.75		Cold pretreatment length	11	98.33	
Interaction	108	191.37	P<0.001	Interaction	50	181.50	P<0.001
Residual	1327	571.42		Residual	1394	879.13	
Genotype	11	196.78		Microspore stage	11	159.37	
Cold pretreatment length	11	112.60		Incubation temperature	3	76.61	
Interaction	71	312.34	P<0.001	Interaction	26	90.93	P<0.001
Residual	1373	696.60		Residual	1426	991.41	
Genotype	11	196.78		Cold pretreatment length	11	119.26	
Incubation temperature	3	139.45		Donor plant environment	20	315.41	
Interaction	28	137.26	P<0.001	Interaction	47	91.48	P<0.001
Residual	1424	844.83		Residual	1388	792.17	
Microspore stage	11	159.37		Incubation temperature	3	77.70	
Sugar	3	29.01		Donor plant environment	20	270.72	
Interaction	30	144.31	P<0.001	Interaction	12	6.08	ns[a]
Residual	1422	985.63		Residual	1431	963.83	
Sugar	3	37.29		Cold pretreatment length	11	119.26	
Donor plant environment	20	342.77		Incubation temperature	3	82.98	
Interaction	21	8.30	ns[a]	Interaction	13	106.82	P<0.001
Residual	1422	929.97		Residual	1439	1009.27	
Cold pretreatment length	11	119.26					
Sugar	3	54.33					
Interaction	18	123.90	P<0.001				
Residual	1434	1020.82					

All main effects P<0.001.

Embryos differentiated to give rise to green and/or albino plants, or they remained undifferentiated. Each factor was considered individually to determine its effect on the regeneration of plants from the embryos. Most factors had no significant effect on the regeneration of green or albino plants (Table 6.4) and only three factors affected the proportion of embryos which differentiated these. The production of green plants was affected by cold pretreatment length and sugar, while that of albino plants was affected by the microspore stage at which anthers were cultured and cold pretreatment length (Table 6.4).

The only interaction which affected the regeneration of plants was genotype x microspore stage ($P<0.05$) which had an effect on albino regeneration.

Table 6.4 Results for the one-way analysis between factors tested for their contribution to regeneration of green and albino plants, for Group 1 genotypes. Where df = degrees of freedom, χ^2 = chi square statistic, ns = not significant, a = residual significant.

Source of deviation	Green plants			Albino plants		
	df	χ^2	Probability	df	χ^2	Probability
Genotype	9	13.29	ns	9	15.09	ns
Residual	33	44.25		33	45.17	
Microspore stage	6	9.66	ns	6	20.44	$P<0.01$
Residual	36	47.87		36	39.83	
Donor plant environment	8	10.64	ns	8	13.87	ns
Residual	34	46.90		34	46.39	
Cold pretreatment length	5	15.22	$P<0.01$	5	13.70	$P<0.05$
Residual	37	42.32		37	46.56	
Sugar	3	14.46	$P<0.01$	3	3.84	ns
Residual	39	43.07		39	56.42	a
Incubation temperature	3	4.71	ns	3	3.33	ns
Residual	39	52.83		39	56.93	a

Genotype

Some genotypes (e.g. Warigal & Oxley) successfully produced green plants whereas others (i.e. Aroona, Egret, Atys) which produced embryos failed to regenerate plants. Still others (e.g. Spear, Halberd, Pavon-76) failed to produce embryos under all experimental conditions (Table 6.1) and although these were included in statistical analyses, they are not always represented in further tabulation.

Most responsive genotypes had certain antecedents in common, particularly WW15, the CIMMYT breeding line, or its progenitors, Mentana, Kentana and Frontana (Table 6.5). Norin 10/Brevor was also a common cross found in many of the responsive wheats and is the source of the Rht_1 and Rht_2 (reduced height) dwarfing genes (Gale & King 1988). Orofen yielded well in culture (Table 6.1) and is of the CIMMYT group. Nevertheless, the lack of response by anthers from Hartog and Pavon-76 showed that not all CIMMYT-bred wheats performed well in culture.

Non-responsive Australian genotypes tended to have Insignia, Gabo or Nabawa-type wheats in their pedigrees.

Three pairs of sister lines were used: Warigal and Aroona, Dagger and Spear, Hartog and Pavon-76. There were differences in embryo and plant regeneration responses between Aroona and Warigal while the other two pairs were non-responsive.

Although genotype played a major role in embryo induction (Tables 6.2, 6.3), it was not found to influence plant regeneration (Table 6.4).

Microspore stage

Since there was a lack of morphological criteria for recognizing some anther stages (Sunderland & Huang 1985), a detailed system for the classification of microspore development was developed on the basis of the shape and position of the nucleus and the presence or absence of the vacuole (Figure 6.3). This system compares well with published

Table 6.5 Pedigrees of the cultivars and breeding lines used (Zeven & Zeven-Hissink 1976). The portions of the pedigrees which are common among the responsive cultivars are indicated. Genotypes which responded to anther culture are in bold print.

Genotype	Pedigree	Pedigree in common
WW15	Lerma Rojo//Norin 10/Brevor (Seln 14)/3/3*Andes	WW15
Warigal	WW15/Raven	WW15
Aroona	WW15/Raven	WW15
Schomburgk	W3589/Oxley//2*Warigal/3/2*Aroona	WW15
Oxley	Penjamo 62/4*Gabo 56//Tezanos Pintos Precoz/Nainari60/4/2*WW15	WW15
Egret	Heron/2*WW15	WW15
Machete	Sonora 64//Tezanos Pintos Precos/Yaktana 54/2*Gabo//Madden	CIMMYT-bred
Kite	Norin10/Brevor(Seln14)//4*Eureka2/3/T.A/3*Falcon/4/T.A/4*Falcon/5/T.A/5*Falcon	
Bindawarra	Mexico-120/Koda//Raven	
Hartog	Vicam 71//Ciano 'S'/Siete Cerros/3/Kalyansona/Bluebird	
Insignia	Ghurka/Ranee	
Dagger	Sabre/MEC3//Insignia	
Spear	Sabre/MEC3//Insignia	
Halberd	Scimitar/Kenya C6042//Bobin/3/Insignia 49	
Gabo	Gaza/2*Bobin-39	
Bencubbin	Gluyas Early/Nabawa	
Federation	?Purple Straw	
Pavon-76	Vicam 71//Ciano'S'/Siete Cerros/3/Kalyansona/Bluebird	
Chinese Spring	local Chinese variety	
Orofen	Yaqui 48//Kenya C9908/Mentana/3/Frontana	CIMMYT-bred
Atys	Aubers//Thatcher/YGA	

where:

- Andes (55) (Colombian)	Kentana 48/Frontana//Mayo 48
- Dundee (Australian)	Hard Federation/Cleveland//Sands
- Eureka (Australian)	Kenya/Florence//Dundee
- Falcon (Australian)	Bencubbin//Dundee/Gular/3/Gular
- Frontana (Brazilian)	Fronteira/Mentana
- Hard Federation	Federation variant
- Heron (Australian)	RDR/Insignia 49
- Kentana 48 (Mexican)	Kenya C9906/Mentana
- Lerma Rojo (Mexican)	Mentana/4*Kentana 48/Yaqui 48//Mario Escobar*2/Supremo 211
- Mexico-120 (Mexican)	Yaktana 52//Norin 10/Brevor
- Purple Straw (Australian)	? Red Straw
- Raven (Australian)	Orfed, Uruguay, Mayo/4?*Dirk 48
- Sonora 64 (Mexican)	Yaktana 54//Norin 10/Brevor/3/2*Lerma Rojo 54
- Yaktana 54 (Mexican)	Yaqui 48/Kentana 48//Frontana

observations for wheat (Ouyang *et al.* 1973; He & Ouyang 1984) with stages 4 - 9 including all stages which are commonly referred to as 'mid-uninucleate'. Variation in the physical appearance of nuclei, microspore wall, cytoplasm and size of the pollen was encountered.

The pollen mother cells were defined as stage 1 and tetrads, enclosed within a fine sac-like wall, as stage 2. Cells separated from tetrads retained their diagnostic triangular form. Stages 3 - 9 were uninucleate microspores.

Very early uninucleate microspores (stage 3) had no germpore or exine and only the fine intine present. Stage 4 cells had a fine germpore and were referred to as early uninucleate microspores. Mid uninucleate microspores (stage 5) were observed to have fully developed germpores and exine (though this tended not to be sculpted) with the nucleus situated centrally within the cytoplasm.

In stage 6 cells (mid-side uninucleate), the nucleus was between the centre and the side of the cell, while at stage 7 (mid-late uninucleate), it was against the wall and opposite the germpore.

Later stages of development were characterized by the nucleus remaining opposite the germpore and by the presence of a large central vacuole, peripheral cytoplasm and pronounced exine. The shape of the nucleus was diagnostic such that a rounded nucleus defined stage 8 cells (late-rounded uninucleate) and one flattened along the wall defined stage 9 cells (late uninucleate) in which the cytoplasm was further reduced and the cell was about to undergo mitosis.

Mitosis resulted in binucleate pollen (stage 10) in which the vegetative cell, situated under the germpore, tended to have an ill defined nucleus, whereas the generative cell and nucleus were opposite the germpore. Stage 10 could be confused with stage 7 if the vegetative nucleus of binucleate pollen could not be resolved.

After a further mitotic division of the generative nucleus, the microspore became trinucleate (stage 12). Maturation included elongation of the generative nuclei and packing of the pollen with starch grains. When the exact stage of the microspore could not be determined it was because the nucleus could not be defined. Such microspores were classified according to the presence (stage 11) or absence of the cytoplasm (sterile). Microspores at stage 11 characteristically had well developed exine and were therefore regarded as a form of mature pollen.

Anthers from all twelve microspore stages were cultured but embryos were formed only from cultures containing anthers at stages 5-10 (Table 6.6). Few embryos were obtained from stages 5 and 6. The optimum stage for embryo production varied widely among genotypes (Table 6.6). Even though a higher percentage of embryos was produced from more mature microspores (stages 8-10), only a small proportion regenerated plants, which tended to be albino (Table 6.7). Conversely, although there was a trend for a smaller percentage of embryos to be produced from younger microspores (stages 5-7), the regeneration of green plants from these was proportionally better. These proportions were strongly dependent on genotype as the interaction between microspore stage and genotype was significant ($P<0.01$) (Table 6.4).

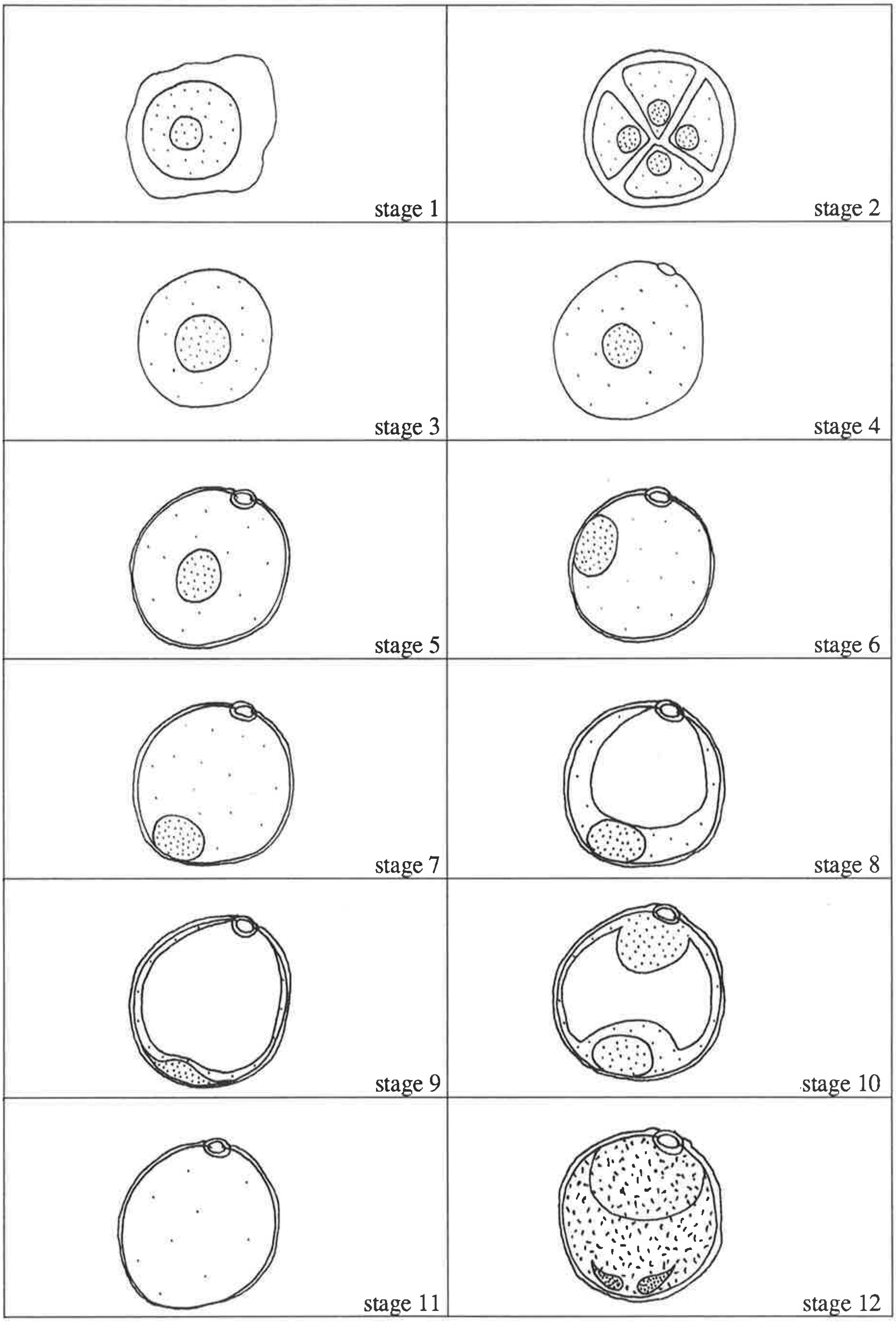

Figure 6.3 The microspore stages which were observed.

Table 6.6 The mean number of embryos produced per 100 plated anthers ± se, for each of the responding genotypes. Only those microspore stages which gave a response are shown.

Genotype	Microspore stage					
	5	6	7	8	9	10
Aroona	0	0	0.13 ± 0.13	0	0	0
Warigal	0	2.07 ± 1.52	0.66 ± 0.66	0	1.76 ± 1.23	19.23
Schomburgk	0	0	0.66 ± 0.47	0.62 ± 0.43	0.48 ± 0.34	0
Oxley	0.27 ± 0.19	0	0	0	0	50.0 ± 41.43
Kite	0.36 ± 0.22	0	0	0	0.23 ± 0.23	0
Machete	0	0	0.32 ± 0.32	0.38 ± 0.26	0	0
WW15	0	0	0	0.95 ± 0.95	0	0
Egret	0.48 ± 0.48	0	0	0	0	5.71 ± 5.71
Federation	0	0.08 ± 0.08	0	0	0	0
Chinese Spring	0	0.08 ± 0.08	0	0	1.10 ± 0.61	0
Orofen	0	0	0	5.99 ± 2.70	0	0
anther Orofen	0	0	8.89 ± 8.89	0	0	0
Atys	0	0.07 ± 0.07	0.90 ± 0.47	0	0	2.38 ± 2.38

Table 6.7 The proportion of green plants (g) and albino plants (a) regenerated ± se from the total number of embryos produced for each of the responding genotypes. Only those microspore stages are shown in which a response was obtained.

Genotype	Microspore stage											
	5		6		7		8		9		10	
	g	a	g	a	g	a	g	a	g	a	g	a
Aroona					0	0						
Warigal			0.11 ± 0.04	0.36 ± 0.07	0.20	0			0.25 ± 0.25	0.61 ± 0.24	0	0.57
Schomburgk					0.63 ± 0.38	0.13 ± 0.13	0.50 ± 0.50	0	0	0		
Oxley	0.50 ± 0.50	0									0.03 ± 0.03	0.17 ± 0.17
Kite	0.17 ± 0.17	0							0	0.50		
Machete					0.50	0.25	0	0.50 ± 0.50				
WW15							0	0.50				
Egret	0	0									0	0
Federation			1.00	0								
Chinese Spring			0	1.00					0.33 ± 0.17	0		
Orofen							0.03 ± 0.03	0.06 ± 0.06				
anther Orofen					0.08	0.17						
Atys			0	0	0	0					0	0

Donor plant environment

Donor plants were grown in two environments: growthroom (GR) and glasshouse (GH). There were major genotypic differences in response to donor plant environment for embryo production (Table 6.8) and plant regeneration (Table 6.9). As there was considerable variation among sowing dates, these were pooled into two seasonal groups - 'winter' (April - September) and 'summer' (October - March). Clearly, more genotypes produced embryos when grown during winter and, in particular, in the glasshouse (Table 6.8) whereas in summer, higher embryo means were obtained.

Analysis showed that neither donor plant environment nor genotype had a significant effect on the proportion of embryos which regenerated green and albino plants (Tables 6.4, 6.9). Nevertheless, embryos from several genotypes regenerated more albino plants than green plants from the glasshouse in both seasons, while Schomburgk, Oxley and Federation regenerated more green plants than albino plants (Table 6.9). The night-day temperature of the growthroom was 10-15°C whereas during winter in the glasshouse it ranged between 7-25°C. The light intensity in the glasshouse was far greater than that in the growthroom.

Alternative analyses, where data were pooled for the number of responding genotypes for each of four environmental sets, namely winter and summer glasshouse, winter and summer growthroom, confirmed that there was a significant difference in the number of genotypes which produced embryos between these (Appendix 3). The interaction between season (winter, summer) and environment (GH, GR) was significant ($P<0.01$) the result of a greater number of genotypes responding in winter and, in particular, in the glasshouse (Appendix 3). Analysis of the number of culture dishes in which embryos were formed, out of the total number of dishes plated for each responsive genotype for each environment was uninformative as the data were unbalanced (Appendix 4).

Table 6.8 The mean number of embryos produced per 100 plated anthers ± se for each of the responding genotypes, from the donor plant environments (growthroom (GR) and glasshouse (GH) during "winter" (Apr - Sep) and "summer" (Oct - Mar)) from which a response was obtained.

Genotype	Donor plant environment								
	winter					summer			
	GH				GR	GH		GR	
	Apr 1	Jun 21	Jul 28	Aug 8	Aug 8	Jan 16	Nov 16	Jan 16	Nov 16
Aroona		0		0	0.74 ± 0.74			0	0
Warigal		1.74 ± 0.98	0	0	0	2.40 ± 2.40	19.69 ± 12.46	1.48 ± 1.48	
Schomburgk		0.99 ± 0.44	0	0	0		0	0	0.33 ± 0.33
Oxley	3.96 ± 3.52	0.19 ± 0.19	0		0				
Kite	0	0.66 ± 0.33	0	0	0	0	0	0	0
Machete		0.57 ± 0.46	0.26 ± 0.26	0	0	0	0	0	0
WW15	0.32 ± 0.32								
Egret	0.95 ± 0.66								
Federation	0.16 ± 0.16								
Chinese Spring	0	0.53 ± 0.53	0.31 ± 0.31	0.99 ± 0.72					
Orofen	0					0	8.65 ± 3.60		0
anther Orofen						0	0		44.44
Atys						0	1.04 ± 0.47	0	0.11 ± 0.11

Table 6.9 The proportion of green plants (g) and albino plants (a) regenerated ± se from the total number of embryos produced for each of the responding genotypes, from the donor plant environments (growthroom (GR) and glasshouse (GH) during "winter" (Apr - Sep) and "summer" (Oct - Mar)) from which a response was obtained.

Genotype	Donor plant environment																	
	winter										summer							
	GH								GR		GH				GR			
	Apr 1		Jun 21		Jul 28		Aug 8		Aug 8		Jan 16		Nov 16		Jan 16		Nov 16	
	g	a	g	a	g	a	g	a	g	a	g	a	g	a	g	a	g	a
Aroona									0	0								
Warigal			0.20 ± 0.20	0.60 ± 0.19							0	0	0.11 ± 0.04	0.36 ± 0.07	0.20	0		
Schomburgk			0.25 ± 0.19	0.05 ± 0.05													1.00	0
Oxley	0.02 ± 0.02	0.12 ± 0.12	1.00	0														
Kite			0.13 ± 0.13	0.13 ± 0.13														
Machete			0.25 ± 0.25	0.63 ± 0.38														
WW15	0	0.50																
Egret	0	0																
Federation	1.00	0																
Chinese Spring			0.50	0	0	1.00	0.25 ± 0.25	0										
Orofen													0.03 ± 0.03	0.06 ± 0.06				
anther Orofen																	0.08	0.17
Atys													0	0			0	0

Cold pretreatment of spikes

A pretreatment of the harvested spikes at 4°C for 3 days was shown to be effective across a range of genotypes (Chapter 5). Therefore, this length was taken as the shortest pretreatment time given and durations up to 40 days were tested but no response past 10 days was obtained. Within the range of 3-10 days pretreatment, different genotypes showed considerable variation in optima for production of embryos (Table 6.10) and plants (Table 6.11). The shortest pretreatment was associated with embryo formation in 8 of 10 genotypes including all Australian genotypes tested but longer pretreatments generally resulted in much larger numbers of embryos from fewer genotypes.

More than 10 embryos/100 anthers were obtained from Orofen with 4 days cold pretreatment and Warigal with 6 days (Table 6.10). Cold pretreatment of spikes was a major determinant for the production of green plants (Table 6.4). All embryos from Oxley with 3 days cold pretreatment differentiated into green plants as did those from Federation with 10 days pretreatment (Table 6.11). The high embryo yield of Warigal after 6 days cold pretreatment was not carried through into plant yield as two thirds of the embryos differentiated into albino plants and none into green plants. The regeneration of albino plants is undesirable as they are of no practical breeding use. Cold pretreatment was also a determinant in the production of albino plants (Table 6.4).

Table 6.10 The mean number of embryos produced per 100 plated anthers ± se for each of the responding genotypes, for cold pretreatment length (days at 4°C). Only those cold pretreatment lengths which gave a response are shown (i.e. 3-10).

Genotype	4°C pretreatment length (days)						
	3	4	6	7	8	9	10
Aroona	0.13 ± 0.13	0	0	0	0	0	0
Warigal	0.16 ± 0.11	0	11.74 ± 5.50	0	4.19 ± 2.20	0	0
Schomburgk	0.41 ± 0.21	1.19 ± 0.83	0	0	0	0	0
Oxley	0.14 ± 0.14	0	0	0	0	5.71 ± 5.07	0
Kite	0.29 ± 0.15	0	0	0	0	0	0
Machete	0.35 ± 0.28	0.24 ± 0.24	0	0	0	0	0
WW15					0	0	2.86 ± 2.86
Egret					0.41 ± 0.29	0	0
Federation					0	0	0.29 ± 0.29
Chinese Spring	0.32 ± 0.22	0	0	1.32 ± 0.93	0	0	0
Orofen	0	15.02 ± 6.97	0	0	0.27 ± 0.27	0	0
anther Orofen	0	0	0	0	0.77 ± 0.77	0	0
Atys	0.77 ± 0.50	0.22 ± 0.22	0	0	0.07 ± 0.07	0.34 ± 0.34	0

Table 6.11 The proportion of green plants (g) and albino plants (a) regenerated ± se from the total number of embryos produced for each of the responding genotypes, for cold pretreatment length (days at 4°C). Only those lengths which gave a response are shown (i.e. 3-10).

Genotype	4°C pretreatment length (days)													
	3		4		6		7		8		9		10	
	g	a	g	a	g	a	g	a	g	a	g	a	g	a
Aroona	0	0												
Warigal	0.50 ± 0.50	0.50 ± 0.50			0	0.67 ± 0.17			0.10 ± 0.04	0.18 ± 0.11				
Schomburgk	0.31 ± 0.24	0.06 ± 0.06	0.50 ± 0.50	0										
Oxley	1.00	0									0.02 ± 0.02	0.12 ± 0.11		
Kite	0.13 ± 0.13	0.13 ± 0.13												
Machete	0.25 ± 0.25	0.63 ± 0.34	0	0										
WW15													0	0.50
Egret									0	0				
Federation													1.00	0
Chinese Spring	0.25 ± 0.25	0.50 ± 0.50					0.25 ± 0.25	0						
Orofen			0.06 ± 0.06	0.11 ± 0.11					0	0				
anther Orofen									0.08	0.17				
Atys	0	0	0	0					0	0	0	0		

Culture incubation temperature

Culture incubation temperature regimes affected the mean number of embryos produced for each genotype. The interaction of incubation temperature with microspore stage and cold pretreatment also affected embryo production (Table 6.3). Genotypes of group 1 responded most strongly when cultures were given an initial shock at 30°C for 3 days followed by maintenance at 25°C (Table 6.12). Clearly, all genotypes responded when exposed to the higher temperature, whether at 30/25°C or 30/18°C and embryo induction was greater at these split temperature regimes than at either of the constant temperatures. Chinese Spring, however, responded under all incubation conditions, though somewhat poorly at 18°C. Analysis showed that both green and albino plant regeneration was not affected by the temperature at which cultures were incubated (Table 6.4). Nevertheless, the highest proportions of green plants were obtained from the cultures incubated at 30/25°C (Table 6.13).

Sugar

Analysis showed that embryo production between the sugars was different, and that different genotypes showed variable responses to the sugars (Table 6.2). The interaction between genotype and sugar was highly significant (Table 6.3). Embryo yields were highest from cultures containing sucrose followed by those containing cellobiose, maltose and gentiobiose (Table 6.14). The number of genotypes responding, however, was greatest on cellobiose, followed by sucrose, maltose and gentiobiose. Analysis showed that the regeneration of green plants but not albino plants was affected by sugar (Table 6.4) where cellobiose generally gave high proportions of green plants regenerated than albino plants (Table 6.15).

While embryos were induced from anthers cultured on maltose, with the cvs Aroona and Chinese Spring responding only to maltose, plant regeneration was poor (Table 6.15) with only Chinese Spring forming green plants. Embryo production and plant regeneration from gentiobiose cultures were restricted to cv. Oxley.

Table 6.12 The mean number of embryos produced per 100 plated anthers ± se for each of the responding genotypes, for each temperature regime in which cultures were incubated.

Genotype	Incubation temperature regime (°C)			
	30/25	30/18	25	18
Aroona	0.09 ± 0.09	0	0	0
Warigal	2.83 ± 1.14	0	0	0
Schomburgk	0.45 ± 0.19	0	0	0
Oxley	0.19 ± 0.19	0	0	1.07 ± 0.96
Kite	0.23 ± 0.12	0	0	0
Machete	0.20 ± 0.16	0	0.29 ± 0.29	0
WW15		2.86 ± 2.86		
Egret		0.41 ± 0.29		
Federation		0.29 ± 0.29		
Chinese Spring	0.31 ± 0.31	0.93 ± 0.93	0.44 ± 0.44	0.02 ± 0.02
Orofen	6.49 ± 2.89	0	0	0
anther Orofen	5.56 ± 5.56	0	0	0
Atys	0.37 ± 0.16	0	0	0

Table 6.13 The proportion of green plants (g) and albino plants (a) regenerated ± se from the total number of embryos produced for each of the responding genotypes, for each temperature regime in which cultures were incubated.

Genotype	Incubation temperature regime (°C)							
	30/25		30/18		25		18	
	g	a	g	a	g	a	g	a
Aroona	0	0						
Warigal	0.16 ± 0.11	0.42 ± 0.13						
Schomburgk	0.38 ± 0.20	0.04 ± 0.04						
Oxley	1.00	0					0.02 ± 0.02	0.12 ± 0.11
Kite	0.13 ± 0.08	0.13 ± 0.13						
Machete	0.25 ± 0.25	0.63 ± 0.38						
WW15			0	0.50				
Egret			0	0				
Federation			1.00	0				
Chinese Spring	0	1.00	0.50	0	0.50	0	0	0
Orofen	0.03 ± 0.03	0.06 ± 0.06						
anther Orofen	0.08	0.17						
Atys	0	0						

The difference in response shown by Australian and non-Australian genotypes to sugar was marked. Australian genotypes produced more embryos on the cellobiose medium than on maltose or sucrose, whereas non-Australian genotypes performed best on the sucrose medium (Table 6.14). Only three of the Australian cultivars produced embryos on sucrose with the best responses from Warigal and Egret. Warigal performed well regardless of whether the sugar was maltose, cellobiose or sucrose. Furthermore, plant regeneration, particularly of green plants, of Australian genotypes was marked compared to the non-Australian material (Table 6.15) with high proportions from Schomburgk and Oxley on cellobiose and Federation on sucrose.

Other interactions

Seven of the ten pair-wise combinations of treatments produced significant interactions for embryo production for group 1 genotypes (Table 6.3) but none did for the regeneration of green and albino plants. The duration of cold pretreatment interacted with sugar (Tables 6.16, 6.17), microspore stage (Tables 6.18, 6.19), donor plant environment (Tables 6.20, 6.21) and incubation temperature (Tables 6.22, 6.23). Microspore stage interacted significantly with sugar (Tables 6.24, 6.25), donor plant environment (Tables 6.26, 6.27) and incubation temperature (Tables 6.28, 6.29).

The optimum duration of cold pretreatment for maltose and cellobiose was 6 days, while for sucrose it was 4 days and for gentiobiose, 9 days (Table 6.16). The best regeneration rate for green plants was obtained on cellobiose after 3 days cold pretreatment (Table 6.17) whereas the highest percentage of green plants produced from the anthers plated was 0.33 embryos/100 anthers on maltose with 7 days pretreatment (Appendix 5).

Embryos were produced from anthers containing microspores at stages 5-10 after 3-10 days of cold pretreatment (Table 6.18). The high value for 9 days cold pretreatment at stage 10 was due to cultivar Oxley but the interaction remained significant when this cultivar was omitted. Other particularly high values were found for stage 8 at 4 days, 9 at 6 days and 10 at 8 days.

Table 6.14 The mean number of embryos produced per 100 plated anthers ± se, for each of the responding genotypes, on each sugar.

Genotype	Sugar			
	maltose	cellobiose	sucrose	gentiobiose
Aroona	0.14 ± 0.14	0	0	0
Warigal	1.05 ± 0.70	1.52 ± 1.18	5.02 ± 3.12	0
Schomburgk	0.10 ± 0.10	1.23 ± 0.54	0	0
Oxley	0	0.55 ± 0.39	0	2.22 ± 2.04
Kite	0.15 ± 0.11	0.37 ± 0.25	0	0
Machete	0.07 ± 0.06	0.52 ± 0.43	0	0
WW15	0	0.20 ± 0.20	0	0
Egret	0	0.57 ± 0.57	0.42 ± 0.42	0
Federation	0	0	0.09 ± 0.09	0
Chinese Spring	0.46 ± 0.23	0	0	0
Orofen	0	2.11 ± 2.11	7.48 ± 3.71	0
anther Orofen	0	0	8.89 ± 8.89	0
Atys	0	0.13 ± 0.13	0.44 ± 0.23	0

Table 6.15 The proportion of green plants (g) and albino plants (a) regenerated ± se from the total number of embryos produced for each of the responding genotypes, for each sugar.

Genotype	Sugar							
	maltose		cellobiose		sucrose		gentiobiose	
	g	a	g	a	g	a	g	a
Aroona	0	0						
Warigal	0	0.67 ± 0.17	0.38 ± 0.31	0.43 ± 0.30	0.09 ± 0.06	0.14 ± 0.14		
Schomburgk	0	0	0.45 ± 0.23	0.05 ± 0.05				
Oxley			0.50 ± 0.50	0			0.03 ± 0.03	0.17 ± 0.17
Kite	0	0	0.25 ± 0.25	0.25 ± 0.25				
Machete	0	0	0.25 ± 0.25	0.63 ± 0.38				
WW15			0	0.50				
Egret			0	0	0	0		
Federation					1.00	0		
Chinese Spring	0.25 ± 0.14	0.25 ± 0.25						
Orofen			0	0	0.03 ± 0.03	0.07 ± 0.07		
anther Orofen					0.08	0.17		
Atys			0	0	0	0		

Table 6.16 The mean number of embryos produced per 100 plated anthers ± se, for each length of cold pretreatment on each sugar, independent of genotype.

Cold pretreatment length (days)	Sugar			
	maltose	cellobiose	sucrose	gentiobiose
3	0.11 ± 0.05	0.56 ± 0.19	0.23 ± 0.23	
4	0.09 ± 0.09	0.61 ± 0.39	1.92 ± 1.35	
5	0			
6	2.59 ± 1.80	0.93 ± 0.93		
7	1.32 ± 0.93	0	0	0
8	0	0.31 ± 0.31	0.99 ± 0.47	0
9	0	0.22 ± 0.22	0.18 ± 0.18	1.33 ± 1.22
10	0	0	0	0
11	0	0	0	0
12			0	0
13				0
14				0
36				0

Table 6.17 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each length of cold pretreatment on each sugar, independent of genotype.

Cold pretreatment length (days)	Sugar							
	maltose		cellobiose		sucrose		gentiobiose	
	g	a	g	a	g	a	g	a
3	0.07 ± 0.07	0.29 ± 0.18	0.43 ± 0.14	0.20 ± 0.10	0	0		
4	0	0	0.33 ± 0.33	0	0.06 ± 0.06	0.11 ± 0.11		
6	0	0.51 ± 0.06	0	1.00				
7	0.25 ± 0.25	0						
8			0.14	0.29	0.04 ± 0.03	0.07 ± 0.05		
9			0	0	0	0	0.03 ± 0.03	0.17 ± 0.17

Table 6.18 The mean number of embryos produced per 100 plated anthers ± se, for each length of cold pretreatment for each microspore stage, independent of genotype.

Length of cold pretreatment (days)	Microspore stage										
	1	2	3	4	5	6	7	8	9	10	12
3	0	0	0	0	0.15 ± 0.08	0.11 ± 0.11	0.49 ± 0.24	0.20 ± 0.12	0.26 ± 0.12		
4				0	0	0.10 ± 0.10	0.31 ± 0.31	3.31 ± 1.94	0.17 ± 0.17		
5		0			0	0	0	0	0		
6	0		0	0	0		0		7.47 ± 5.77	4.27 ± 4.27	
7						0	0	0	1.98 ± 1.32		
8	0	0	0	0	0	0.42 ± 0.31	0.62 ± 0.44	0.20 ± 0.12	0	6.41 ± 6.41	0
9	0		0	0	0.11 ± 0.11	0	0	0	0	50.40 ± 14.43	0
10					0	0	0	0	0	0	
11					0	0	0	0	0		
12					0	0	0				
13						0					
14					0	0					
35						0					

Table 6.19 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each length of cold pretreatment for each microspore stage, independent of genotype.

Length of cold pretreatment (days)	Microspore stage											
	5		6		7		8		9		10	
	g	a	g	a	g	a	g	a	g	a	g	a
3	0.38 ± 0.24	0	0	1.00	0.15 ± 0.10	1.00 ± 0.06	0.33 ± 0.33	0.33 ± 0.33	0.30 ± 0.20	0.30 ± 0.20		
4			0	0	1.00	0	0.04 ± 0.04	0.08 ± 0.08	0	0		
6									0.25 ± 0.25	0.72 ± 0.07	0	0.57
7									0	0		
8			0.11 ± 0.04	0.36 ± 0.07	0.09 ± 0.06	0.06 ± 0.06	0	0			0	0
9		0.11									0.03 ± 0.03	0.17 ± 0.17

All embryos produced from microspores at stage 7 and after 4 days cold pretreatment differentiated into green plants (Table 6.19). Good green plant regeneration was also obtained after 3 days pretreatment using stages 5, 8 and 9. The highest percentage of green plants was produced from stage 10 and 9 days (Appendix 5).

The three highest mean embryo productions were 5.71 from a 9-day length of cold pretreatment and an April sowing (= June/July flowering) (winter), 3.91 from a 6-day and June sowing and 3.76 from a 4-day and November (summer). These figures suggest a trend towards a shorter pretreatment length from donor plants grown during warmer months and a longer pretreatment for those from cooler months (Table 6.20). The best proportions of green plants regenerated from the embryos produced were from the cold pretreatment of 4 days and the November sowing (50%), and 7 days and August (25%). Both these occurrences coincided with no regeneration of albino plants (Table 6.21). The highest percentage of green plants was produced from the glasshouse in winter with 7 days pretreatment (Appendix 5).

Cultures given a preliminary incubation for 2-3 days at 30°C (30/18, 30/25) after between 4 and 8 days cold pretreatment produced the highest embryo means/100 anthers (Table 6.22). The highest embryo yield at 30/25°C with 6 days pretreatment (Table 6.22) was not carried through as green plant yield with only albinos regenerated (Table 6.23). Analysis showed that the duration of cold pretreatment affected both green and albino plant regeneration (Table 6.4) with best regeneration of green plants obtained at 30/18°C and constant 25°C (Table 6.23).

Embryo production occurred from anthers containing microspores from all stages between 5-10 across the range of sugars tested (Table 6.24). The high mean embryo number obtained with gentiobiose did not affect the significance of the interaction. Cellobiose produced roughly equal numbers from stages 6-9 and sucrose from stages 6-8. The clear optimum for maltose and sucrose, however, was stage 10.

Table 6.20 The mean number of embryos produced per 100 plated anthers ± se, for each season and donor plant environment, for each cold pretreatment length, independent of genotype.

Season	Donor plant environment and sowing date	Length of cold pretreatment (days)												
		3	4	5	6	7	8	9	10	11	12	13	14	35
	Growthroom													
Winter	Jul 28	0	0	0										
	Aug 8	0.31 ± 0.31	0	0	0		0	0						
	Aug 23								0					
Summer	Jan 16						0.87 ± 0.87	0	0	0				
	Nov 16	0	0.21 ± 0.16				0.74 ± 0.74	0	0					
	Glasshouse													
Winter	Apr 1						0	5.71 ± 5.07	0	0	0			0
	Apr 16						0	0						
	Jun 18						0	0		0	0			
	Jun 21	0.42 ± 0.12	0.31 ± 0.31	0	3.91 ± 2.35									
	Jul 12							0						
	Jul 28	0.06 ± 0.06	0.48 ± 0.48	0		0								
	Aug 8	0	0			1.98 ± 1.32	0	0	0					
	Aug 17						0	0	0	0		0	0	
	Aug 23						0		0					
	Sep 13						0							
Summer	Oct 8						0	0	0	0	0			
	Nov 2						0	0	0	0	0			
	Nov 14							0						
	Nov 16	1.03 ± 0.65	3.76 ± 2.29				2.45 ± 1.42	0.95 ± 0.95						
	Dec 10						0	0						
	Jan 14						0	0		0				
	Jan 16						0.92 ± 0.92	0		0				

Table 6.21 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each season and donor plant environment, for each cold pretreatment length, independent of genotype.

Season	Donor plant environment and sowing date	Length of cold pretreatment (days)											
		3		4		6		7		8		9	
		g	a	g	a	g	a	g	a	g	a	g	a
	Growthroom												
Winter	Aug 8	0	0										
Summer	Jan 16									0.20	0		
	Nov 16			0.50 ± 0.50	0					0.08	0.17		
	Glasshouse												
Winter	Apr 1											0.02 ± 0.02	0.11 ± 0.11
	Jun 21	0.34 ± 0.11	0.21 ± 0.10	0	0	0	0.67 ± 0.17						
	Jul 28	0	1.00	0	0								
	Aug 8							0.25 ± 0.25	0				
Summer	Nov 16	0	0	0.06 ± 0.06	0.11 ± 0.11					0.04 ± 0.02	0.12 ± 0.08	0	0
	Jan 16									0	0		

Table 6.22 The mean number of embryos produced per 100 plated anthers ± se, for each length of cold pretreatment for each incubation temperature, independent of genotype.

Length of cold pretreatment (days)	Culture incubation temperature (°C)			
	30/25	30/18	25	18
3	0.30 ± 0.08	0	0.06 ± 0.06	0
4	1.01 ± 0.55	0	0.41 ± 0.41	0
5	0		0	
6	2.76 ± 1.70		0	
7		1.85 ± 1.85		0.26 ± 0.26
8	1.27 ± 0.54	0		0
9	0.19 ± 0.19	0		0.95 ± 0.85
10	0	0		0
11	0	0		0
12				0
13				0
14				0
35				0

Table 6.23 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each length of cold pretreatment for each incubation temperature, independent of genotype.

Length of cold pretreatment (days)	Culture incubation temperature (°C)							
	30/25		30/18		25		18	
	g	a	g	a	g	a	g	a
3	0.25 ± 0.10	0.24 ± 0.09			0.50	0		
4	0.19 ± 0.16	0.06 ± 0.06			0	0		
6	0	0.67 ± 0.17						
7			0.50	0			0	0
8	0.06 ± 0.02	0.10 ± 0.05						
9	0	0					0.02 ± 0.02	0.11 ± 0.11

The highest proportion of green plants regenerated from the embryos produced was on cellobiose at stage 5 and although sugar was not found to affect plant regeneration (Table 6.4), regeneration of green plants was best on cellobiose (Table 6.25). Plants were only regenerated from stage 9 on maltose, stage 10 on gentiobiose but between stages 6 and 8 on sucrose. The highest percentage of green plants/100 anthers was produced from stage 10 on gentiobiose (Appendix 5) and this was using cultivar Oxley, from the glasshouse in winter, after 9 days cold pretreatment and incubation at 18°C.

Regardless of sowing time, microspore stages 1-4 failed to generate any embryos. The optimum microspore stage varied across donor plant environments, with high values obtained from stage 10 in 3 sowings but from stages 6, 7 and 8 in one each (Table 6.26). This interaction remained significant following the removal of Oxley (stage 10) from the data for analysis, though significance was reduced to $P<0.01$. The highest proportion of green plants regenerated from embryos was from microspore stage 7, from the growthroom in summer (November) (Table 6.27). Analysis showed that both green and albino plant regeneration was not affected by the donor plant environment but that the regeneration of albino plants was affected by microspore stage (Table 6.4). Nevertheless, all embryos produced across microspore stages from donor plants sown in the glasshouse in June (winter) regenerated plants and the proportions of green plants regenerated from microspore stage 7 were better than those for albino plants (Table 6.27).

Both spike pretreatment and culture incubation affected the regeneration of albino plants from embryos and incubation also affected green plant regeneration (Table 6.4). A temperature shock of cultures at 30°C before maintenance at 25°C (30/25) produced embryos from the widest microspore range, stages 5-10 (Table 6.28). The two highest means were both obtained with microspore stage 10. This temperature regime supported the greatest amount of plant regeneration and no albino plants were regenerated at 30/18°C and 25°C.

Table 6.24 The mean number of embryos produced per 100 plated anthers ± se, for each microspore stage on each sugar, independent of genotype.

Microspore stage	Sugar			
	maltose	cellobiose	sucrose	gentiobiose
1-4	0	0	0	0
5	0.07 ± 0.05	0.23 ± 0.13	0	0
6	0.05 ± 0.05	0.46 ± 0.46	0.76 ± 0.73	0
7	0.10 ± 0.10	0.53 ± 0.28	1.12 ± 0.77	0
8	0.11 ± 0.08	0.44 ± 0.28	1.60 ± 0.90	0
9	0.55 ± 0.35	0.50 ± 0.22	0	0
10	8.54 ± 8.54	0	5.21 ± 4.80	50.00 ± 41.43
12		0	0	0

Table 6.25 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each microspore stage on each sugar, independent of genotype.

Microspore stage	Sugar							
	maltose		cellobiose		sucrose		gentiobiose	
	g	a	g	a	g	a	g	a
5	0	0	0.50 ± 0.29	0				
6	0	1.00	0.15	0.29	0.04 ± 0.04	0.21 ± 0.21		
7	0	0	0.440.21 ±	0.130.07 ±	0.07 ± 0.05	0.04 ± 0.04		
8	0	0	0.33 ± 0.33	0.33 ± 0.33	0.03 ± 0.03	0.07 ± 0.07		
9	0.20 ± 0.12	0.29 ± 0.20	0.20 ± 0.20	0.30 ± 0.20				
10	0	0.57			0	0	0.03 ± 0.03	0.17 ± 0.17

Table 6.26 The mean number of embryos produced per 100 plated anthers ± se, for each donor plant environment for each microspore stage, independent of genotype.

Season	Donor plant environment and sowing date	Microspore stage										
		1	2	3	4	5	6	7	8	9	10	12
	Growthroom											
Winter	Jul 28	0	0	0		0	0	0	0	0		
	Aug 8	0		0	0	0	0	0.98 ± 0.98	0	0		
	Aug 23					0	0					
Summer	Jan 16				0	0	0	0.92 ± 0.92	0	0	19.23	
	Nov 16			0		0	0.08 ± 0.08	1.67 ± 1.49	0	0	0	0
	Glasshouse											
Winter	Apr 1	0		0		0.16 ± 0.16	0	0	0	0	50.00 ± 41.43	0
	Apr 16		0		0	0	0	0	0			
	Jun 18	0	0			0	0		0			
	Jun 21		0		0	0.37 ± 0.19	0	0.51 ± 0.34	0.44 ± 0.26	1.01 ± 0.50	4.27 ± 4.27	
	Jul 12					0						
	Jul 28		0	0	0	0	0.36 ± 0.36	0	0.14 ± 0.14	0		
	Aug 8				0	0	0	0	0	0.57 ± 0.42		
	Aug 17				0	0	0	0	0	0		
	Aug 23					0	4.15		0			
	Sep 13						0	0			0	
Summer	Oct 8			0		0	0	0	0	0	0	
	Nov 2	0	0	0	0	0	0	0	0			
	Nov 14										0	
	Nov 16				0	0	4.00 ± 3.00	0.82 ± 0.43	5.56 ± 2.54	0	1.19 ± 1.19	
	Dec 10	0				0	0	0	0	0	0	
	Jan 14	0				0	0	0	0			
	Jan 16		0			0	0	0	0			

Table 6.27 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each donor plant environment for each microspore stage, independent of genotype.

Season	Donor plant environment and sowing date	Microspore stage											
		5		6		7		8		9		10	
		g	a	g	a	g	a	g	a	g	a	g	a
	Growthroom												
Winter	Aug 8					0	0						
Summer	Jan 16					0.20	0					0	0
	Nov 16			0	0	0.54 ± 0.46	0.08 ± 0.08						
	Glasshouse												
Winter	Apr 1	0	0									0.03 ± 0.03	0.17 ± 0.17
	Jun 21	0.38 ± 0.24	0			0.38 ± 0.13	0.25 ± 0.00	0.33 ± 0.33	0.33 ± 0.33	0.19 ± 0.13	0.37 ± 0.16	0	0.57
	Jul 28			0	1.00			0	0				
	Aug 8									0.25 ± 0.25	0		
	Aug 23			0	0								
Summer	Nov 16			0.11 ± 0.04	0.36 ± 0.07	0	0	0.03 ± 0.03	0.06 ± 0.06			0	0

Table 6.28 The mean number of embryos produced per 100 plated anthers ± se, for each microspore stage at each incubation temperature, independent of genotype.

Microspore stage	Culture incubation temperature (°C)			
	30/25	30/18	25	18
1-4	0	0	0	0
5	0.14 ± 0.07	0	0	0.02 ± 0.02
6	0.90 ± 0.61	0	0	0
7	0.90 ± 0.42	0	0	0
8	0.95 ± 0.41	0	0.15 ± 0.15	0
9	0.52 ± 0.28	0.69 ± 0.69	0.28 ± 0.28	0.09 ± 0.09
10	3.85 ± 3.85	0	0	2.27 ± 2.08
12	0	0	0	0

Table 6.29 The proportion of green (g) and albino (a) plants regenerated/embryos ± se for each microspore stage at each incubation temperature, independent of genotype.

Microspore stage	Culture incubation temperature (°C)							
	30/25		30/18		25		18	
	g	a	g	a	g	a	g	a
5	0.38 ± 0.24	0					0	0
6	0.05 ± 0.03	0.43 ± 0.21						
7	0.23 ± 0.11	0.07 ± 0.04						
8	0.13 ± 0.11	0.15 ± 0.11			0	0		
9	0.14 ± 0.14	0.42 ± 0.17	0.50	0	0.50	0	0	0
10	0	0.29 ± 0.29					0.03	0.17

F_1 hybrids

Although the wheats Schomburgk and Machete responded on WM2 with maltose (Table 6.14) there was no embryo production from any of the F_1 hybrids or other parents (Table 6.30). The parent NapHal was unavailable for culture.

Table 6.30 The response to culture of other wheat cultivars and their F_1 hybrids.

Genotype	Total number of anthers plated	Embryos
Frocor	242	0
Frocor x Schomburgk	477	0
Frocor x Sunfield	200	0
Najah	67	0
Najah x Sunco	234	0
Najah x Sunfield	190	0
NapHal x Hartog	411	0
NapHal x Machete	279	0
Sunco	308	0
Sunfield	280	0

Discussion

Anther culture response in wheat was found to be affected by the genotype, health and maturity of the microspores as well as the cold and warm temperature shocks early in culture and the sugar in the medium on which anthers were plated. The generation of embryos from anthers and the regeneration of green and albino plants from those embryos were all affected by these six factors and furthermore, many of the interactions between factors were also significant. These components of response will be discussed below.

Genotype

The results demonstrate that genotype had a pivotal influence on anther culture response, with many genotypes failing to produce embryos regardless of culture conditions.

The responsive Australian wheats had a considerable number of parents in common. WW15 itself was responsive and four of the other responsive genotypes contain it in their published pedigrees (Table 6.5). A further responsive cultivar, Schomburgk, is the progeny of three responsive WW15 derivatives. WW15 in turn contains Lerma Rojo in its pedigree as does Sonora 64, a parent of Machete, another responsive cultivar. Lerma Rojo has Mentana, Kentana and Yaqui 48 in its pedigree and one or another of these also features in the pedigree of the responsive cultivar Orofen and Yaktana 54, another parent of Machete.

The responsiveness of Orofen confirms previous results with this cultivar in a variety of conditions (Shimada 1981; Ouyang *et al.* 1983, 1987; Datta & Wenzel 1987; Last & Brettell 1990; Ziegler *et al.* 1990).

The only common antecedent that Kite has with this group of responsive genotypes is Norin 10/Brevor 14, which could be related to anther culture performance. In reality, it would be difficult to verify the relationship because Norin 10/Brevor is so widely found in modern wheats since it is the source of the Rht_1 and Rht_2 (reduced height) dwarfing genes (Gale & King 1988). Perhaps Kite is a representative of a second type of responsive wheat. Results for the cultivar Kite are of interest in relation with other reports where Kite has been used (Last & Brettell 1990; Ling *et al.* 1991; Luckett *et al.* 1991) since these offer the only other information about the response of Australian cultivars to anther culture.

Kite failed to respond on sucrose, as was also found by Last and Brettell (1990) but it did respond on maltose, in contrast to those researchers' results despite the use of the same medium and similar growing conditions for the donor plants. Others have obtained a response from Kite to maltose but in a different medium and only after either a gamma irradiation (Ding *et al.* 1991) or with the use of membrane rafts (Luckett *et al.* 1991).

Three other responsive lines, however, showed no known common antecedents with this set or with each other, namely Federation, Chinese Spring and Atys.

Taken together, these results suggest the presence of a major gene affecting anther culture response in an antecedent of WW15 which is probably linked with an agronomically important factor for it to have been maintained in so many crosses. A similar gene is also clearly present in some other backgrounds. The gene may be lost in breeding, as shown by the non-responsiveness of Yaktana 54-derived lines such as Bindawarra, Hartog and Pavon-76. Responses differed among the responding lines, with some, for example Warigal, able to produce embryos under a range of conditions but others, for example the sister line of Warigal, Aroona, requiring quite specific culture conditions. Such variation suggests the presence of minor modifying genes in addition to a major gene. As some embryogenic lines were apparently unable to regenerate plants, the ability to regenerate may be independent of the ability to form embryos confirming previous reports (Agache *et al.* 1989; Tuvesson *et al.* 1989; Ekiz & Konzak 1991c).

Similarly, many of the non-responsive cultivars had Insignia either in their own pedigree or in the pedigree of one of their parents. This may indicate a suppressive factor associated with Insignia, or merely the absence of the promotive factor. Egret was the poorest of the responsive genotypes contrary to a recent report (Luckett *et al.* 1991) and included Heron, an Insignia progeny, in its pedigree. Given that Egret is also a WW15 derivative, this suggests the presence of a weak suppressor rather than a null factor in this background.

The lack of response shown by Pavon-76 and its sister line Hartog was notable particularly as this genotype has been responsive in a number of major anther culture studies (Zhou & Konzak 1989; Yuan *et al.* 1990; Ekiz & Konzak 1991c; Zhou *et al.* 1991; Ball *et al.* 1992). All of these studies except that by Yuan *et al.* (1990) were conducted by the same group of researchers and thus presumably with a consistent protocol and seed stock. Differences in anther culture response for this genotype could be partly attributable to the use of different seed stocks, the use of a cold treatment, the use of different liquid media (WM2 and Potato) or the inevitable variation in culture protocols. These results and those with Kite, support the observation that replication of results in anther culture is inconsistent between research laboratories even when apparently the same material and protocol are involved.

These results have allowed the development of a testable hypothesis on the genetic basis of anther culture response. The simplest model is that there is a major gene promoting embryo development which is present in the WW15 family, with several minor modifying elements possible. The same gene may be present in the other responsive genotypes or it may be another gene. There is evidence for a suppressor of embryo response in the Insignia family and a promoter of green plant regeneration in the WW15 family. Parts of this model are in agreement with previous reports showing independent inheritance and polygenic control for embryo production and plant regeneration (Deaton *et al.* 1987; Lazar *et al.* 1987; Szakács *et al.* 1988; Knudsen *et al.* 1989).

To test these hypotheses, a number of crosses have been made between responsive WW15 relatives (Warigal x Aroona, Warigal x Schomburgk, Warigal x Orofen, Aroona x Schomburgk) and unrelated cultivars (Atys x Machete) as well as between responsive and unresponsive cultivars (Orofen x Hartog). F_1 hybrid seed has been produced but has not been grown for culturing. Hybrid vigour in anther culture (Picard & de Buyser 1977; Bullock *et al.* 1982; Lazar *et al.* 1984a, b) may make these results hard to interpret genetically so F_2 generation plants should be screened as well. Nevertheless, there was no evidence of heterosis promoting anther culture in the range of hybrids already evaluated in this chapter.

The widespread use of WW15 in Australian wheat breeding programmes and its evident positive effect on anther culture response make it reasonable to propose a culture protocol suitable to a wide range of breeding material. The following sections will discuss the components of this protocol.

Microspore stage

Flowering development in a spike of wheat is asynchronous, with distal and proximal spikelets behind central ones and distal florets behind proximal ones within the same spikelet. Thus, the evaluation of microspore staging used here is a guide-line rather than critical. This variability may account for the development of embryos from spikes containing apparently mature pollen, which conflicts with the established concept presented in the literature review (Chapter 2) that, to be responsive, pollen must be between tetrad stage and the final mitosis. As expected, however, microspores younger than uninucleate failed to respond.

A broad range of suitable microspore stages was found for most genotypes, covering most of the uninucleate stages. Therefore, to streamline anther culture for the broadest applicability, the most important information required about the microspores is whether or not they are uninucleate. The most appropriate stage within this range can then be chosen to optimize response for a particular genotype.

In several genotypes, higher yields of embryos were obtained from later microspore stages whereas higher proportions of embryos regenerated into green plants from earlier stages, as has been reported before (Sunderland & Huang 1985; Huang 1987). This result provides further circumstantial evidence in favour of the hypothesis of independent genetic control of embryo generation and plant regeneration.

No broad conclusions can be drawn from the interactions of microspore stage with donor plant environment, cold pretreatment length or incubation temperature as there were no consistent trends in the data. Thus it can not be determined whether, for example, more advanced microspores required a longer cold pretreatment to alter their development.

Donor plant environment

Maximum embryo production occurred when anthers were taken from healthy, vigorous plants, which showed the importance of a suitable environment for the growth of the donor plants. The importance of the physiological condition and growth environment of the donor plants and seasonality to the realization of the genotypic potential for anther culture has been well recognized (Sunderland 1971, 1978; Picard & de Buyser 1973; Dunwell 1976; Foroughi-Wehr *et al.* 1976, 1982; Keller & Stringam 1978; Heberle-Bors & Reinert 1979, 1981; Wang & Chen 1980; Lazar *et al.* 1984a; Heberle-Bors 1985; Luckett & Smithard 1992). As shown by the significant interaction between genotype and donor plant environment, the optimum conditions varied among cultivars.

The controlled-environment growthroom was seldom an optimum environment, which may be attributed to the low light levels in comparison to those of the glasshouse. Improved embryo yields for several cereals have been associated with higher light levels (Lazar *et al.* 1985). The uniformity of conditions within the growthroom implies that the seasonal variation in response within genotypes may be attributable either to other experimental differences such as length of cold pretreatment or to random variation.

Light and temperature levels were naturally more variable through the year in the glasshouse than in the growthroom. Despite the greater environmental control provided by growthrooms, superior anther cultures are commonly obtained from plants grown in glasshouses (Bjørnstad *et al.* 1989) or the field (Ouyang *et al.* 1983, 1987; Lazar *et al.* 1984a; Andersen *et al.* 1987). Most Australian genotypes produced more embryos when grown in 'winter' conditions, providing similar conditions to their normal growing season, than in 'summer' conditions.

Cold pretreatment of spikes

Cold pretreatment of spikes was used following widespread reports on its effectiveness in promoting wheat anther culture. Responses have been reported from pretreatments of 3 days (Pan *et al.* 1975; Xu 1985) to 8 days (Picard & de Buyser 1977; Shimada 1981; Datta & Wenzel 1987) or 14 days (Lazar *et al.* 1985). Greater durations were not effective in wheat, confirming a previous report (Li et al. 1988) although they are in barley (Huang & Sunderland 1982; Hunter 1985; Powell 1988).

Other authors, however, have reported success without cold pretreatment (Liang *et al.* 1982; Marsolais *et al.* 1984; Hu 1986a; Müller *et al.* 1989). Vernalization promotes a number of cold-response genes, some of which may also be involved in the effect of cold pretreatments of winter wheat (Müller *et al.* 1989). Spring wheat, in contrast, receives no vernalization so any cold-response genes would still need triggering. Nevertheless, recent reports (Venkatanagappa & Darvey, unpublished) showed that several Australian cvs, including Grebe, have no cold pretreatment requirements. A further advantage of cold pretreatment is that it allows non-detrimental storage of spikes between harvesting and culture.

The effects of cold pretreatment may be expressed later rather than earlier. Plant regeneration, rather than embryo formation, was promoted by cold pretreatment, which is consistent with results in rye (Flehinghaus *et al.* 1991). Nevertheless, longer pretreatments were in fact associated with reduced green plant formation.

A mechanism for the effect of cold pretreatment has been proposed (Wilson *et al.* 1978) in which the cold causes nucleo-cytoplasmic disturbances which in turn stimulate abnormal mitoses of the microspore nuclei. Osmotic shock with mannitol (Roberts-Oehlschlager & Dunwell 1990; Ziauddin *et al.* 1990) and gamma-irradiation (Ling *et al.* 1991) are other pretreatments which may have similar mechanisms.

Culture incubation temperature

The stimulation of embryogenesis in most genotypes by an initial interval at a higher temperature (30°C) confirms previous reports for wheat (Ouyang *et al.* 1983, 1987; Huang 1987; Li *et al.* 1988; Simmonds 1989; McGregor & McHughen 1990). Some researchers, in contrast, report good performance without a heat shock (McGregor & McHughen 1990; Orshinsky *et al.* 1990) and this was confirmed by the results for cv. Oxley.

The choice of maintenance temperature appeared relatively unimportant if a prior heat shock had been given. However, maintenance of cultures at the higher temperatures may enhance response (Orshinsky *et al.* 1990), so it might be worthwhile testing higher maintenance temperatures. The interactions of culture temperature with cold pretreatment and microspore stage revealed no trends towards, for example, longer pretreatments or older microspores being more or less sensitive to heat shock or incubation temperature. Therefore, no information on the mode of action of the heat shock can be derived from them. Despite the widespread use of heat shock to commence the incubation of anther cultures, its mode of action remains obscure.

A lower temperature (18°C) was used for incubation of regenerant cultures as it was comparable to winter-spring glasshouse temperatures. Thus it was expected that this temperature would facilitate acclimation and growth of the regenerated plants. Therefore, a culture protocol was adopted which incorporated a high cultural shock for embryo induction followed by cool temperatures for regeneration.

Sugar

The choice of sugar for the anther culture medium was very important to the successful promotion of embryo formation, as different genotypes responded best on different sugars. The causes of these varying responses are difficult to identify, as the sugar has two well defined roles in the medium - as a nutrient source and as an osmoticum. It can also be the source of toxic by-products. Furthermore, plant regeneration is determined more by the sugar in the induction medium than by the sugar in the regeneration medium, as shown both in the current results and by Chu *et al.* (1990).

Sucrose, the major sugar translocated in plants, has been the major sugar used in anther culture media and it was the most effective sugar for the non-Australian genotypes in these experiments. Recent reports have shown that it is not always the best sugar for some wheat genotypes (Chu *et al.* 1990; Last & Brettell 1990; Roberts-Oehlschlager *et al.* 1990), possibly because of a toxic effect of fructose released from sucrose hydrolysis (Raquin 1983; Last & Brettell 1990). Fructose has, however, supported good embryo production in some wheats (Chu *et al.* 1990) and barley (Finnie *et al.* 1989), so its toxicity is not universal.

The reduced number of Australian genotypes found to respond on sucrose could be related to genotypes of this group possessing a sensitivity to fructose. This inhibition of androgenesis can be reduced if culture pH is increased prior to autoclaving (Owen *et al.* 1991). However, the WM2 medium used was filter sterilized (Chapter 4) to prevent degrading of the sugar as a consequence of autoclaving (de Lange 1989; Hsiao & Bornman 1989). The discovery of this has recently been substantiated (Hsiao & Bornman 1991; Schenk *et al.* 1991). For these reasons, it is less likely that fructose toxicity due to medium preparation is the primary cause for the reduction in the number of Australian cultivars responding on sucrose. If fructose sensitivity is limiting the ability of certain genotypes to respond on sucrose, even though the medium has been filter sterilized, then the alternative is to find a culture sugar for these genotypes other than sucrose and not one containing fructose. The results show that cellobiose presents such an alternative.

Glucose has been shown to be an effective substitute for sucrose (Chu *et al.* 1990; Last & Brettell 1990) and various papers have identified various glucose disaccharides as suitable for anther culture (Hunter 1987; Sorvari & Schieder 1987; Orshinsky *et al.* 1990).

Maltose has been widely used for anther culture of several cereals including barley (Hunter 1987; Finnie *et al.* 1989) and rye (Flehinghaus *et al.* 1991) as well as wheat (Last & Brettell 1990; Orshinsky *et al.* 1990). The present results, however, showed that maltose was a poor sugar for the culture of most Australian genotypes and even those that responded on it showed poor regeneration of green plants.

Cellobiose was the sugar on which the greatest range of Australian genotypes responded. There are only two other reports where cellobiose has been used in anther culture. In these, it was shown that cellobiose promoted high embryo production in barley over sucrose and maltose (Hunter 1987) and improved the proportion of embryos which differentiated into green plants for wheat (Orshinsky *et al.* 1990). The lower use of cellobiose may be attributed to its expense, ten-fold that of maltose (Sigma). The use of gentiobiose was novel for wheat and was associated with excellent response in one genotype. These sugars clearly offer excellent potential in expanding the range of genotypes responsive to anther culture.

These experiments have provided a set of recommendable standard conditions for the anther culture of Australian wheats. In general, the donor plants should be vigorous and healthy and from adequately illuminated environments which most closely as possible resemble natural growing conditions. Detailed investigation of the microspore stage beyond simple determination of uninucleate status requires more time than is, in most cases, warranted. Spikes can be stored for a short period in cold conditions (up to 10 days) before plating the anthers which then should be given a short (2 days) incubation at 30°C. The subsequent maintenance temperature of the cultures is of little importance. The culture medium itself should be based on cellobiose for most Australian genotypes.

While the frequency of embryo production and yield of green plants is still too low to be used in breeding programmes, adoption of this protocol should assure the detection of response from the widest possible range of Australian genotypes. Where attention is to be focused on a specific genotype, some variation in sugar or length of cold pretreatment may be warranted.

Chapter 7 Anther culture of wheat-barley disomic chromosome addition lines

Introduction

Substantial improvements in plant yields have been attained in anther culture of barley, compared to wheat (Table 1.1). The improvements are the result of recent protocol modifications combined with an apparently inherent receptiveness of barley anthers to culture. The comparatively recalcitrant nature of wheat has meant that most wheat anther culture procedures are based on protocols developed for barley.

The greater response of barley may be attributable to genes on a single chromosome. Therefore, it was hypothesized that a genetic line incorporating barley into the wheat genome would improve anther response to culture (in terms of embryogenesis or plant regeneration) above that of the euploid wheat parent. Wheat-barley disomic chromosome addition lines were available for such an investigation. The lines are composed of individual pairs of barley chromosomes, from cv. Betzes, added to the chromosome complement of euplasmic wheat cv. Chinese Spring. A response from any member of the series would implicate the action of an individual barley chromosome, thus giving insight into several aspects of the genetic and physiological control of anther culture response for barley.

Experiments were therefore designed to test the culturability of these lines. The design incorporated culture of all lines and parents using two protocols, one designed primarily for wheat and one for barley. The medium of the wheat protocol was a liquid medium using Ficoll as the support matrix (WM2, Appendix 2) whereas that in the barley protocol was agar-solidified (FHG, Appendix 2) as is standard practice for barley (Dr S Finnie pers.

comm.). In addition, the sugar in the induction medium was varied using maltose, cellobiose and melibiose.

Materials and Methods

Seeds of the wheat-barley disomic chromosome addition line series (dt-1HS, 2H, 3H, 4H, 5H, 6H, 7H) (*Triticum aestivum* cv. Chinese Spring background with *Hordeum vulgare* L. cv. Betzes chromosome additions) were obtained from Dr Rafiq Islam, Waite Agricultural Research Institute, South Australia. The addition line 1H can not be obtained as a self-fertile addition because the presence of this chromosome in a wheat background causes extreme meiotic abnormalities and male sterility of the plant. It has been established that the cytological abnormalities and sterility are due to gene(s) located on the long arm of this chromosome (1HL) and the ditelosomic addition 1HS is self-fertile. Nonetheless, donor plants of this line grew poorly and no anthers were able to be collected. Germination, planting and care are described in Chapter 4.

Experiment 1 Cellobiose and melibiose

Seeds of the addition lines and the wheat parent, cv. Chinese Spring, were germinated at room temperature. Standard root tip cytological techniques were used to check that seedlings had the correct disomic addition number of 44 ($2n=42+2$) before plants were put into pots in the glasshouse and cared for as described in Chapter 4.

Spikes were prepared for cold pretreatment as outlined in Chapter 4 and given 8 days pretreatment at 4°C. Anthers were cultured on liquid WM2 medium (modified from Datta & Wenzel (1987), see Appendix 2) where the sugar component had been altered by incorporating either cellobiose or melibiose at 6%. Culture was according to the protocol described in Chapter 4, where 35 anthers were placed on each plate, with anthers from one side of the spike assigned to a petri-dish of medium containing cellobiose and those from the other side to melibiose. Cultures were sealed and incubated in darkness at 30/18°C.

Experiment 2 Maltose and culture protocols

Germination of the addition lines plus the parental materials and root tip squashes were as described for experiment 1. The spikes of Chinese Spring and of the addition lines were collected as described for wheat (Chapter 4). Repeated poor growth of some of the addition line donor plants, particularly 1H and 6H, meant some lines were unavailable for culture. Spikes of the barley parent cultivar Betzes were collected when the distance of emergence of the flag leaf (interligule length) was between 35 and 60 mm, which correlated with anthers containing microspores around the early to mid uninucleate stage of development (Finnie *et al.* 1989). Preparation for cold pretreatment for the two species was the same. Tillers were trimmed and swabbed with ethanol. Spikes were removed aseptically from the sheath and placed in one half of a 90 mm split compartment petri-dish. In the other half was placed a small square of sterile absorbent cloth (Wettex) moistened with sterile water - this maintained humidity but prevented overflow of water into the compartment holding the spike. The spikes were divided into two groups containing similar sets of the controls and of each of the addition lines but exact replication was not possible due to the variation in maturity and availability of spikes for each addition line.

Both species were cultured under both protocols (Table 7.1).

Table 7.1 The anther culture protocols used, listing treatment differences in the experimentation.

Protocol designed for:	Pretreatment at 4°C (days)	Medium		Incubation temperature (°C)
wheat	8	liquid	WM2	30/18
barley	28	solid	FHG	25

The WM2 medium was modified from Datta and Wenzel (1987) (see Appendix 2). The barley medium was based on FHG medium (Kasha *et al.* 1990) with the addition of 1 mg/l of BAP and IAA and with agar replacing the Ficoll (see Appendix 2). Both media contained 6% w/v maltose. Fifteen anthers were plated per 35 mm petri-dish (plastic UV sterilized) where anthers from one side of the spike were assigned to a petri-dish containing FHG medium and those from the other side to WM2. Cultures were sealed and incubated in darkness at 30/18°C or 25°C.

Embryos from all cultures were transferred for regeneration on to a solid medium which was a further modified FHG induction medium with reductions in the concentrations of IAA and BAP (to 0.4 mg/l) and maltose (3%). Regeneration cultures were maintained in light at either 18°C or 25°C.

The count of embryos produced could not be compared between the two procedures because of differences in determining the number of embryos produced. For the liquid cultures, individual embryos are counted and given per plate (as it is not always possible to assign each embryo to its originating anther as the embryos tend to separate from the anthers and float freely on the medium) whereas for the solid cultures, embryogenic response is given as the number of responding anthers (discrete numbers of embryos can not be defined because these structures tend to grow *en masse* and stay attached to the anther rather than separate as individuals). Therefore, the number of regenerated plants were recorded together with whether they were green or albino plants.

Results

Experiment 1

The wheat parent Chinese Spring responded to culture on cellobiose with the formation of embryos. None of the wheat-barley addition lines responded on liquid medium containing either cellobiose or melibiose (Table 7.2). The 1HS addition and the barley parent Betzes were not available for this experiment.

Table 7.2 Anther response (embryo production) for each of the wheat-barley addition lines cultured on liquid medium (WM2) containing either cellobiose or melibiose, after 8 days cold pretreatment.

Genotype	Anthers cultured per sugar	Embryo production	
		cellobiose	melibiose
Chinese Spring	595	+	0
2H	260	0	0
3H	340	0	0
4H	682	0	0
5H	583	0	0
6H	525	0	0
7H	700	0	0

where: (+) = response (embryo production)

Experiment 2

Barley embryogenesis occurred in all treatments. Green plants were regenerated from liquid medium regardless of pretreatments, but some albino plants were also regenerated following 28 days cold pretreatment. Anthers cultured on solid medium regenerated both green and albino plants from 28 days cold pretreatment but neither type from 8 days.

Wheat did not respond to any treatment and 2H was the only addition line of those cultured to show a response (Table 7.3). Embryos and albino plants were obtained only on liquid medium containing maltose (wheat medium) and after 28 days cold pretreatment (barley protocol).

Table 7.3 Plants regenerated per 100 anthers for the wheat-barley chromosome addition lines and their controls after culture on liquid (WM2) and solid (FHG) media containing maltose and following either 8 or 28 days cold pretreatment.

Pretreatment	Genotype	Embryo response	Anthers cultured per medium	Plants regenerated/100 anthers			
				green		albino	
				WM2	FHG	WM2	FHG
8 days	Betzes	+	75	5.3	0.0	0.0	0.0
	Chinese Spring	0	90				
	2H	0	60				
	4H	0	345				
	5H	0	75				
	7H	0	30				
28 days	Betzes	+	165	7.3	5.5	11.5	12.1
	Chinese Spring	0	121				
	2H	+	45	0.0		2.2	
	3H	0	15				
	4H	0	105				
	5H	0	75				

Discussion

Both parent materials of these lines responded best to their own protocols and media (with the exception that barley in fact did better on the wheat medium with the barley protocol than on the barley medium). The addition lines generally seemed to be even more poorly responding than the Chinese Spring wheat background, so there was little indication of a promotive effect of any portion of the barley genome. The exception was line 2H, which produced a single albino plant on the liquid (wheat) medium with maltose (barley) and a long (barley) cold pretreatment. The lack of response by the addition lines in experiment 1 (Table

7.2) and experiment 2 (Table 7.3) was possibly due to the poorer growth of the donor plants of these lines which affected anther development and so the number of anthers obtained for culture (Table 7.3). However, since the response by the Chinese Spring parent was also limited, it is further possible that genetic factors may have contributed to the lack of response by the addition lines. These results will be discussed below.

There were several differences between the culture protocols, namely, the length given for cold pretreatment, the temperature at which cultures were incubated and most particularly, the culture media themselves. Barley responded better to the longer cold pretreatment (barley protocol) before plating whereas wheat responded to the shorter one, confirming previous results (i.e. < 10 days) (Chapter 6).

It is common practice to subject wheat cultures to a period at high temperatures (around 30°C) either continually or for a short period before maintenance at a lower temperature and this procedure was found effective in Chapter 6. In barley, on the other hand, anther cultures are generally kept at 25°C without a post-culture incubation temperature shock. The only addition line to respond (2H) did so after 28 days cold pretreatment and this was perhaps attributable to influence from the barley chromosome (Table 7.2). After 28 days cold pretreatment, Betzes responded on both media but after 8 days, it responded only on WM2. Albinos were produced only after the longer pretreatment and the ratio of green to albino regenerants was best after the shorter exposure; these results are consistent with other findings (Genovesi & Magill 1979).

Although liquid medium has been used for barley (Kao 1981, 1988), the standard media adopted for these investigations were solid for barley (Kasha *et al.* 1990) and liquid for wheat (Chapter 6). The liquid medium, in spite of many compositional differences, was more successful for barley than the solid medium. The results obtained on the liquid medium may in part be due to the use of Ficoll, which raised the buoyancy of the cultured anthers while the liquid nature of the medium allowed circulation which aids nutrient exchange, aeration and waste dispersal. The wheat Chinese Spring responded only on the liquid medium.

Since wheat-barley lines all failed to respond to a standard barley anther culture protocol (Finnie 1990) the present results showed that a wheat protocol was a more appropriate basis for evaluating such lines.

As discussed in Chapter 6, sugar has a major effect on culture response. Incorporation of maltose into media in experiment 2 was an adoption from barley protocol to test the hypothesis that maltose would effect a response from the barley component of the wheat-barley addition lines. Cellobiose was used as the standard sugar for the wheat protocol based on the results obtained on it with Australian cultivars (Chapter 6). The choice to use melibiose as another experimental sugar, as used in conjunction with the wheat-developed liquid medium (experiment 1), was in response to results which showed significant improvements in embryo and plant formation in barley using melibiose as the sugar (Sorvari & Schieder 1987). However, this medium involved the use of barley starch which can be hydrolysed by amylases in the microspores (Roberts-Oehlschlager *et al.* 1990) to produce maltose. The lack of response by any of the barley additions or Chinese Spring and other wheat cultivars (Last & Brettell 1990) on WM2 medium with melibiose suggest that the response (Sorvari & Schieder 1987) relied heavily upon the presence of the starch. Alternatively, barley may be better suited to metabolize both starch and maltose than wheat. This would strengthen the concept that improvements in anther response are obtained when the composition of the induction medium is tailored to suit the physiological requirements of the plant in use.

These experiments showed that sugar and length of pretreatment were major factors differentiating the responses of the two species. Line 2H produced embryos when given barley conditions for sugar and cold pretreatment while on a wheat medium. This result suggests that important factors associated with anther culture response are located on chromosome 2H. If the background wheat of the wheat-barley addition lines was more responsive to anther culture it would allow a more comprehensive evaluation of specific chromosomal effects.

The transfer of genetic material to hexaploid wheat from species with genomes other than A, B or D is a means by which desirable alien characters can be utilized. However, such transferral is complicated by the presence of the *Ph* pairing mechanism on chromosome 5B of wheat that prevents recombination between homoeologous chromosomes in hybrids (Sears 1981). Wild wheats have frequently been used as sources for disease resistance in wheat breeding. When such transfers have been made, however, it has often been found that linked, deleterious genes have been transferred as well (Knott 1989). Furthermore, this alien genetic material can cause a reduction in male transmission, thus upsetting expression of the characters under investigation (Kerber & Dyck 1990). It is possible that similar mechanisms from the present wheat are preventing the expression of the barley genes. It would be desirable to test the culture ability of these lines in different background cultivars. Alternative approaches to this experiment, including using larger numbers of addition line plants while adopting the suggested protocol recommendations, may well yield a better outcome.

Several published analyses have included the use of various alloplasmic and euplasmic substitution and addition lines and there is conflicting evidence for the effects of several chromosomes on cultured response (Chapter 2), particularly those of homeologous group two.

Kaleikau *et al.* (1989b) found controlling genes on 2DL, 2AL and 2BS which were involved in culture response of immature wheat embryos such that an increased dosage of 2A and 2D could not compensate for the absence of 2B. Felsenburg *et al.* (1987) reported the presence of 2BS as essential for shoot differentiation in wheat cultures and Szakács *et al.* (1988) recorded a strong influence of 2D on green plant regeneration in anther cultures.

A final barley-like feature of the responsive addition line was the regeneration of an albino rather than a green plant. Albinism has been commonly found in barley anther culture

(Finnie *et al.* 1989; Finnie 1990) and may be a result of plastid damage from the extended cold pretreatments (Day & Ellis 1985; Aubry *et al.* 1989). Short cold pretreatments were correspondingly associated with an enhanced proportion of green plants produced in these experiments.

The ratio of green to albino regenerants is strongly affected by genotype in both wheat and barley (Andersen *et al.* 1987; Knudsen *et al.* 1989; Larsen *et al.* 1991) and little affected by medium (Zhou & Konzak 1989; Chu *et al.* 1990) and although the data obtained allows no conclusions to be made, there is a possibility that the alien chromosomes may have affected this ratio with the wheat.

Apparently, cold pretreatment length needs to be short enough to maximize green plant production by minimizing tissue damage but long enough to promote a response in the barley component of the genome. Further studies in this area should thus use a responsive wheat background and appropriate sugar in a liquid medium, while attempting to optimize this problem of pretreatment time. It would also be worthwhile to optimize the growing conditions of the donor plants so an increase could be made in the number of both anthers and genotypes available for culture. It will then be possible to conduct more definitive experiments on addition lines.

Chapter 8 Anther culture of male sterile wheats and pentaploids

Introduction

The effects of male sterility on anther response have been investigated but controversy exists with regard to the nature and mode of action of the sterility in culture. There has been indication of nucleo-cytoplasmic interactions (Foroughi-Wehr *et al.* 1982; Charmet & Bernard 1984) and that cytoplasmic genes from cytoplasmic male sterile lines in wheat affect induction of embryogenic pollen (Heberle-Bors & Odenbach 1985). Furthermore, higher pollen plant yields were found in some cytoplasmic male sterile lines of rice compared with those found in male fertile lines (Ling *et al.* 1978).

The present study aimed to extend the above observations by investigating the effect of three forms of wheat sterility on anther culture response, namely cytoplasmic male sterility (CMS) in wheat with Australian backgrounds, pentaploid lines and chemical gametocides. Firstly, it was postulated that the sterility exhibited was due to pollen dysfunction, in terms of immaturity or malformation at the time of anthesis. If this were so, then despite impairment of fertilizing ability, the pollen from these anthers would not only be responsive in culture but might even show an enhanced response since it is immature pollen grains which normally respond. Secondly, many alloplasmic wheats are male sterile and such crosses can produce spontaneous haploids, so these wheats may possess characteristics conducive to haploidy. Such a tendency could be both advantageous in and promoted through anther culture which is itself an induction process for haploidy. Thirdly, it was probable that the reported effects of male sterility on anther response were genotype-dependent and also influenced by the mode of action of the sterility of the donor plant. This prompted the use of stock from an Australian hybrid wheat breeding programme using cytoplasmic male sterility to investigate culture response and control.

Pentaploids are derived from crosses of *Triticum durum* Desf. tetraploids (AABB) by *Triticum aestivum* hexaploids (AABBDD) and the unbalanced genome makes a proportion of the pollen sterile (Kihara 1982). A selection of pentaploids was used as an alternative source of sterile material with which to investigate anther culture response in wheat.

Gametocides have been shown to cause pollen abnormalities and have been associated with improved anther culturability in wheat (Schmid & Keller 1986; Picard *et al.* 1987). The reportedly effective gametocides CGA and RH0007 were unobtainable for experimentation, but since Ethrel has also been reported to induce male sterility through abnormal microspore development (Bennett & Hughes 1972; Reynolds 1987; Thakur & Rao 1988), it was proposed that pretreatment with this chemical might improve culturability. The effect of gibberellic acid was also investigated since it has shown gametocidal properties in some plants (Kaul 1988).

Materials and Methods

Seeds of breeding lines for the production of cytoplasmic male sterile wheats were supplied by Mr Peter Wilson, Cargill Seeds Pty Ltd, Tamworth, NSW. Sterility was derived from certain alloplasmic lines of *Triticum aestivum* with either of the tetraploids *T. timopheevi* or *Aegilops variabilis*. To allow for possible comparisons, the selection of wheats included representatives from the maintainer (B-lines/females) and restorer lines (R-lines/pollen donors) as well as the sterile lines (A & VA-lines). The *T. timopheevi* cytoplasms used were identical, as were the *A. variabilis* cytoplasms (Mr P Wilson pers. comm.). The pentaploid lines, which were *Triticum durum* cv. Langdon substitution lines x Australian hexaploid crosses, were supplied by Mr Chaoyin Liu, Waite Agricultural Research Institute.

Seed germination, plant care, spike collection, pretreatment preparation, culture, regeneration procedures and statistical analyses are as detailed in Chapter 4. As shown in Chapter 6, donor plant environment had a major effect on success, so the optimum conditions, as defined there, were used. Thus, CMS seeds were sown in 'winter' (April) in the glasshouse but pentaploids were sown in summer also in the glasshouse. Spikes were pretreated at 4°C for 6, 8 or 10 days and anthers with microspores between stages 5 - 9 were cultured (see Chapter 6). All anthers were cultured 35 per 1 ml plate of liquid WM2 medium containing 6% cellobiose as the sugar (see Appendix 2). Induction and regeneration cultures were incubated at 30/18°C. The regeneration medium used was 190-2 (see Appendix 2). Thus, the main variable was genotype.

The level of male fertility ascribed to each line (Table 8.2) was the result of previous assessment (Mr P Wilson pers. comm.) but was cytologically checked by staining with acetocarmine the anthers from mature heads of each plant.

Ethrel (Ciba-Geigy) which contains 480 g/l ethephon was used at the rate of 240 ppm and the solution neutralized with NaOH to pH 7. Gibberellic acid (GA_3) was made as a 1 mg/l solution. Cultivars Aroona, Kite, Machete, Schomburgk and Spear were used. Pre-meiotic spikes were cut from donor plants, kept in the sheath leaf and stood in a conical flask of the solution (100 ml) which was sealed with Nescofilm and placed back in the growthroom with the donor plants. The control was deionized, distilled water. Treatments continued until the spikes had developed sufficiently to be at the stage for collection for anther culture (as detailed in Chapter 4).

Results

Cytoplasmic male steriles

Cytology

Pollen of all fertile donor plants appeared cytologically normal at anthesis with anthers containing high densities of trinucleate, starch-filled pollen grains. Pollen development was similar between lines with *T. timopheevi* and *A. variabilis* cytoplasm.

Sterile plants exhibited shrivelled, pale and sickle-shaped anthers which at flowering tended not to dehisce. Various forms of pollen abnormalities were observed (Figure 8.1). The samples shown are representative of the pollen at anthesis for the depicted lines and thus represent the stage of pollen development for each plant at maturation. Pollen from lines designated as having male fertility below 50% stained weakly with acetocarmine, contained few or no starch grains and rarely contained three nuclei. Binucleate pollen was commonly observed (Figure 8.1.F). Some samples contained a mixture of mature and immature pollen (Figures 8.1.D, E) or only dead pollen (Figure 8.1.H). Germpore deformation was prevalent (Figures 8.1.A-C, G) amongst the sterile lines.

Embryo production

The genotype of the breeding line, the microspore stage at which anthers were plated and the length of time at which spikes were pretreated at 4°C all strongly influenced the production of embryos from the cultures (Table 8.1). There were no significant interactions among these factors.

Four genotypes responded in culture, representing all three cytoplasms (*T. aestivum*, *T. timopheevi* & *A. variabilis*) (Table 8.2). Nuclear genotypes responded differently in different cytoplasms and cytoplasmic genotypes differently with different nucleotypes. Line 2521 in *T. timopheevi* and *A. variabilis* cytoplasm responded by producing embryos but not in the euplasm or when crossed with B 2317. Line 2372 K31 responded as a euplasmic but not when in the *T. timopheevi* cytoplasm. The euplasmic 2652 K61 responded similarly but not the F_1 hybrid of the alloplasmic line with R 535. The male fertility restorer line (R 535) was also unresponsive in culture (Table 8.2).

The highest embryo production was obtained from the euplasmic maintainer line B 2372 K31 (Table 8.2) but the best performance in the alloplasmic lines was line 2521 in *T. timopheevi* cytoplasm followed by the same line in *A. variabilis* cytoplasm. The maintainer line B 2652 K61 had the lowest embryo production and was the only responsive line which failed to regenerate plants.

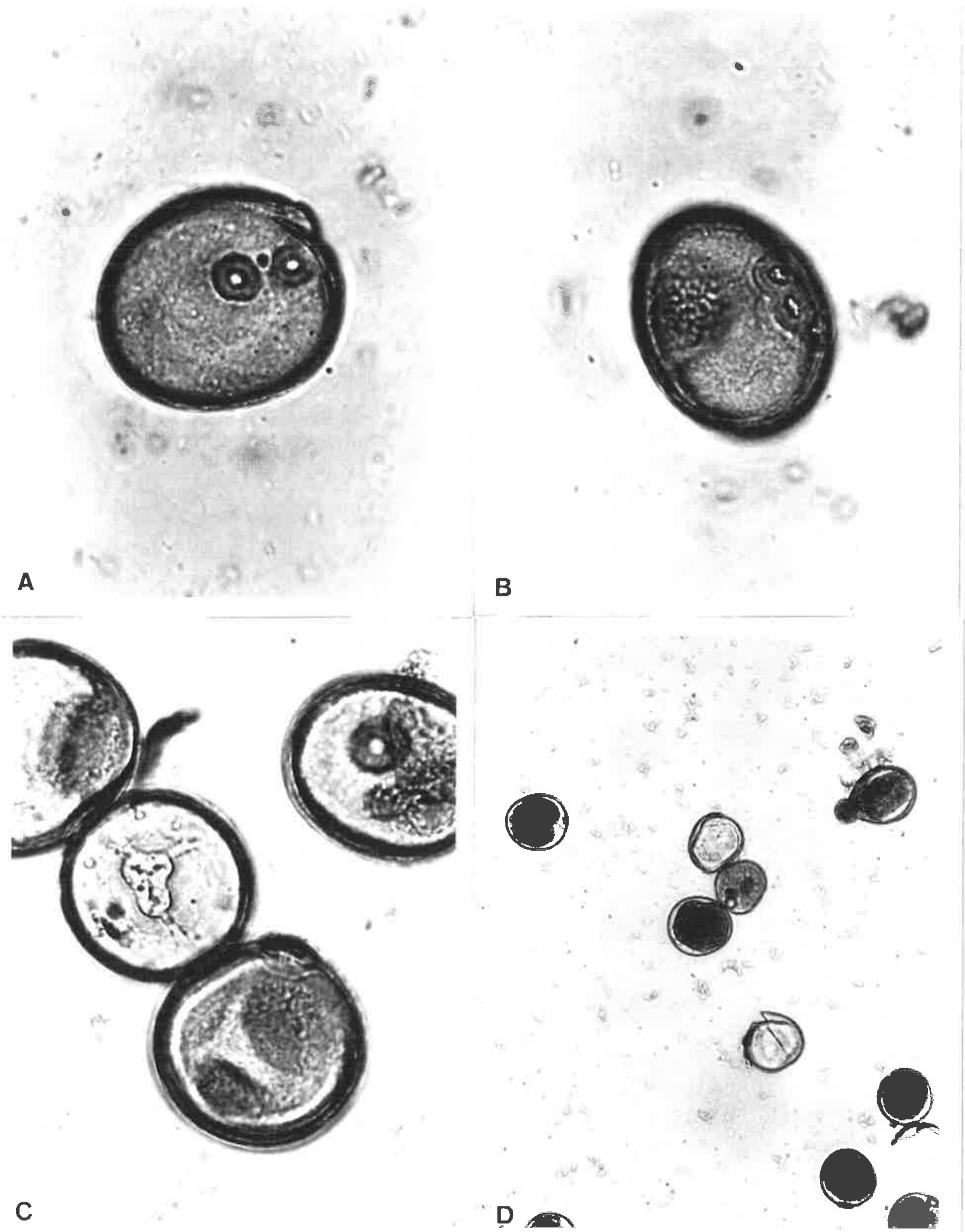

Figure 8.1 Anomalous pollen from mature heads of the male sterile lines. (*A*) A 2521 pollen grain with two germpores, x268; (*B*) A 2521 pollen grain with three germpores, x268; (*C*) A 2521 binucleate pollen showing malformed germpore, x268; (*D*) VA 2521/B 2317 mature, properly formed and functional pollen grains together with sterile, empty pollen from the same anther, x67.

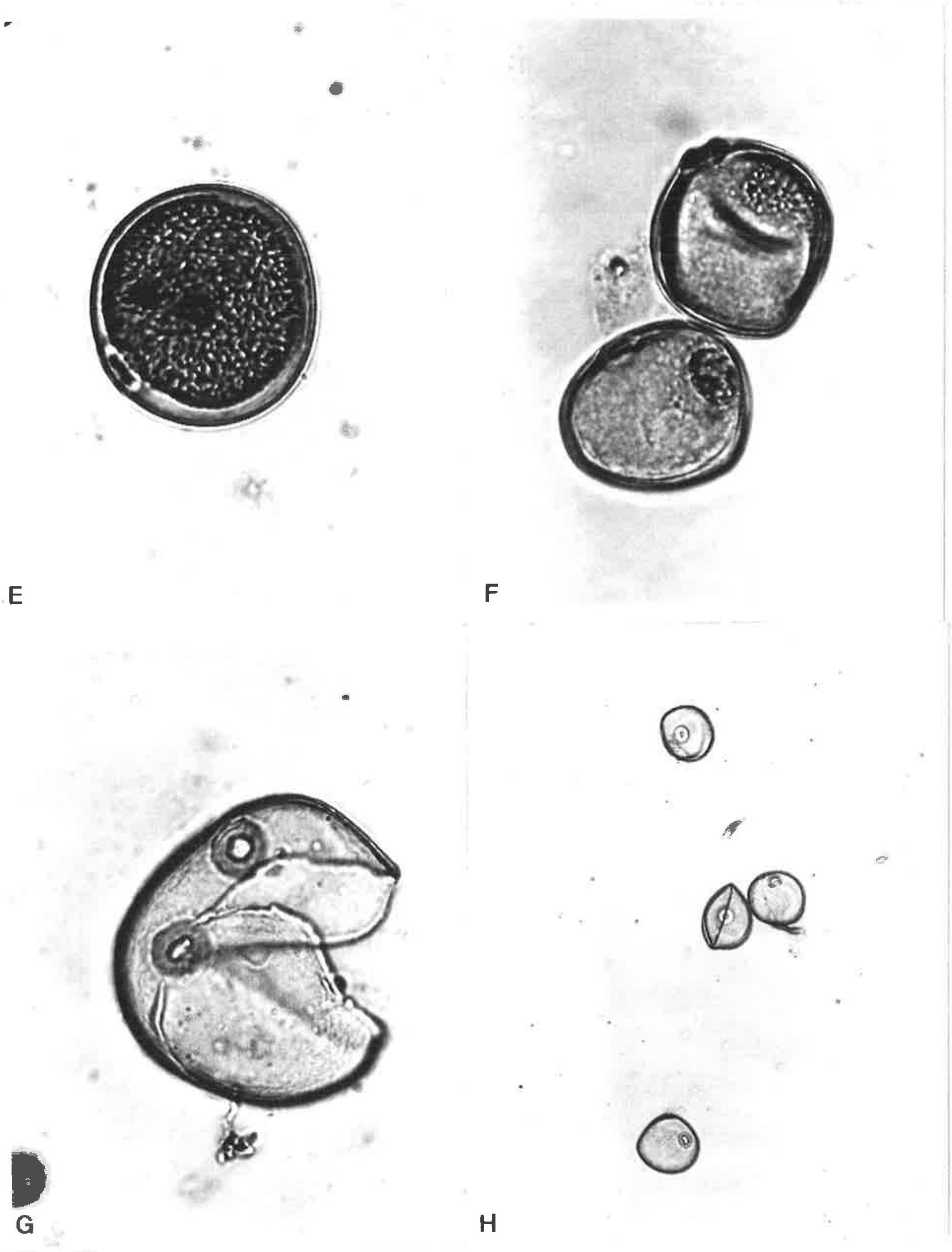

Figure 8.1 continued: (*E*) VA 2521 mature trinucleate pollen grain full of starch granules, x268; (*F*) VA 2521 underdeveloped pollen grains released at anthesis as sterile non-functional binucleate microspores, x268; (*G*) A 2372 K31 sterile, damaged pollen grain with two germpores, x268; (*H*) A 2372 K31 sterile, empty pollen, x67.

Table 8.1 The effect of factors on embryo production for all of the male sterile breeding lines cultured. Where df = degrees of freedom, χ^2 = chi square statistic.

Source of deviation	df	χ^2	Probability
Genotype	8	41.53	$P<0.001$
Residual	197	111.14	
Microspore stage	8	37.08	$P<0.001$
Residual	197	115.59	
Cold pretreatment length	7	17.42	$P<0.05$
Residual	198	135.25	

Plant regeneration

As for embryo production, all cytoplasm types were represented among the green plant regenerants (Table 8.2) since all of the embryos differentiated into green plants except the euplasmic line B 2652 K61 (Table 8.2). No albino plants were regenerated from any cultures (Table 8.2).

All of the factors under consideration (genotype, microspore stage and cold pretreatment) were found to have a significant influence on the regeneration of the green plants, however as yield was low in these experiments no meaningful comparisons can be made among these factors ($P = 0.01 - 0.001$, $\chi^2_{2\text{-}3} = 16.22$). Embryos were produced in only 3 of the 8 pretreatment lengths (8, 10 & 12 days) and plants from only two of these (8 & 12 days). Similarly, green plants were regenerated from cultures initiated at microspore stages 6 and 7 only.

Table 8.2 Anther culture response in euplasmic and alloplasmic wheats. Male fertility, number of anthers plated, number of embryos produced per 100 plated anthers and the proportion of green and albino plants regenerated for the euplasmic and alloplasmic breeding lines of wheat (*Triticum aestivum* genome in *T. timopheevi* or *Aegilops variabilis* cytoplasms).

Line	Cytoplasm	% male fertility	Anthers plated	Mean number of embryos / 100 anthers plated	Proportion of plants / embryos	
					Green	Albino
B 2521	T. a	90-100	665			
A 2521	T. t	0	1050	0.49	1.00	0
VA 2521	A. v	0-30	910	0.22	1.00	0
VA 2521/B 2317	A. v	5-50	705			
B 2372 K31	T. a	90-100	630	1.59	1.00	0
A 2372 K31	T. t	0	687			
B 2652 K61	T. a	90-100	945	0.11	0	0
A 2652 K61/R 535	T. t	5-30	910			
R 535	T. t	90-100	735			
Total			7237			

Where:

A = A-line *T. timopheevi* cytoplasm (T. t)
VA = A-line *A. variabilis* cytoplasm (A. v)
B = B-line *T. aestivum* cytoplasm (T. a)
R = R-line *T. timopheevi* cytoplasm (T. t)

Pentaploids

The pentaploids displayed pollen sterility similar to that observed for the cytoplasmic male sterile lines. There was no culture response from any of the pentaploid wheats (Table 8.3).

Table 8.3 Pedigrees of the pentaploid lines cultured and their response to culture.

Pentaploid genotype	# anthers plated	embryos produced
Langdon 1D(1A) x Raven	258	0
Langdon 1D(1A) x Sabre-3-5-2	1164	0
Langdon 1D(1A) x Teal-3-4	1042	0
Langdon 1D(1B) x Festiguay-5-5	1610	0
Langdon 1D(1B) x Wren	647	0

Ethrel and GA_3-treated plants

Cytological examination of the cut spikes after treatment revealed that ethrel-treated plants in general had withered, yellowing anthers which contained a mixture of normal pollen, empty microspores and some pollen with atypic nuclei numbers and no starch development. Pollen dimorphism was also observed. GA_3-treated plants had soft, small white anthers which lacked microspores or contained empty microspores with poor wall development and occasionally two germpores. Controls had normal pollen.

Although abnormal pollen was sometimes observed, the majority of the pollen from the cut spikes was empty so anthers were not cultured.

Discussion

Cytoplasmic male steriles

Male sterility is the result of microspore malformation, impaired pollen release and female reception, failed gametic union or impaired gametophyte development. Nuclear and cytoplasmic genes control the expression of all these functions. However, for these genes to be expressed during the culture of sterile anthers and so govern culture response, the microspores themselves must be androgenically viable. Unlike for fertilization, for successful culture, it is not important whether the anther can dehisce naturally or that at the time of pollen release, the pollen is immature, but rather that the microspores within have androgenic capabilities. This being so, anthers from sterile plants can contain microspores capable of embryogenesis.

Not all of the pollen observed from the CMS lines was necessarily abnormal. A proportion of healthy, mature grains (Figure 8.1.D, E) was sometimes observed among the sterile grains which were immature, malformed or dead. Moreover, at anthesis, much of the pollen was not fully developed but, nonetheless, was healthy (Figure 8.1.F) and so had the potential to be androgenic. The response from anthers which contained mixed pollen (Figure 8.1.D, E) would have depended upon whether functional or non-functional microspores became androgenic. This observed variability in pollen development, even within one anther, could contribute to the observed differences in embryo production between cultures from the same plant and between different genotypes, and for the fertility range observed. The sterility of the breeding lines was confirmed by the presence of incomplete pollen at anthesis (Figure 8.1.F) but this did not correlate necessarily with culture performance. The cytological observations of pollen from the male sterile wheats at flowering are consistent with those of others (Joppa *et al.* 1966; Heberle-Bors & Odenbach 1985; Becraft & Taylor 1989). Characteristics of male sterile wheat such as stunted anthers, immature pollen grains and a lack of or reduction in starch grain filling have been suggested to be the result of reduced solute transport into the stamens due to poorly differentiated vascular bundles (Joppa *et al.* 1966). It would be necessary to follow the development of individual isolated microspores in order to determine whether or not abnormal microspores develop into embryos and the outcome would be further regulated by the genotype of each microspore. As yet, even in isolated microspore culture, such specialized culture has not been achieved. Development of such a technique will be an ultimate goal for the breeding of plants *in vitro* .

The high level of significance for the effect of cold pretreatment length on the regeneration of green plants was probably due more to the presence of a response from only two pretreatment classes than to any specific effect of length of cold on physiology. Cold pretreatment was nonetheless effective in promoting embryo formation. Similarly, the effect of microspore stage on green plant regeneration would be reliant on those stages which affected embryo induction since regeneration is reliant on embryo formation.

Alloplasmic lines of wheat are cytoplasmic substitution lines where the same nuclear genome has been placed in different cytoplasms of wild wheat relatives. Since anther culture response in wheat is influenced by both nuclear (Bullock *et al.* 1982; Henry & de Buyser 1985; Becraft & Taylor 1989) and cytoplasmic (Lazar *et al.* 1984b; Ekiz & Konzak 1991a,b,c) genes, this material is unique in allowing separation of nuclear and extra-nuclear effects on responsiveness to anther culture. Variation in embryo production between alloplasmic and euplasmic plants with genome 2521 also suggested influence from cytoplasmic factors. These results confirm that both nuclear and cytoplasmic genotypes affect embryogenic response. They also show that both components affect regeneration of green plants, in contrast to other reports that cytoplasm had no effect (Sági & Barnabás 1989).

Since genotype was a principal factor determining androgenesis, valuable information about genetic control can be found in the cultivar pedigrees (Table 8.5). The Russian cultivar Skorospelka carries the 1B/1R translocation chromosome (Mr G Hollamby pers. comm.) and was common to three of the four responding lines (Table 8.5). Lines 2521 and 2372 K31 carry 1B/1R (Mr P Wilson pers. comm.) whereas line B 2652 K61, showing the lowest embryo production and no green plant regeneration, does not.

It has been indicated that the presence of 1B/1R may be a basic requisite for haploid production in certain alloplasmic wheats (Kihara & Tsunewaki 1962; Kobayashi & Tsunewaki 1980) with the gene responsible located on the rye arm. Furthermore, investigations into mechanisms of male sterility found that nearly 90% of parthenogenically derived haploids had the 1B/1R chromosome (Kobayashi & Tsunewaki 1980) suggesting that some gene(s) control haploid induction gametophytically but not sporophytically. The same might be true for androgenically derived haploids.

Nevertheless, other non-responsive genotypes were derived from cv. Skorospelka and not all 1B/1R wheats show good androgenic capabilities (Foroughi-Wehr & Zeller 1990). Therefore, while the presence of this chromosome is strongly correlated with haploid induction, it is not a necessary condition for haploid production in wheat (Kaul 1988) indicating that there is more than one gene controlling anther response.

This translocation chromosome also may be involved in the control of regeneration as only those lines derived from Skorospelka produced plants, plus only green plants were regenerated. However, since no albinos were produced, this chromosome may, in some unknown way, also be affecting albino regeneration. Regeneration resulting entirely in green plants has also been reported for cv. Siete Cerros in the cytoplasm of *A. variabilis* which did not carry 1B/1R (Sági & Barnabás 1989). The absence of the 1B/1R chromosome might, in part, be why the euplasmic line 2652 K61 failed to regenerate any plants.

Although nuclear genotype had a strong effect on embryogenesis, cytoplasm was also influential. Embryos were produced from the line 2521 with both *T. timopheevi* and *A. variabilis* cytoplasms but not in *T. aestivum* cytoplasm, suggesting influence from cytoplasmic factors. Similarly, differences between similar euplasmic- and A-lines suggested that cytoplasm affected embryo induction.

A. variabilis cytoplasm has previously shown significant stimulative effects on embryogenic anther response when compared with *T. aestivum* cytoplasm (Sági & Barnabás 1989) and a high culture response has been obtained from a combination of *A. variabilis* cytoplasm and the 1B/1R translocation chromosome *in vivo* (Kihara & Tsunewaki 1962) and *in vitro* (Kobayashi & Tsunewaki 1980). Since androgenic potential has been attributed to the presence of alien genes (Chapter 2, 6, 7; Lazar *et al.* 1987) it is possible that the presence of

Agropyron (3 Ag 14) in the pedigree of B 2652 K61 (Table 8.5) might have been involved in culture response of this genotype. Plants have been regenerated from anther cultures of an octoploid *Triticum-Agropyron* intermediate (Miao *et al.* 1988) including the regeneration of wheat-alien addition lines.

Table 8.5 Pedigrees of the breeding lines used.

Line	Pedigree
2521	RAC311*3//Lance*4/Skorospelka
2372 K31	Skorospelka/4*Condor
2652 K61	B 2277//B 355/B 2310
R 535	R 893//R 900/B 2190

where:

B 2317 (A)	Sunstar: Condor/4/2*WW15/3/Steinwedel/WC356//La Prevision
A 2652 K61 (T)	B 2277//B 355/B 2310
B 2277 (A)	Federation/McMurachy//Kentana/Yaqui/3/Chris/4/Norteno66/Ciano67//Olesen
B 355	Sun 9E: Gamut/5/Gabo*6/Mentana//Gabo*2/Eureka/W1656/3/Gabo*7/Mentana//Gabo*2/ W2077/4/Mengavi/3/Frontana//Kenya 58/Newthatch/6/Sonora 64A
B 2310	B1916/B1801
B 1916	Sun 9E*4/3Ag14
B 1801	Inia Reselection/Ciano67//Kite Sib
R 893 (T)	R 649/R 617/4/Timgalen/3/Mex 8156//R 515/Primepi
R 649 (T)	US HRW R-line
R 617 (T)	Mex 8156//R 515/Primepi
R 515 (T)	US HRS R-line
R 900 (T)	Kite Sib (T)//Dirk R-line/R 617
Dirk R-line (T)	R-line based on Dirk (Canadian)
B 2190 (A)	S948A1/7*Santa Elena

where: (T) = *T. timopheevi* cytoplasm; (A) = *T. aestivum* cytoplasm; RAC311 is a breeding line from Roseworthy Agricultural College, University of Adelaide, SA.

Refer to Table 8.2 for notation of B, A, VA & R.

Embryos and green plants were obtained from line 2521 carrying 1B/1R in *A. variabilis* cytoplasm. Nevertheless, a hybrid from this line in this cytoplasm yielded nothing in culture. The difference suggests that response was influenced by nuclear rather than cytoplasmic genes or that a gene responsible for response was recessive or that an interaction existed. The embryogenic performance of the CMS nuclear genotype 2521 in *A. variabilis* cytoplasm was apparently not, however, as high as the same nuclear genotype in *T. timopheevi* cytoplasm (Table 8.2) which is consistent with other reports using *Aegilops* spp (Becraft & Taylor 1989). Good anther culture performance has been reported for wheat with *T. timopheevi* cytoplasm (Picard & de Buyser 1973; Felsenburg *et al.* 1987; Picard *et al.* 1987) and also triticale (Charmet & Bernard 1984). In embryo cultures, an alloplasmic line with *T. timopheevi* cytoplasm exceeded the euplasmic line in all parameters (Mathias *et al.* 1986).

As the embryo response between line 2521 in both *T. timopheevi* and *A. variabilis* was not equal, together with the above 2652 K61 relationship, then perhaps the response obtained by anther culture was moderated by the species from which the cytoplasm originated.

The cytoplasm of *Aegilops variabilis* is probably more closely related than that of *T. timopheevi* to *T. aestivum* (Tsunewaki *et al.* 1976) and this is supported by the greater sterility of the *T. timopheevi* lines than the *A. variabilis* lines (Table 8.2). Furthermore, high male sterility has been correlated with high genetic distance (Kaul 1988) and the present results with line 2521 confirm this correlation. Finally, increasing sterility was positively correlated with embryogenic response in line 2521, as was found elsewhere (Heberle-Bors & Odenbach 1985). Therefore, it seems probable that increasing genetic diversity of alloplasmic wheats would also correlate with embryogenic response (Mathias *et al.* 1986). Nevertheless, line 2372 K31 produced conflicting results, with higher response arising from the male fertile cytoplasm rather than the male sterile cytoplasm. Clearly, no generalization can be made about the relationship between sterility and culture response, and the importance of specific nucleo-cytoplasmic interactions is again emphasized.

In sum, this study found that both cytoplasm and nuclear genotypes affect anther response, supporting recent findings by Ekiz and Konzak (1991a). Breeding lines with *Triticum timopheevi* and *Aegilops variabilis* cytoplasms were responsive and are consistent with other reports (Picard & de Buyser 1973; Felsenburg *et al.* 1987; Picard *et al.* 1987; Becraft & Taylor 1989; Ekiz & Konzak 1991a) as were some of the euplasmic *T. aestivum* lines tested (Chapter 8). Three of the four responsive CMS lines also had the 1B/1R chromosome and perhaps its presence contributed to the results obtained. The cytological reasons why male sterile pollen does not effect fertilization probably do not inhibit androgenesis but rather facilitate gametophytic development in culture. However, the results do not conclusively show that male sterility was the influencing trait in these lines since not all the breeding lines screened responded and, furthermore, only half of these were CMS. The response by the CMS lines could be due to alloplasmy or male sterility but further testing of CMS lines is needed in order to better understand the relationship between male sterility and anther culture ability (Ling *et al.* 1978; Heberle-Bors & Odenbach 1985). Although no response was obtained from plants treated with ethrel, further experimentation into the effect of gametocides would be valuable if use of such chemicals can indeed effectively remove genotypic constraints on culture (Schmid & Keller 1986; Picard *et al.* 1987). It would then be necessary to establish whether any improvement in culture ability is because of the resultant sterility or directly due to stimulation by the chemical.

Pentaploids

There are many possible reasons for the failure of the pentaploids to respond in culture. Since pentaploids characteristically exhibit varying levels of pollen malformation (Kihara 1982), one reason might be due to the presence of empty, degenerated or similarly non-androgenic microspores. Furthermore, the donor plants were grown in summer which is known to be unfavourable for embryogenesis (Chapter 6).

The negative response might also be purely or partially genetic with influences from either or both the hexaploid or tetraploid parents. None of the parents were available for culture, nor had any been reported in the literature although three of the lines (Sabre, Teal and Wren) have Gabo derivatives in their pedigrees (Zeven & Zeven-Hissink 1976) which have already been shown to be unresponsive in culture (Chapter 6). A considerable effect on embryogenic induction of anther cultures has been attributed to chromosomes 1D (Agache *et al.* 1989) and the lines cultured were all 1D substituted. Therefore, the removal of chromosome 1D from these lines might have in turn, removed their capability to promote embryo formation. In general, durum wheats have seldom been cultured. Embryos have been generated but the production of green plants is less common (Hadwiger & Heberle-Bors 1986; Foroughi-Wehr & Zeller 1990). The expression of genes for androgenesis in either parent could be masked in the hybrid. The response might also be negative rather than be lacking or masked. If so, inhibition of androgenesis might be the result of interactions between the alien genomes or simply that either parent expresses this trait.

Chapter 9 General Discussion

Anther culture has great potential for plant breeding (Chapter 1) yet the full utilization of this technology has been prevented by many problems (Chapter 2). The culture of wheat anthers has been particularly problematic. Research and development with wheat anthers world wide has been hampered by the overall recalcitrance of wheat in culture, genotypic differences and low yields. Indeed, the results of this thesis concur with those from other laboratories and a major finding of this thesis is that cultural factors affecting anther culture success contribute the recalcitrance of wheat in culture. The major objective of this thesis was to specifically identify the difficult areas and the means by which to improve the response of anthers in culture (Chapter 3). The outcomes of these investigations are discussed and their implications for the improvement of wheat anther culture are considered.

The initiation of embryogenesis

Since wheat remains a relatively recalcitrant species in terms of anther culture, the numbers of responding anthers was relatively small. Most petri-dishes contained no embryos (Appendix 6) while a few contained small numbers. One of the questions examined in detail in this thesis was whether the formation of embryos was random, in which case it would be expected that the data would fit a Poisson distribution (with the variance equal to the mean) (Sokal & Rohlf 1981). Or, alternatively, that anther response was non-random, such that if one anther responded in a particular dish, then others would have been likely to have followed. In this case it would be expected that the data would follow an over-dispersed distribution in which the variance was significantly larger than the mean (Sokal & Rohlf 1981), with the over-dispersion arising from a contagion acting among the plated anthers.

Such contagious factors could include minor variations in media composition from dish to dish, variations in anther placement and compounds released from the developing embryos.

Clearly, if such a contagious factor were acting and could be identified, then its routine and deliberate use could lead to a substantial increase in the number of responding anthers in wheat. Overall analyses of the data presented here indicated that anther response was random. In view of this, it is concluded that there was no contagion effect and embryos occurred independently with respect to each other.

The results showed that if embryo-inducing factors or 'anther factors' (Xu & Huang 1984) released from the plated anthers existed, then they were weak and perhaps much higher densities of anthers are needed to detect them. Indeed, Xu and Huang (1984) found that by increasing plating density to 80 anthers/ml (double that used in this thesis), embryo production was substantially increased. All the reports of the effect of conditioning media with anthers have been for barley and the results obtained with wheat (Chapter 5) suggest that perhaps the action of anther factors is a phenomenon specific to barley. However, in the experiment with wheat, conditioning anthers were autoclaved during media preparation as a way of eliminating certain organic compounds which could have been the source of an influence. The lack of response with and without autoclaved conditioned media is indicative of the lack of evidence for both heat labile and non-labile anther factors.

The results were based on the number of embryos produced irrespective of the number of anthers responding, so increasing the plating density of anthers is one way that embryo production could be increased. The density at which microspores are plated in microspore culture is far greater than in anther culture (Lörz *et al.* 1988), so microspore culture is an alternative way to achieve such an increase and demonstrates that it is the microspore which is responsive not the anther. However, increasing anther density would avoid the need for extra processing which is involved in microspore culture preparation (Ziauddin *et al.* 1990) in order to obtain a pure suspension of cells.

A complexity of factors that control androgenesis

With the knowledge that genotype, season, donor plant environment, microspore stage, sugar and cultural treatments strongly affect embryo production, the primary form of anther culture response of wheat, and that operative ranges for these have been established, future experiments can be streamlined. The probability of getting an anther culture response from wheats of unknown performance should increase since the ranges identified were those in which a response was obtained by all responsive genotypes.

An investigation of the factors affecting anther culture response had to be designed to include all these factors, which made both analyses of the results and conclusions from them complex. In order to facilitate the understanding of such a complex group of factors, three main groups were defined - genetic, environmental and cultural.

Since all factors exhibited main effects for embryo production and there was a considerable amount of interaction, it was not possible to eliminate any factors. The randomness of embryo production meant that any of the processes and treatments under consideration could still be responsible. This combination of randomness of embryo production and the strong main effects from genetic, environmental and cultural factors is an indication of the complexity of the factors that control androgenesis in wheat and means that it would be difficult to isolate the effect of one factor over another as well as to determine accurately the extent of its influence. Had embryo production been under the influence of a contagion of some kind or another, it would have been much easier to design experiments to determine the cause or causes.

The variability in anther culture response is an additional reason why it is difficult to reproduce results in anther culture. This is particularly so between research institutions, even when the same or similar conditions and materials are used. Furthermore, it is apparent that in order to obtain optimum response, each genotype requires specific culture conditions (eg. cv. Oxley (Chapter 6); Yuan *et al.* 1990). Nevertheless, a culture protocol suited to a wide range of genotypes would mean that optimum responses would be replaced by responses from as many genotypes as possible. With this in mind and despite the consequence of low embryo yields, developing such a protocol was made a primary aim of this thesis.

How yield is affected

Low yield in association with the screening of a large number of genotypes using anther culture has been reported elsewhere for wheat (Andersen *et al.* 1987; Foroughi-Wehr & Zeller 1990) and there is no doubt that such studies are an important progression towards using anther culture for wheat breeding and research. The final results confirm the value of defining three main areas of control - genetic, environmental, cultural.

Most research and developments in anther culture have been achieved using only a small number of responsive cultivars. Indeed research into anther culture of barley has been dependent, in the main, upon only the one responsive cultivar, Igri (Hunter 1987; Olsen 1987; Kasha *et al.* 1990). The applicability of anther culture decreases when research is limited to such a small selection of responsive material. For practical purposes, it is equally important to establish which breeding materials fail to respond since conventional breeding relies on a broad range of wheats expressing diverse traits. Yield depends on having responsive genotypes. If a greater proportion of genotypes screened are unresponsive then yields can be expected to be low. Conversely, if the proportion of responsive genotypes is larger, there should be a corresponding increase in yield. The number of genotypes found to be highly responsive would further increase yield. Since research into increasing the number of known responsive genotypes involves the culture of genotypes of previously unknown culture ability, and because the number of known highly responsive genotypes is relatively low, the likelihood of finding recalcitrant wheats must be greater than that of finding responsive or highly responsive genotypes.

The right start - genotype forecasting

Genotype is of primary importance in determining anther culture response. It determines whether or not a wheat is responsive in culture.

The results of experiments reported in this thesis provide supporting evidence for the strong genetic basis of anther culture response. Both nuclear and cytoplasmic genotypes contribute to the induction of embryos with indication that both CIMMYT-bred wheats and the translocation chromosome 1B/1R carry promotory genes. In addition to *T. aestivum* cytoplasm, those of *Triticum timopheevi* and *Aegilops variabilis* were responsive which is consistent with other reports (Picard & de Buyser 1973; Felsenburg *et al.* 1987; Picard *et al.* 1987; Becraft & Taylor 1989; Ekiz & Konzak 1991a). Of the euplasmic wheats, a majority were CIMMYT-bred of which most of the Australian cultivars were derived from the Australian breeding line WW15 (Chapter 6).

The success in culture of other WW15-derived Australian cultivars (Luckett *et al.* 1991) lends additional weight to the theory that Australian WW15 wheats show improved efficacy and productivity in anther culture. In addition to 1B/1R, there are CIMMYT-bred and WW15 wheats in the pedigrees of the responsive cytoplasmic male sterile lines (Chapter 8) which may have had some bearing on their performance. The value of CIMMYT-bred wheats is further supported by results with Ciano 67 (Datta & Wenzel 1987; Ouyang *et al.* 1987), Pavon-76 (Zhou & Konzak 1989; Yuan *et al.* 1990; Ekiz & Konzak 1991c; Zhou *et al.* 1991; Ball *et al.* 1992), Penjamo-62 (Sági & Barnabás 1989; Ekiz & Konzak 1991a), Pitic-62 (Marsolais *et al.* 1984; Datta & Wenzel 1987), Siete Cerros 66 (Sági & Barnabás 1989; Ekiz & Konzak 1991b) and Yecora Rojo (Ekiz & Konzak 1991c).

Anther culture of wheats carrying the 1B/1R translocation chromosome were found to be responsive (Chapter 8), lending support to the potential of this chromosome in culture (Henry & de Buyser 1985; Foroughi-Wehr & Zeller 1990). The cv. Skorospelka was the source of the translocation chromosome in these lines and it is also the parent of the Australian cv. Grebe in which improved performance in anther culture has been attributed to the 1B/1R translocation chromosome (Ding *et al.* 1991; Luckett *et al.* 1991). Egret, the other parent of Grebe, is a WW15 derivative (Chapter 6), suggesting that excellent performance can be achieved when a cultivar carries both these components (1B/1R & WW15).

There is indication that 1B/1R could be involved in green plant production since all of the responsive CMS lines with 1B/1R regenerated only green plants whereas the line which failed to regenerate plants lacked it (Chapter 8). The capacity for green plant formation has been found to be inherited via the chromosome both in barley (Larsen *et al.* 1991) and wheat (Zhou *et al.* 1991). However, the lack of embryo numbers and thus low plant yields obtained in this study make the basis for such speculation uncertain, so further testing of this hypothesis is required.

The effect of chromosome 1B on embryogenic induction in anther cultures is controversial with both progressive (Szakács *et al.* 1988) and regressive (de Buyser et al. 1992) effects reported. The improved culture ability of the translocation chromosome has been attributed to the rye chromosome (Lazar *et al.* 1987; Müller *et al.* 1989) with two distinct genetic regions found on 1RS which were involved in embryo culture response (Langridge *et al.* 1991). Furthermore, this improved effect was restricted to the short arm since the presence of the entire 1R chromosome in the addition line resulted in significantly reduced embryogenic performance compared to the parental wheat, Chinese Spring (Langridge *et al.* 1991). Although the genetic control of embryo and anther culture has been suggested to be different (Lazar *et al.* 1987; Agache *et al.* 1988), similar regions on this rye arm could be involved in response in anther culture. These results suggest that 1RL perhaps limited the expression of the beneficial effects of the rye chromosome since enhanced culture ability was also shown by the 1DL/1RS translocation chromosome and more so by the 1BL/1RS (Langridge *et al.* 1991), in turn suggesting some influence from chromosomes 1B (Szakács *et al.* 1988) and 1D (Agache *et al.* 1989). It would be worthwhile comparing the anther culture response of 1RS telesomic additions with 1BL/1RS translocation lines.

1B/1R was introduced for rust resistance and yield improvement and is now a component of many commercial wheats. Its link with genes controlling androgenesis increases the possibility that these will also be incorporated into agronomically important wheats; in turn increasing the response of Australian wheats in culture.

The contribution of rye to wheat anther culture response could be tested through the culture of 1R, 1RS and 1RL substitution lines. The source of the rye genes having the effect requires further clarification and, since these genetic lines can be obtained in a Chinese Spring background with Imperial, Pektus and King II rye (Dr N Singh pers. comm.), determination would be possible. There is no stock of Chinese Spring with Imperial rye 1BL/1RS but this line is available in Gabo. The cultivars Kavkay, Aurora and Veery-3 and -S contain 1BL/1RS with Pektus rye. Chromosome 1 substitutions with King II rye are available with Chinese Spring and Holdfast. Culture of these genetic lines would determine the effect of group 1 chromosomes in wheat and rye and determine from which rye stock the gene(s) originated.

The link between enhanced anther culture response and 1B/1R is indicative of the potential alien germplasm offers as a source for genes involved in androgenesis. Alien addition lines to wheat offer even greater potential since there is the chance that the effect of individual alien chromosomes can be defined. Although no improvements were found with the culture of the series of wheat-barley addition lines, perhaps because of genetic masking of expression, there is still potential for using these and other genetic lines to investigate the differences in anther culture response between species. The results did indicate that the current better performance of barley over wheat could be due to media modifications. These avenues were investigated and are discussed below. Equally, improvements in the culture of other cereals such as euplasmic rye (Flehinghaus *et al.* 1991) and triticale (Schumann 1990) may lead to a discovery of other alien chromosomes conveying androgenic ability with culture of wheat-rye addition lines finding rye chromosome 4 also to have a positive effect upon culture responses (Lazar *et al.* 1987). Since culture ability is a heritable trait, these chromosomes could be incorporated into the make-up of new cultivars. Such breeding for anther culture response could complement the use of anther culture in plant breeding.

Extensive genetic studies by Ekiz and Konzak (1991a,b,c) showed that culture ability is a heritable trait and that the anther culture ability of recalcitrant genotypes can be improved through crossing with responsive genotypes so that the filial generations can be used in anther culture. Clearly, it is essential to know which genotypes are likely to be recalcitrant before anther culture can be of real value in commercial plant breeding. Hence seeds from the reciprocal crosses made between various responsive and non-responsive lines (Chapter 6) will be useful in testing whether or not there are heritable genetic factors promoting or inhibiting anther culture ability between CIMMYT-bred and Insignia-type wheats. If, by screening the seeds from these parents and crosses, enzyme and/or DNA markers distinguishing responsive and non-responsive material could be found, then future wheats and F_1 hybrid seed could be routinely screened to test for their potential to respond in culture. Such a system would facilitate the accumulation of a library of wheats of known culture capacity for use in breeding via anther culture.

This study has demonstrated the value of screening both wheats of unknown culture ability and local breeding stocks. The genetic commonalities proposed for genotypes of responsive and non-responsive groups can henceforth be used to forecast the aptitude of other wheats to anther culture. These lineal relationships should be expanded by screening more local commercial wheats and breeding lines. The criteria formulated could then be used to apply anther culture technology in current Australian breeding programmes.

Albinism

In general, the low yields obtained in this thesis preclude any meaningful discussion about how to improve the problem of albinism in wheat anther culture. Certain genotypes appeared less prone to regenerate albino plants, particularly those carrying 1B/1R but as genotype was not found to affect the outcome of regeneration, it can only reasonably be suggested that the formation of albinos is specific to pollen development and not a result of culture. Albinism is not a problem in all cereal species as it is rare in maize (Dr D Barloy pers. comm.) but routine at high frequencies in barley (Kasha *et al.* 1990), suggesting that it may be a plant specific phenomenon. Furthermore, it is not a problem in the anther culture of dicotyledons. So, if albinism is related to the normal cytological activity of the pollen, then there could be fundamental differences in pollen development between cereals and non-cereals as well as between species. If the mechanisms governing these processes are translated and expressed during anther culture then differences in genic control between species exhibiting high and low frequencies of albinism may exist. A screen of the DNA of members of each group could be used to identify the genes, specific to each group, which are involved in pollen development and the control of albinism.

A support matrix - medium and sugar

The choice of medium on which to culture anthers is another important consideration for performance improvement since differences were observed between species and among genotypes of the same species to media. Modification of the components of a single medium further altered genotype performances. There are three main properties a culture medium provides the plated anthers, they are, physical, osmotic and nutritional. These will be discussed below.

This study found that sugar was a significant contributor to anther response among the genotypes tested (Chapter 6) supporting earlier findings with barley (Hunter 1987; Olsen 1987; Sorvari & Schieder 1987). More recently, its importance in the growth of wheat has been observed (Chu *et al.* 1990; Last & Brettell 1990; Orshinsky *et al.* 1990). This includes its importance with barley (Finnie *et al.* 1989; Roberts-Ohelschlager *et al.* 1990) and for *in vitro* wheat pollen germination (Cheng & McComb 1992). The present results indicate that cellobiose should be the principal sugar for anther culture of Australian wheat.

Physical support

There has been a trend towards liquid media for wheat anther culture, with the advantage of increased aeration and nutrient dispersal (Ekiz & Konzak 1991a,b,c; Kao *et al.* 1991; Luckett *et al.* 1991). An associated problem, however, is the buoyancy of the plated anthers. Increased aeration and nutrient dispersal through the use of membrane rafts, which also maintain anthers on the surface of the liquid medium, probably contributed to recent anther culture improvements (Luckett *et al.* 1991). Membrane rafts on liquid medium may prevent developing embryos from submersion and thus remove potential yield losses as a result of embryo necrosis. Their use with WM2 would be worthy of trial, particularly with a wider range of genotypes.

The recent success of some CIMMYT-bred wheats by Ekiz and Konzak (1991a,b,c) using a liquid Potato medium plus the results of Chapter 5 suggest that this medium could be developed as an alternative for the culture of Australian wheat. Similarly, the results obtained on C-17 medium and its recent development in liquid form (Luckett *et al.* 1991) indicate its potential, although the use of membrane rafts (Luckett *et al.* 1991; Luckett & Darvey 1992) may account for the increased yields.

WM2 was the only medium on which all of the genotypes which were tested responded, suggesting that it has a composition in which androgenesis is favoured. It is likely that the inclusion of Ficoll contributes to the better effect of WM2 since it increases the viscosity of the medium thereby increasing the buoyancy of the plated anthers thus facilitating aeration and respiration (Simmonds 1989; Kao *et al.* 1991). Regeneration of green plants from cultivars which previously only produced albinos has also been attributed to the use of Ficoll (Kao *et al.* 1991).

Osmotic support

It has been suggested that high Ficoll concentrations are inhibitory to androgenesis (Luckett & Darvey 1992). Nevertheless, such concentrations are needed to maintain anther buoyancy. Recent determination that the osmotic potential of the induction medium may have profound effects on callus induction as well as on green plant production (Zhou *et al.* 1991) can be related to Ficoll concentration since osmotic potential is directly correlated with the concentration of the major source of sugar (Ball *et al.* 1992). Ficoll is a polymer of sucrose so due to colligative properties, an increase in Ficoll concentration would result in an increase in osmotic pressure of the induction medium resulting in a dehydrating environment. In turn, this would result in a stress which could inhibit androgenesis for anthers plated on such a medium. Membrane rafts might therefore resolve such osmoticum problems by allowing the use of a liquid medium with a lower osmotic pressure while maintaining buoyancy through structural means.

Nutritive support

The effect of sugar in the induction medium must also be partly nutritional. In barley, high sugar concentrations have been associated with improved embryogenesis whereas lower concentrations have been associated with embryo greening (Sorvari & Schieder 1987). Given that the role of sugar differs with androgenic development, as embryos differentiate into plants, better utilization is made of the carbohydrate source for establishment of sugar transport and thus photosynthesis. This concept suggests that, as in any finite system, a gradation in sugar concentration from high concentrations of the induction medium through intermediate steps (Dr D Barloy pers. comm.) to the low concentrations required for regeneration may facilitate successful plant regeneration and reduce non-differentiation of embryos.

It has been proposed that it is the ability to metabolize the sugar in the medium that determines whether a genotype will produce embryos on it (Sorvari & Schieder 1987; Finnie *et al.* 1989; Last & Brettell 1990). The number of disaccharides which are effective in anther culture shows that many enzymes can be involved in the development of embryos. However, wheat may possess less than barley since barley has produced embryos on melibiose (Sorvari & Schieder 1987) whereas wheat has not (Chapter 5, 7; Last & Brettell 1990). This suggests that the performance of wheat may be even more closely linked with the use of all-glucose disaccharides (Hunter 1987).

The marked differences in the results from the Australian group to maltose and cellobiose contradict recent suggestion that maltose is a superior sugar for the anther culture of wheat (Last & Brettell 1990; Orshinsky *et al.* 1990) even though this might be true for barley (Olsen 1987; Finnie *et al.* 1989). Although cellobiose and maltose are both glucose disaccharides, they differ in the interglucose link configuration such that maltose has an α–1,4 link whereas cellobiose has a β–1,4 link. Thus, cellobiose is a structural sugar and stands in relationship to cellulose as maltose, a storage sugar, does to starch. Enzymes that hydrolyze one do not affect the other, so the performance of genotypes which showed a response to only one of these sugars might be controlled by a single gene whereas multiple genes are implicated for genotypes able to respond to several sugars. A differential ability to hydrolyse the interglucose links may account for the differences in response shown amongst the genotypes. This would imply that responsive genotypes possess a gene and enzyme with the capacity to hydrolyse and utilise a particular sugar more readily than another.

Interesting genotype x sugar interactions exhibited by the sister lines Aroona and Warigal may be a result of such gene-enzyme control and these results show the strong genetic basis of culture ability. Although this could be due to variation in culture treatments, it should be possible to test the genetic hypothesis since F_1 hybrid seed was produced between these parents.

Accepting that sugar is partly nutritional, embryo yields from the Australian group of responsive genotypes were lower on cellobiose than yields on sucrose by the non-Australian group (Chapter 6). A reduced genetic ability of the wheats to initiate and sustain embryo development would result in both lower yields and a need for an extended time for sugar availability in order to establish embryo development. A carbon source of slower degradation than sucrose would provide an extension of the availability of sugar. Cellobiose and maltose degrade at a slower rate than sucrose in barley anther cultures (Roberts-Oehlschlager *et al.* 1990). Thus the lower yields on cellobiose are perhaps a function of sugar availability, perhaps in turn related to whether or not the sugar is structurally based or nutritionally based, as well as genotype. If this hypothesis is valid, it may account in part for the overall better regeneration rates on cellobiose than sucrose (Chapter 6).

An alternative and opposing theory for the function of sugar in the induction medium is that it acts more an osmoticum than as an essential carbon source (Zhou *et al.* 1991). If this is so, then the ability of the anther to utilise available sugar could be independent of concentration. Generally, sugar starvation is promotive for embryo induction (Wei *et al.*

1986; Kao *et al.* 1991) and the response on cellobiose could be from a lack of a suitable hydrolyzing enzyme, leading to starvation. As neither theory has been resolved, there may be two genetic systems involved in the utilization of sugar in a medium.

Ethylene production by anthers in culture (Kasha *et al.* 1990) may constitute another form of stress acting in a similar manner to sugar starvation and cold pretreatments (Chapter 6) which induce microspore division and thus embryo formation. Ethylene released by the application of ethrel (Kaul 1988), which has been associated with inducing male sterility (Chapter 8; Kasha *et al.* 1990), may also contribute to the positive relationship between male sterility and improved culture ability (Chapter 8).

In sum

This thesis establishes that an embryo formed through androgenesis by anther culture is a rare and independent event. Nevertheless, it is one which is governed by genotype and regulated by a number of physiological and cultural factors. The factors which affect efficacy and productivity (culture ability) in anther culture are determined. A responsive range in each is identified and specific treatments are recommended. The culture ability of some Australian cultivars is established, identifying both responsive and non-responsive groups of genotypes. Investigation into the effect of male sterility and the role of alien germplasm provides additional information for the mechanisms controlling response in wheat anther culture. Together with the only other information on Australian cultivars (Last & Brettell 1990; Ding *et al.* 1991; Luckett *et al.* 1991) the research presented in this thesis forms the basis for future research into the culture ability of Australian wheat, an area unresearched at the commencement of this investigation and now still in its infancy.

The protocol developed from the ranges which have been determined can now be used to maximize efficiency for future culture of wheat anthers and in particular, that of Australian wheat.

Chapter 10 Summary

1. Embryo production was a random event.
2. The greatest number of genotypes responded on liquid WM2 medium.
3. Genotype was a dominant character affecting culture response.
4. There was a relationship between embryo response and CIMMYT-bred wheats, in particular, genotypes derived from the Australian breeding line WW15.
5. There was a relationship between embryo response and the translocation chromosome 1B/1R.
6. There was little to no promotive effect from the barley genome of wheat-barley chromosome addition lines.
7. Cytoplasmic and nuclear effects were found for anther culture response of male sterile, alloplasmic wheats.
8. There were environment and seasonal influences on the donor plants which were reflected in differences in embryo production amongst the genotypes. Donor plants grown in the glasshouse, particularly in winter, performed best.
9. Best embryo response was obtained from microspores between stages 5-10.
10. Spikes cold pretreated between 3-10 days gave the best anther culture response.
11. Australian wheats responded best on medium containing the sugar cellobiose.
12. The non-Australian cultivars responded better on sucrose.
13. The best culture incubation temperature found was 30/25°C.

Appendices

Appendix 1 Abbreviations

CIMMYT	Centro Internacional de Mejoramiento de Maiz y Trigo International maize and wheat improvement centre, Mexico.

Chemicals

2,4-D	2,4-dichlorophenoxyacetic acid
BAP	benzylamino purine
GA_3	gibberellic acid
IAA	indole-3-acetic acid
kinetin	6-furfurylaminopurine
NAA	napthaleneacetic acid
zeatin	6-(4-hydroxy-3-methyl but-2-enyl) amino purine

University of California soil mix

60% sand (AAA washed concrete sand)

40% peat moss

+ hydrated lime to pH 6.5

Microspore stages

1	pollen mother cells
2	tetrads
3	very early uninucleate
4	early uninucleate
5	mid uninucleate
6	mid-side uninucleate
7	mid-late uninucleate
8	late-rounded uninucleate
9	late uninucleate
10	binucleate
12	trinucleate
11	sterile or no distinguishable nucleus

Factors in statistical analyses

geno	Genotype
mspst	Microspore stage
DPenv	Donor plant environment
sug	Sugar
pT	Pretreatment (4°C)
incubT	Incubation temperature
sig	significant (statistically)
ns	not significant / no significance (statistically)
ME	main effects

Appendix 2 Media

Media

MS	Murashige & Skoog (1962)
N_6	Chu (1981)
190-2	Zhuang & Xu (1983)
C-17	Xu (1985)
WM2	Datta & Wenzel (1987)
Melibiose	Sorvari & Schieder (1987) (EDAM I)
W5	Feng & Ouyang (1988)
Ficoll	Kao (1988)
Potato-2	Shimada & Otani (1988)
FHG	Kasha, Ziauddin & Cho (1990)

MS induction medium

chemical	g/l
KNO_3	1.9
NH_4NO_3	1.65
$MgSO_4$	0.37
KH_2PO_4	0.17
$CaCl_2$	0.44
$MnSO_4$	0.0223
$ZnSO_4$	0.0086
H_3BO_3	0.0062
$CuSO_4$	0.000025
$CoCl_2$	0.000025
KI	0.00083
Na_2MoO_4	0.00025
Na_2EDTA	0.0373
$FeSO_4$	0.0278
inositol	0.1
glycine	0.002
nicotinic acid	0.0005
pyridoxine HCl	0.0005
thiamine HCl	0.0001
IAA	0.001
kinetin	0.0005
sucrose	30.0
agar	10.0
H_2O	upto 1000 ml

pH : 5.7-5.8

Murashige, T and Skoog, F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum <u>15</u>: 473-497.

N_6 induction medium

chemical	**g/l**
KNO_3	2.83
$(NH_4)_2SO_4$	0.463
$MgSO_4$	0.185
KH_2PO_4	0.4
$CaCl_2$	0.166
$MnSO_4$	0.0044
$ZnSO_4$	0.0015
H_3BO_3	0.0016
KI	0.0008
FeEDTA	0.0421
glycine	0.002
nicotinic acid	0.0005
pyridoxine HCl	0.0005
thiamine HCl	0.001
sucrose	50.0 - 120.0
agar	8.0
H_2O	upto 1000 ml

pH : 5.8

Chu, CC (1981) The N_6 medium and its applications to anther culture of cereal crops. Plant Tissue Culture; Symposium of plant tissue culture. Beijing, China 1978. p 43-50.

190-2+ Regeneration medium

chemical	**g/l**
KNO_3	1.0
$(NH_4)_2SO_4$	0.2
$MgSO_4$	0.2
KH_2PO_4	0.3
KCl	0.04
$Ca(NO_3)_2$	0.1
$MnSO_4$	0.008
$ZnSO_4$	0.003
H_3BO_3	0.003
KI	0.0005
$FeSO_4$	0.0278
Na_2EDTA	0.0373
inositol	1.0
glycine	0.002
thiamine HCl	0.001
nicotinic acid	0.0005
pyridoxine HCl	0.0005
NAA	0.0005
kinetin*	0.0015
†sucrose	30.0
agar	7.0
myo-inositol	0.1
H_2O	upto 1000 ml

pH : 5.8

Zhuang, JJ and Xu, J (1983) Increasing differentiation frequencies in wheat pollen callus. Cell and tissue culture techniques for cereal crop improvement Hu, H and Vega, MR (eds). Science Press. Beijing, China. p 431-432.

* 1.5x original recipe
† maltose was substituted

C-17 induction medium

chemical	**g/l**
KNO_3	1.4
NH_4NO_3	0.3
$MgSO_4$	0.15
KH_2PO_4	0.4
$CaCl_2$	0.15
$MnSO_4$	0.0112
$ZnSO_4$	0.0086
H_3BO_3	0.0062
$CuSO_4$	0.000025
$CoCl_2$	0.000025
KI	0.00083
Na_2EDTA	0.03725
$FeSO_4$	0.02785
inositol	0.1
glycine	0.002
nicotinic acid	0.0005
pyridoxine HCl	0.0005
folic acid	0.0005
thiamine HCl	0.001
d-biotin	0.0015
2,4-D	0.002
kinetin	0.0005
sucrose*	90.0
agar	5.5
H_2O	upto 100 ml

pH : 5.8

Xu, H (1985) Studies on raising the induction frequency of green plantlets in anther culture of wheat. Crops 3: 30-33.

Pei, W *et al* (1986) A study on the application of C-17 medium for anther culture. Acta Botanica Scinica 28(1): 33-45.

*maltose was substituted

WM2 induction medium

chemical	**g/l**
KNO_3	2.8
$(NH_4)_2SO_4$	0.463
$MgSO_4$	0.185
KH_2PO_4	0.4
$CaCl_2$	0.166
$MnSO_4$	0.0044
$ZnSO_4$	0.0015
H_3BO_3	0.0016
KI	0.0008
FeEDTA	0.0421
L-glutamine	0.16
serine	0.1
glycine	0.002
nicotinic acid	0.0005
pyridoxine HCl	0.0005
folic acid	0.0005
thiamine HCl	0.001
2,4-D	0.005
inositol	5.0
sugar	60.0
Ficoll-400	100.0
H_2O	upto 1000 ml

pH : 5.8

Datta, SK and Wenzel, G (1987) Isolated microspore derived plant formation via embryogenesis in *Triticum aestivum* L. Plant Science 48: 49-54.

EDAM I induction medium

chemical	**g/l**
KNO_3	2.83
$(NH_4)_2SO_4$	0.463
$MgSO_4$	0.185
KH_2PO_4	0.4
$CaCl_2$	0.166
$MnSO_4$	0.0044
$ZnSO_4$	0.0015
H_3BO_3	0.0015
KI	0.0008
Na_2EDTA	0.0373
$FeSO_4$	0.0278
glycine	0.002
nicotinic acid	0.0005
pyridoxine HCl	0.0005
thiamine HCl	0.001
IAA	0.001
BAP	0.001
melibiose	120.0
barley starch	60.0
H_2O	upto 1000 ml

pH : 5.8

Sorvari, S and Schieder, O (1987) Influence of sucrose and melibiose on barley anther cultures in starch media. Plant Breeding 99: 164-171.

W5 induction medium

chemical	**g/l**
KNO_3	2.022
$(NH_4)_2SO_4$	0.3
$MgSO_4$	0.25
KH_2PO_4	0.4
$CaCl_2$	0.125
$MnSO_4$	0.008
$ZnSO_4$	0.003
H_3BO_3	0.003
KI	0.0005
Na_2EDTA	0.03725
$FeSO_4$	0.02785
glycine	0.002
nicotinic acid	0.0005
pyridoxine HCl	0.0005
thiamine HCl	0.001
2,4-D	0.002
kinetin	0.0005
sucrose*	9.0
agar	4.5
H_2O	upto 1000 ml

pH : 6

Feng, GH and Ouyang, J (1988) The effects of KNO_3 concentration in callus induction medium for wheat anther culture. Plant Cell, Tissue and Organ Culture 12: 3-12.

* maltose was substituted.

Ficoll induction medium

chemical	**g/l**
KNO_3	2.2
$(NH_4)_2SO_4$	0.67
NH_4NO_3	0.6
$MgSO_4$	0.31
KH_2PO_4	0.17
$CaCl_2$	0.445
NaH_2PO_4	0.075
KCl	0.15
$MnSO_4$	0.01
$ZnSO_4$	0.002
H_3BO_3	0.003
KI	0.00075
Na_2MoO_4	0.00025
$CuSO_4$	0.000025
$CoCl_2$	0.000025
FeEDTA	0.03725
inositol	0.1
nicotinic acid	0.001
pyridoxine HCl	0.001
thiamine HCl	0.002
d-calcium pantothenate	0.0005
folic acid	0.0002
p-aminobenzoic acid	0.00001
d-biotin	0.000005
ascorbic acid	0.001
sodium pyruvate	0.005
citric acid	0.01
malic acid	0.01
fumaric acid	0.01
2,4-D	0.0005
zeatin riboside	0.001
casamino acids	0.25
coconut water	10.0ml
glucose	2.5
sucrose	42.5
xylose	0.15
Ficoll-400	300
H_2O	upto 1000 ml

pH : 6.0
filter sterilize

Kao, KN (1988) In vitro culture in barley breeding. In: Semi-dwarf cereal mutants and their use in cross breeding III. Proceedings of the final research coordination meeting on evaluation of semi-dwarf mutants for cross breeding. International Atomic Energy Agency (IAEA), Vienna 1988.

Potato-2 induction medium

chemical	**g/l**
KNO_3	1.0
$(NH_4)_2SO_4$	0.1
$MgSO_4$	0.0125
KH_2PO_4	0.2
$Ca(NO_3)_2$	0.1
KCl	0.035
Fe EDTA	0.0421
thiamine HCl	0.001
2,4-D	0.0015
kinetin	0.0005
sucrose	90.0
potato (10%)	100.0
agar	7.0
H_2O	upto 1000 ml

pH : 5.8

Shimada, T and Otani, M (1988) Efficiency of potato medium on induction of pollen embryoids in anther culture of Japanese wheat cultivars. Japanese Journal of Breeding 38: 212-222.

FHG induction medium

chemical	**g/l**
KNO_3	1.9
NH_4NO_3	0.165
$MgSO_4$	0.37
KH_2PO_4	0.17
$CaCl_2$	0.44
$MnSO_4$	0.0223
$ZnSO_4$	0.0086
H_3BO_3	0.0062
$NaMoO_4$	0.00025
$CuSO_4$	0.000025
$CoCl_2$	0.000025
KI	0.00083
$FeNa_2$	0.04
L-glutamine	0.73
thiamine HCl	0.0004
2,4-D*	0.005
inositol	0.1
maltose	62.0
Ficoll-400*	200.0
H_2O	upto 1000 ml

pH : 5.6

Kasha, KJ; Ziauddin, A and Cho, U-H (1990) Haploids in cereal improvement: anther and microspore culture. In: Gene Manipulation in Plant Improvement II, Gustafson, JP (ed). Plenum Press, USA. p 213-235.

*Modified:- Agar for Ficoll. Addition of 1mg/l BAP & IAA.

Appendix 3 Donor plant environment

The mean number of embryos produced per 100 plated anthers for each of the responding genotypes, from the growthroom (GR) and glasshouse (GH) during "winter" (Apr - Sep) and "summer" (Oct - Mar). The number of genotypes which produced embryos out of the total number of responsive genotypes from which anthers were cultured for each environment is shown.

Genotype	Donor plant environment			
	winter		summer	
	GR	GH	GR	GH
Aroona	0.29 ± 0.29	0	0	0
Warigal	0	0.92 ± 0.53	1.48 ± 1.48	2.88 ± 1.77
Schomburgk	0	0.44 ± 0.21	0.29 ± 0.29	0
Oxley	0	1.44 ± 1.22		0
Kite	0	0.24 ± 0.12	0	0
Machete	0	0.26 ± 0.18	0	0
WW15		0.09 ± 0.09		0
Egret		0.26 ± 0.18		0
Federation		0.03 ± 0.03		0
Chinese Spring	0	0.27 ± 0.14		0
Orofen		0	0	1.13 ± 0.57
anther Orofen	0	0	44.44	0
Atys	0	0	0.08 ± 0.08	0.24 ± 0.12
Total	1/9	9/13	4/8	3/13

The proportion of green plants (g) and albino plants (a) regenerated from the total number of embryos produced for each of the responding genotypes from the growthroom (GR) and glasshouse (GH) during "winter" (Apr - Sep) and "summer" (Oct - Mar).

Genotype	Donor plant environment							
	winter				summer			
	GR		GH		GR		GH	
	g	a	g	a	g	a	g	a
Aroona	0	0						
Warigal			0.20 ± 0.20	0.60 ± 0.19	0.20	0	0.07 ± 0.04	0.24 ± 0.13
Schomburgk			0.25 ± 0.19	0.05 ± 0.05	1.00	0		
Oxley			0.27 ± 0.25	0.09 ± 0.09				
Kite			0.13 ± 0.13	0.13 ± 0.13				
Machete			0.17 ± 0.17	0.42 ± 0.30				
WW15			0	0.50				
Egret			0	0				
Federation			1.00	0				
Chinese Spring			0.25 ± 0.14	0.25 ± 0.25				
Orofen							0.03 ± 0.03	0.06 ± 0.06
anther Orofen					0.08	0.17		
Atys					0	0	0	0

Results of analysis for the difference between the numbers of genotypes which responded, by producing embryos, between the four environments: winter growthroom, winter glasshouse, summer growthroom, summer glasshouse.

	df	Deviance	Level of significance
Regression	3	10.25	$P<0.05$
Residual	0	0	
Total	3	10.25	

Appendix 4 Donor plant environment

The number of plates in which an embryo/s was formed out of the total number of plates for each genotype for each donor plant environment (winter glasshouse (GH), winter growthroom (GR), summer glasshouse, summer growthroom).

Genotype	Donor plant environment			
	winter		summer	
	GR	GH	GR	GH
Aroona	1/20			
Warigal		5/57	1/13	3/34
Schomburgk		5/67	1/19	
Oxley		4/60		
Kite		4/72		
Machete		3/59		
WW15		1/39		
Egret		2/52		
Federation		1/50		
Chinese Spring		4/66		
Orofen				6/69
anther Orofen			1/1	
Atys			1/28	4/60

Appendix 5 Green plant production

The highest percentage of green plants produced for a significant interaction between culture variables for group 1, showing the combination which yielded that percentage.

Interaction (combination involved)	mean green plants /100 anthers	se
Donor plant environment x genotype (GR summer x Anther Orofen)	3.70	
Donor plant environment x microspore stage (GH winter x stage 10)	2.86	2.86
Donor plant environment x cold pretreatment length (GH winter x 7 days)	0.69	0.69
Genotype x incubation temperature (Anther Orofen x 30/25°C & Chinese Spring x 30/18°C)	0.46	0.46
Genotype x sugar (Anther Orofen x sucrose)	0.74	0.74
Microspore stage x genotype (stage 10 x Oxley)	2.86	2.86
Microspore stage x sugar (stage 10 x gentiobiose)	2.86	2.86
Cold pretreatment length x genotype (4 days x Schomburgk)	0.79	0.79
Cold pretreatment length x incubation temperature (7 days x 30/18°C)	0.93	0.93
Cold pretreatment length x microspore stage (9 days x stage 10)	2.86	2.86
Cold pretreatment length x sugar (7 days x maltose)	0.46	0.46

Appendix 6 Factor combinations for data of Chapter 6 which produced no response

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
cellobiose	18	5	GH1/4	9	Oxley
cellobiose	18	5	GH2/11	8	Chinese Spring
cellobiose	18	6	GR23/8	10	Atys
cellobiose	18	6	GH1/4	9	Chinese Spring
cellobiose	18	6	GH1/4	10	Oxley
cellobiose	18	6	GH10/12	8	Chinese Spring
cellobiose	18	6	GH17/8	8	Chinese Spring
cellobiose	18	6	GH2/11	8	Chinese Spring
cellobiose	18	6	GH2/11	9	Chinese Spring
cellobiose	18	6	GH23/8	10	Atys
cellobiose	18	7	GH10/12	8	Chinese Spring
cellobiose	18	7	GH17/8	8	Chinese Spring
cellobiose	18	7	GH2/11	8	Chinese Spring
cellobiose	18	8	GH1/4	7	Oxley
cellobiose	18	8	GH10/12	8	Chinese Spring
cellobiose	18	8	GH23/8	8	Orofen
cellobiose	18	9	GH10/12	8	Chinese Spring
cellobiose	18	9	GH8/8	10	Warigal
cellobiose	18	12	GH1/4	9	Chinese Spring
cellobiose	30/18	1	GH14/1	8	Gabo, Pavon-76
cellobiose	30/18	1	GH16/4	8	Pavon-76
cellobiose	30/18	1	GH2/11	9	Bencubbin
cellobiose	30/18	3	GH1/4	12	Egret
cellobiose	30/18	3	GH10/12	8	Egret, Insignia
cellobiose	30/18	3	GH16/4	8	Bindawarra
cellobiose	30/18	3	GH16/4	9	Pavon-76
cellobiose	30/18	4	GH1/4	9	Bindawarra
cellobiose	30/18	4	GH10/12	8	Bindawarra, Insignia
cellobiose	30/18	4	GH16/4	8	Hartog
cellobiose	30/18	4	GH17/8	8	Bencubbin
cellobiose	30/18	5	GH1/4	8	Federation, Hartog, WW15
cellobiose	30/18	5	GH10/12	8	Bencubbin
cellobiose	30/18	5	GH10/12	9	Federation
cellobiose	30/18	5	GH13/9	8	Pavon-76
cellobiose	30/18	5	GH14/1	8	Insignia
cellobiose	30/18	5	GH16/4	8	WW15
cellobiose	30/18	5	GH17/8	8	Bindawarra, Dagger, Egret, Federation, Gabo, Hartog, Insignia
cellobiose	30/18	5	GH17/8	9	Bindawarra
cellobiose	30/18	5	GH2/11	11	Pavon-76
cellobiose	30/18	5	GH8/10	10	Insignia

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
cellobiose	30/18	6	GR23/8	10	Atys
cellobiose	30/18	6	GH1/4	7	WW15
cellobiose	30/18	6	GH1/4	8	Dagger
cellobiose	30/18	6	GH1/4	8	WW15
cellobiose	30/18	6	GH1/4	10	Dagger, Federation
cellobiose	30/18	6	GH10/12	8	Bindawarra, Egret, Federation
cellobiose	30/18	6	GH10/12	9	Bindawarra
cellobiose	30/18	6	GH14/1	8	Gabo, Pavon-76
cellobiose	30/18	6	GH17/8	8	Bencubbin, Dagger, Egret, Federation, Gabo, Hartog, Insignia, WW15
cellobiose	30/18	6	GH17/8	9	Bencubbin, Dagger
cellobiose	30/18	6	GH2/11	8	Bencubbin, Dagger, Federation, Pavon-76
cellobiose	30/18	6	GH2/11	10	Bindawarra
cellobiose	30/18	6	GH23/8	10	Atys
cellobiose	30/18	6	GH8/10	8	Bindawarra
cellobiose	30/18	7	GH1/4	8	Federation, WW15
cellobiose	30/18	7	GH1/4	10	Insignia
cellobiose	30/18	7	GH1/4	36	Federation
cellobiose	30/18	7	GH10/12	8	Bindawarra, Egret
cellobiose	30/18	7	GH13/9	8	Pavon-76
cellobiose	30/18	7	GH17/8	8	Bencubbin, Bindawarra, Dagger, Federation, Gabo, Hartog, WW15
cellobiose	30/18	7	GH18/6	8	Hartog
cellobiose	30/18	8	GH1/4	8	Dagger
cellobiose	30/18	8	GH1/4	10	WW15
cellobiose	30/18	8	GH10/12	8	Federation
cellobiose	30/18	8	GH17/8	8	Bencubbin, Dagger, Federation, WW15
cellobiose	30/18	8	GH23/8	8	Orofen
cellobiose	30/18	9	GH14/1	8	Gabo
cellobiose	30/18	9	GH14/1	10	Federation
cellobiose	30/18	9	GH2/11	8	WW15
cellobiose	30/18	9	GH8/8	10	Warigal
cellobiose	30/18	10	GH8/10	8	Bindawarra
cellobiose	30/18	11	GH17/8	8	Egret
cellobiose	30/18	12	GH1/4	8	Egret
cellobiose	30/18	12	GH1/4	11	Egret
cellobiose	30/25	1	GR28/7	3	Halberd
cellobiose	30/25	3	GR16/11	8	Halberd
cellobiose	30/25	3	GR28/7	3	Halberd

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
cellobiose	30/25	3	GH21/6	3	Halberd
cellobiose	30/25	4	GR16/1	8	Halberd
cellobiose	30/25	4	GH16/11	4	anther Orofen
cellobiose	30/25	4	GH16/11	8	Halberd
cellobiose	30/25	4	GH21/6	3	Halberd
cellobiose	30/25	4	GH21/6	3	Warigal
cellobiose	30/25	5	GR16/1	8	Halberd, Machete, Spear, Warigal
cellobiose	30/25	5	GR16/1	9	Machete
cellobiose	30/25	5	GR16/1	10	Atys, Machete
cellobiose	30/25	5	GR16/1	11	Halberd
cellobiose	30/25	5	GR16/11	3	Halberd
cellobiose	30/25	5	GR16/11	4	Atys, Halberd, Machete, Spear
cellobiose	30/25	5	GR16/11	8	Atys, Halberd, Kite, Machete, Spear
cellobiose	30/25	5	GR28/7	3	Kite, Schomburgk
cellobiose	30/25	5	GH16/1	8	anther Orofen
cellobiose	30/25	5	GH16/11	3	Halberd
cellobiose	30/25	5	GH16/11	8	Atys, Halberd
cellobiose	30/25	5	GH21/6	3	Aroona, Halberd, Kite, Oxley, Schomburgk
cellobiose	30/25	5	GH21/6	4	Aroona
cellobiose	30/25	5	GH21/6	6	Aroona, Kite
cellobiose	30/25	6	GR16/1	8	Kite, Machete, Spear, Warigal
cellobiose	30/25	6	GR16/1	9	Halberd
cellobiose	30/25	6	GR16/1	10	Atys, Halberd, Machete
cellobiose	30/25	6	GR16/1	11	Atys, Machete, Schomburgk, Warigal
cellobiose	30/25	6	GR16/11	4	Atys, Halberd, Spear
cellobiose	30/25	6	GR16/11	8	Atys, Halberd, Kite, Machete, Spear
cellobiose	30/25	6	GR16/11	9	Schomburgk
cellobiose	30/25	6	GR28/7	3	Kite
cellobiose	30/25	6	GH16/1	8	Warigal
cellobiose	30/25	6	GH16/1	9	Warigal
cellobiose	30/25	6	GH16/1	11	Warigal
cellobiose	30/25	6	GH16/11	4	Atys, Machete
cellobiose	30/25	6	GH16/11	8	Atys, Halberd, Warigal
cellobiose	30/25	6	GH16/11	9	Atys
cellobiose	30/25	6	GR16/1	10	Aroona
cellobiose	30/25	6	GR16/11	3	Aroona
cellobiose	30/25	6	GR16/11	8	Aroona
cellobiose	30/25	6	GR28/7	3	Aroona
cellobiose	30/25	7	GR16/1	8	Machete, Spear, Warigal
cellobiose	30/25	7	GR16/1	9	Atys, Kite, Spear

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
cellobiose	30/25	7	GR16/1	10	Halberd, Warigal
cellobiose	30/25	7	GR16/1	11	Machete, Spear
cellobiose	30/25	7	GR16/11	3	Schomburgk, Spear
cellobiose	30/25	7	GR16/11	4	Machete, Spear
cellobiose	30/25	7	GR16/11	8	Machete, Schomburgk, Spear
cellobiose	30/25	7	GR16/11	9	Schomburgk
cellobiose	30/25	7	GR16/11	10	Schomburgk
cellobiose	30/25	7	GR28/7	3	Spear
cellobiose	30/25	7	GH16/1	9	Warigal
cellobiose	30/25	7	GH16/11	8	Atys, Schomburgk, Spear
cellobiose	30/25	7	GH16/11	9	Atys, Orofen
cellobiose	30/25	7	GH21/6	3	Aroona, Machete, Oxley, Warigal
cellobiose	30/25	7	GR16/1	8	Aroona
cellobiose	30/25	7	GR16/11	4	Aroona
cellobiose	30/25	7	GR16/11	8	anther Orofen
cellobiose	30/25	7	GR28/7	3	Aroona
cellobiose	30/25	8	GR16/1	8	Halberd, Spear, Warigal
cellobiose	30/25	8	GR16/1	9	Warigal
cellobiose	30/25	8	GR16/1	10	Halberd, Spear
cellobiose	30/25	8	GR16/11	3	Atys
cellobiose	30/25	8	GR16/11	4	Atys
cellobiose	30/25	8	GR16/11	8	Kite, Orofen, Schomburgk
cellobiose	30/25	8	GR16/11	9	Atys, Schomburgk
cellobiose	30/25	8	GR28/7	3	Schomburgk, Spear
cellobiose	30/25	8	GH16/11	4	Halberd, Machete, Orofen, Spear
cellobiose	30/25	8	GH16/11	8	Orofen
cellobiose	30/25	8	GH21/6	3	Aroona, Halberd, Kite, Oxley, Schomburgk, Warigal
cellobiose	30/25	8	GR28/7	3	Aroona
cellobiose	30/25	9	GR16/11	3	Atys, Schomburgk
cellobiose	30/25	9	GR16/11	4	Atys, Spear
cellobiose	30/25	9	GR16/11	8	Kite, Machete, Spear
cellobiose	30/25	9	GH16/11	3	anther Orofen
cellobiose	30/25	9	GH16/11	8	anther Orofen, Halberd, Spear
cellobiose	30/25	9	GH21/6	3	Aroona, Kite, Machete, Schomburgk, Warigal
cellobiose	30/25	9	GH21/6	4	Aroona, Warigal
cellobiose	30/25	9	GH21/6	6	Halberd
cellobiose	30/25	9	GR16/1	11	Aroona
cellobiose	30/25	10	GR16/11	8	Kite
cellobiose	30/25	10	GR16/11	9	Atys

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
cellobiose	30/25	10	GR16/11	10	Schomburgk
cellobiose	30/25	10	GH16/11	8	Kite
cellobiose	30/25	10	GH16/11	9	Atys
cellobiose	30/25	10	GH21/6	3	Oxley
cellobiose	30/25	10	GH21/6	6	Schomburgk, Warigal
cellobiose	30/25	10	GR16/11	8	Aroona
cellobiose	30/25	11	GH16/1	8	Warigal
cellobiose	30/25	12	GR16/11	8	Aroona
gentiobiose	18	1	GH1/4	35	Orofen
gentiobiose	18	1	GH10/12	8	Orofen, Oxley
gentiobiose	18	1	GH10/12	9	anther Orofen, Orofen
gentiobiose	18	1	GH14/1	8	Orofen
gentiobiose	18	1	GH18/6	8	anther Orofen
gentiobiose	18	1	GH2/11	8	Atys, Spear
gentiobiose	18	2	GH16/4	8	Machete
gentiobiose	18	2	GH18/6	8	anther Orofen
gentiobiose	18	3	GH1/4	8	Orofen
gentiobiose	18	4	GH16/4	8	anther Orofen, Orofen
gentiobiose	18	5	GH1/4	8	Chinese Spring, Oxley
gentiobiose	18	5	GH1/4	9	Chinese Spring
gentiobiose	18	5	GH1/4	10	Oxley
gentiobiose	18	5	GH1/4	11	Kite
gentiobiose	18	5	GH10/12	8	Chinese Spring, Orofen
gentiobiose	18	5	GH10/12	9	anther Orofen
gentiobiose	18	5	GH12/7	9	Atys
gentiobiose	18	5	GH14/1	8	Aroona, Orofen
gentiobiose	18	5	GH14/1	9	Aroona
gentiobiose	18	5	GH16/4	8	Oxley
gentiobiose	18	5	GH16/4	9	Schomburgk
gentiobiose	18	5	GH17/8	8	Machete, Schomburgk
gentiobiose	18	5	GH17/8	9	Spear
gentiobiose	18	5	GH17/8	14	Halberd
gentiobiose	18	5	GH18/6	8	Orofen
gentiobiose	18	5	GH18/6	9	Atys
gentiobiose	18	5	GH2/11	8	Chinese Spring, Halberd, Kite, Oxley
gentiobiose	18	5	GH2/11	9	Oxley
gentiobiose	18	5	GH2/11	10	Spear
gentiobiose	18	5	GH2/11	11	Machete, Orofen
gentiobiose	18	5	GH8/10	8	Atys
gentiobiose	18	5	GH8/10	10	Machete, Orofen

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
gentiobiose	18	5	GH8/10	11	Spear
gentiobiose	18	5	GH8/10	12	Atys
gentiobiose	18	6	GH1/4	7	Chinese Spring
gentiobiose	18	6	GH1/4	8	Chinese Spring, Oxley
gentiobiose	18	6	GH1/4	9	Chinese Spring, Kite, Oxley
gentiobiose	18	6	GH1/4	10	Chinese Spring, Kite
gentiobiose	18	6	GH10/12	8	anther Orofen, Kite, Oxley
gentiobiose	18	6	GH13/9	8	Kite
gentiobiose	18	6	GH14/1	8	Aroona, Orofen
gentiobiose	18	6	GH14/1	9	Aroona
gentiobiose	18	6	GH16/4	8	Machete, Warigal
gentiobiose	18	6	GH16/4	9	Orofen
gentiobiose	18	6	GH16/4	13	Schomburgk
gentiobiose	18	6	GH17/8	8	Aroona, Machete, Schomburgk, Spear, Warigal
gentiobiose	18	6	GH17/8	9	Warigal
gentiobiose	18	6	GH17/8	10	Warigal
gentiobiose	18	6	GH17/8	11	Schomburgk
gentiobiose	18	6	GH17/8	13	Aroona, Schomburgk
gentiobiose	18	6	GH17/8	14	Aroona, Machete, Schomburgk, Spear
gentiobiose	18	6	GH18/6	8	Kite, Warigal
gentiobiose	18	6	GH18/6	11	Schomburgk, Warigal
gentiobiose	18	6	GH18/6	12	Atys
gentiobiose	18	6	GH2/11	8	Aroona, Chinese Spring, Halberd, Kite, Machete, Oxley, Spear, Warigal
gentiobiose	18	6	GH2/11	9	Machete, Spear
gentiobiose	18	6	GH2/11	10	Schomburgk, Spear
gentiobiose	18	6	GH8/10	8	Halberd, Machete, Spear
gentiobiose	18	6	GH8/10	9	Atys, Machete, Orofen, Spear
gentiobiose	18	6	GH8/10	10	Machete, Warigal
gentiobiose	18	7	GH1/4	12	Kite
gentiobiose	18	7	GH10/12	8	Orofen
gentiobiose	18	7	GH10/12	9	Kite
gentiobiose	18	7	GH13/9	8	Kite
gentiobiose	18	7	GH14/1	8	Aroona, Orofen
gentiobiose	18	7	GH14/1	11	Orofen
gentiobiose	18	7	GH16/4	8	Kite
gentiobiose	18	7	GH17/8	8	Aroona, Halberd, Kite, Machete, Spear, Warigal
gentiobiose	18	7	GH17/8	10	Warigal
gentiobiose	18	7	GH2/11	8	Aroona, Spear

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
gentiobiose	18	7	GH2/11	10	Schomburgk
gentiobiose	18	7	GH2/11	11	Kite, Spear
gentiobiose	18	7	GH2/11	12	Machete
gentiobiose	18	7	GH8/10	8	Machete
gentiobiose	18	7	GH8/10	9	Warigal
gentiobiose	18	7	GH8/10	10	Machete
gentiobiose	18	8	GH10/12	8	anther Orofen, Aroona, Chinese Spring, Kite
gentiobiose	18	8	GH10/12	9	Chinese Spring
gentiobiose	18	8	GH14/1	8	Aroona
gentiobiose	18	8	GH14/1	9	Aroona
gentiobiose	18	8	GH16/4	8	Kite
gentiobiose	18	8	GH17/8	8	Aroona, Atys, Machete, Spear
gentiobiose	18	8	GH18/6	8	Atys
gentiobiose	18	8	GH2/11	8	Oxley
gentiobiose	18	8	GH2/11	11	Kite
gentiobiose	18	8	GH8/10	8	Atys, Chinese Spring
gentiobiose	18	9	GH1/4	8	Oxley
gentiobiose	18	9	GH10/12	8	Chinese Spring
gentiobiose	18	9	GH10/12	9	Oxley
gentiobiose	18	9	GH17/8	8	Halberd
gentiobiose	18	9	GH8/10	8	Oxley
gentiobiose	18	9	GH8/10	10	Atys
gentiobiose	18	10	GH10/12	8	Chinese Spring
gentiobiose	18	10	GH14/1	8	Aroona
gentiobiose	18	10	GH2/11	8	Kite, Machete, Oxley, Spear
gentiobiose	18	10	GH2/11	9	Machete
gentiobiose	18	10	GH8/10	8	Machete, Oxley
gentiobiose	18	10	GH8/10	9	Kite, Machete, Warigal
gentiobiose	18	10	GH8/10	10	Warigal
gentiobiose	18	12	GH1/4	9	Chinese Spring
gentiobiose	30/18	1	GH1/4	36	Pavon-76
gentiobiose	30/18	1	GH2/11	8	Pavon-76
gentiobiose	30/18	2	GH16/4	8	Bindawarra, Egret
gentiobiose	30/18	3	GH1/4	9	Insignia
gentiobiose	30/18	3	GH14/1	8	Pavon-76
gentiobiose	30/18	3	GH16/4	16	Egret
gentiobiose	30/18	4	GH10/12	8	Insignia
gentiobiose	30/18	4	GH2/11	8	Egret, Federation
gentiobiose	30/18	4	GH26/7	9	Gabo
gentiobiose	30/18	5	GH1/4	8	Bindawarra, Dagger, Hartog

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
gentiobiose	30/18	5	GH10/12	8	Bencubbin, Federation
gentiobiose	30/18	5	GH12/7	9	Federation
gentiobiose	30/18	5	GH13/9	8	Pavon-76
gentiobiose	30/18	5	GH14/1	8	Hartog
gentiobiose	30/18	5	GH16/4	8	Dagger, Egret
gentiobiose	30/18	5	GH16/4	9	Egret
gentiobiose	30/18	5	GH17/8	8	Dagger, Egret, Federation, Hartog, Insignia
gentiobiose	30/18	5	GH2/11	8	Bencubbin, Bindawarra, Dagger
gentiobiose	30/18	5	GH2/11	9	Bencubbin
gentiobiose	30/18	5	GH8/10	9	Dagger
gentiobiose	30/18	5	GH8/10	11	Bindawarra
gentiobiose	30/18	6	GH1/4	7	Pavon-76
gentiobiose	30/18	6	GH1/4	8	Bindawarra, Dagger, Gabo, Pavon-76
gentiobiose	30/18	6	GH1/4	9	Dagger, WW15
gentiobiose	30/18	6	GH1/4	10	Bindawarra
gentiobiose	30/18	6	GH1/4	11	Pavon-76
gentiobiose	30/18	6	GH1/4	12	Dagger
gentiobiose	30/18	6	GH10/12	8	Bencubbin, Egret, Federation, Gabo, Insignia
gentiobiose	30/18	6	GH10/12	9	Egret
gentiobiose	30/18	6	GH12/7	8	Hartog
gentiobiose	30/18	6	GH12/7	10	Pavon-76
gentiobiose	30/18	6	GH12/7	12	Bindawarra, Egret
gentiobiose	30/18	6	GH13/9	8	Pavon-76
gentiobiose	30/18	6	GH14/1	8	Hartog
gentiobiose	30/18	6	GH14/1	9	WW15
gentiobiose	30/18	6	GH16/4	8	Pavon-76
gentiobiose	30/18	6	GH17/8	8	Bindawarra, Egret, Hartog
gentiobiose	30/18	6	GH2/11	8	Bencubbin, Bindawarra, Gabo, Insignia, WW15
gentiobiose	30/18	6	GH8/10	8	Bindawarra, Insignia
gentiobiose	30/18	6	GH8/10	9	Bindawarra, Federation
gentiobiose	30/18	7	GH1/4	8	Bencubbin, Dagger
gentiobiose	30/18	7	GH1/4	10	Hartog, Insignia
gentiobiose	30/18	7	GH1/4	12	Dagger
gentiobiose	30/18	7	GH14/1	8	Hartog
gentiobiose	30/18	7	GH17/8	8	Bindawarra
gentiobiose	30/18	7	GH18/6	10	Gabo
gentiobiose	30/18	7	GH8/10	10	Bindawarra
gentiobiose	30/18	8	GH1/4	8	WW15

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
gentiobiose	30/18	8	GH1/4	11	Dagger
gentiobiose	30/18	8	GH10/12	8	Pavon-76
gentiobiose	30/18	8	GH14/1	8	Gabo
gentiobiose	30/18	8	GH17/8	8	Egret
gentiobiose	30/18	9	GH1/4	8	Dagger, Federation
gentiobiose	30/18	9	GH13/9	8	Pavon-76
gentiobiose	30/18	9	GH14/1	8	WW15
gentiobiose	30/18	10	GH14/1	8	WW15
gentiobiose	30/18	10	GH8/10	8	Insignia
gentiobiose	30/18	10	GH8/10	9	Bencubbin
gentiobiose	30/18	11	GH13/9	8	Pavon-76
gentiobiose	30/18	11	GH2/11	11	Pavon-76
gentiobiose	30/18	11	GH8/10	8	Insignia
gentiobiose	30/18	12	GH1/4	12	Egret
maltose	18	1	GH10/12	8	Atys
maltose	18	1	GH10/12	9	Orofen
maltose	18	1	GH2/11	8	anther Orofen, Atys, Orofen
maltose	18	2	GH2/11	8	Atys
maltose	18	3	GR8/8	9	Oxley
maltose	18	3	GH2/11	8	Orofen
maltose	18	3	GH28/7	3	Halberd
maltose	18	3	GH8/10	8	Atys
maltose	18	4	GR8/8	9	Machete
maltose	18	4	GH17/8	8	anther Orofen
maltose	18	4	GH8/8	4	Halberd
maltose	18	5	GR28/7	3	Chinese Spring, Oxley
maltose	18	5	GR8/8	3	Oxley
maltose	18	5	GR8/8	9	Machete
maltose	18	5	GH10/12	8	Orofen
maltose	18	5	GH14/1	8	Orofen
maltose	18	5	GH14/1	9	Orofen
maltose	18	5	GH17/8	8	anther Orofen, Atys, Orofen
maltose	18	5	GH2/11	8	Atys
maltose	18	5	GH8/8	3	Kite
maltose	18	5	GH8/8	9	Halberd
maltose	18	6	GR8/8	3	Warigal
maltose	18	6	GR8/8	4	Spear, Warigal
maltose	18	6	GR8/8	8	Machete
maltose	18	6	GR8/8	9	Oxley, Warigal
maltose	18	6	GH1/4	10	Oxley

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	18	6	GH10/12	9	Orofen
maltose	18	6	GH17/8	8	anther Orofen, Orofen
maltose	18	6	GH2/11	8	anther Orofen
maltose	18	6	GH2/11	10	Atys
maltose	18	6	GH28/7	3	Oxley
maltose	18	6	GH8/10	8	Atys, Machete
maltose	18	6	GH8/10	11	Atys
maltose	18	6	GH8/8	3	Chinese Spring, Machete
maltose	18	6	GH8/8	8	Spear
maltose	18	6	GH8/8	9	Halberd
maltose	18	7	GR8/8	3	Warigal
maltose	18	7	GH10/12	8	Orofen
maltose	18	7	GH16/4	8	Kite
maltose	18	7	GH17/8	8	anther Orofen, Atys, Orofen
maltose	18	7	GH8/10	8	Atys
maltose	18	7	GH8/8	4	Warigal
maltose	18	8	GR8/8	4	Warigal
maltose	18	8	GH14/1	8	Orofen
maltose	18	8	GH17/8	8	anther Orofen, Atys
maltose	18	8	GH28/7	3	Oxley
maltose	18	8	GH8/8	3	Machete
maltose	18	8	GH8/8	7	Chinese Spring
maltose	18	8	GH8/8	8	Spear
maltose	18	8	GH8/8	9	Chinese Spring
maltose	18	9	GR8/8	4	Warigal
maltose	18	9	GH28/7	7	Oxley
maltose	18	9	GH8/8	3	Spear
maltose	18	9	GH8/8	4	Machete
maltose	18	9	GH8/8	8	Warigal
maltose	18	9	GH8/8	9	Spear
maltose	25	1	GR28/7	3	Halberd
maltose	25	1	GR8/8	6	Schomburgk
maltose	25	3	GR28/7	3	Halberd, Machete
maltose	25	3	GR28/7	5	Halberd
maltose	25	3	GH28/7	3	Kite
maltose	25	3	GH8/8	6	Aroona, Schomburgk
maltose	25	4	GH28/7	3	Chinese Spring, Halberd
maltose	25	5	GR28/7	3	Chinese Spring, Halberd, Kite, Machete, Schomburgk, Spear
maltose	25	5	GR28/7	4	Warigal

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	25	5	GR8/8	3	Halberd
maltose	25	5	GR8/8	6	Schomburgk
maltose	25	5	GH21/6	3	Aroona, Chinese Spring, Kite, Oxley, Schomburgk, Warigal
maltose	25	5	GH28/7	3	Chinese Spring, Halberd, Kite, Schomburgk, Spear
maltose	25	5	GH28/7	5	Kite, Machete
maltose	25	5	GH8/8	3	Aroona, Kite
maltose	25	5	GR8/8	3	Aroona
maltose	25	6	GR28/7	3	Schomburgk, Warigal
maltose	25	6	GR28/7	5	Schomburgk
maltose	25	6	GR8/8	3	Schomburgk, Spear
maltose	25	6	GR8/8	4	Schomburgk
maltose	25	6	GH21/6	3	Chinese Spring, Machete, Oxley
maltose	25	6	GH28/7	3	Chinese Spring, Halberd, Machete
maltose	25	6	GH28/7	4	Kite
maltose	25	7	GR28/7	3	Schomburgk
maltose	25	7	GR8/8	4	Kite
maltose	25	7	GH21/6	3	Chinese Spring, Machete
maltose	25	7	GH28/7	3	Kite
maltose	25	7	GH8/8	3	Aroona, Schomburgk
maltose	25	7	GR28/7	5	Aroona
maltose	25	7	GR8/8	3	Aroona
maltose	25	8	GR28/7	3	Kite, Schomburgk
maltose	25	8	GR8/8	3	Halberd
maltose	25	8	GR8/8	5	Kite, Warigal
maltose	25	8	GH21/6	3	Kite
maltose	25	8	GH28/7	3	Halberd, Kite, Machete, Schomburgk, Warigal
maltose	25	8	GH28/7	4	Chinese Spring
maltose	25	8	GH8/8	3	Aroona
maltose	25	8	GR28/7	3	Aroona
maltose	25	9	GR28/7	3	Kite, Spear, Warigal
maltose	25	9	GR8/8	3	Kite
maltose	25	9	GH21/6	3	Machete, Oxley
maltose	25	9	GH21/6	5	Kite
maltose	25	9	GH28/7	3	Chinese Spring, Schomburgk, Spear
maltose	25	9	GH8/8	4	Aroona
maltose	25	9	GH8/8	6	Aroona
maltose	25	9	GR28/7	5	Aroona
maltose	25	11	GH28/7	3	Chinese Spring

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	30/18	1	GH16/4	8	Bindawarra, Pavon-76
maltose	30/18	1	GH2/11	8	Pavon-76
maltose	30/18	1	GH2/11	9	Bencubbin
maltose	30/18	2	GH18/6	8	Hartog
maltose	30/18	2	GH2/11	11	Federation
maltose	30/18	3	GR8/8	9	Oxley
maltose	30/18	3	GH10/12	8	Egret
maltose	30/18	3	GH16/4	8	Bindawarra
maltose	30/18	3	GH16/4	9	Pavon-76
maltose	30/18	3	GH18/6	11	Federation
maltose	30/18	3	GH2/11	8	Bindawarra
maltose	30/18	3	GH28/7	3	Halberd
maltose	30/18	4	GR8/8	9	Machete
maltose	30/18	4	GH16/4	8	Hartog
maltose	30/18	4	GH17/8	8	Bencubbin, Egret
maltose	30/18	4	GH2/11	8	Federation
maltose	30/18	4	GH8/10	8	Insignia
maltose	30/18	4	GH8/8	4	Halberd
maltose	30/18	5	GR28/7	3	Chinese Spring, Oxley
maltose	30/18	5	GR8/8	3	Oxley
maltose	30/18	5	GR8/8	9	Machete
maltose	30/18	5	GH1/4	7	Egret
maltose	30/18	5	GH1/4	8	Egret
maltose	30/18	5	GH1/4	9	Bencubbin
maltose	30/18	5	GH1/4	10	Hartog, Insignia
maltose	30/18	5	GH10/12	8	Egret
maltose	30/18	5	GH10/12	9	Insignia
maltose	30/18	5	GH12/7	12	Bindawarra
maltose	30/18	5	GH13/9	8	Pavon-76
maltose	30/18	5	GH14/1	8	Hartog, WW15
maltose	30/18	5	GH16/4	8	Dagger, WW15
maltose	30/18	5	GH16/4	9	Pavon-76
maltose	30/18	5	GH17/8	8	Bindawarra, Dagger, Egret, Federation, Gabo, Hartog, Insignia
maltose	30/18	5	GH18/6	8	Gabo
maltose	30/18	5	GH2/11	8	Bindawarra
maltose	30/18	5	GH2/11	11	Pavon-76
maltose	30/18	5	GH8/8	3	Kite
maltose	30/18	5	GH8/8	9	Halberd
maltose	30/18	6	GR8/8	4	Spear, Warigal
maltose	30/18	6	GR8/8	8	Machete

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	30/18	6	GR8/8	9	Oxley, Warigal
maltose	30/18	6	GH1/4	9	Dagger
maltose	30/18	6	GH1/4	11	Bencubbin, Federation
maltose	30/18	6	GH1/4	12	Federation
maltose	30/18	6	GH10/12	8	Bencubbin, Federation, Gabo, Pavon-76
maltose	30/18	6	GH10/12	9	Egret
maltose	30/18	6	GH12/7	8	Pavon-76
maltose	30/18	6	GH12/7	9	Bindawarra
maltose	30/18	6	GH14/1	9	Hartog
maltose	30/18	6	GH16/4	8	WW15
maltose	30/18	6	GH17/8	8	Bindawarra, Dagger, Egret, Federation, Gabo, Hartog, Insignia
maltose	30/18	6	GH2/11	8	Bencubbin, Dagger, Gabo, Insignia, WW15
maltose	30/18	6	GH2/11	9	Federation
maltose	30/18	6	GH2/11	10	Bencubbin, Hartog
maltose	30/18	6	GH2/11	11	Insignia
maltose	30/18	6	GH28/7	3	Oxley
maltose	30/18	6	GH8/10	8	Bindawarra, Dagger, Insignia
maltose	30/18	6	GH8/8	3	Chinese Spring, Machete, Warigal
maltose	30/18	6	GH8/8	8	Spear
maltose	30/18	6	GH8/8	9	Halberd
maltose	30/18	7	GR8/8	3	Warigal
maltose	30/18	7	GH1/4	8	Bindawarra
maltose	30/18	7	GH1/4	10	Dagger
maltose	30/18	7	GH1/4	12	Dagger
maltose	30/18	7	GH10/12	8	Pavon-76
maltose	30/18	7	GH10/12	9	Bencubbin
maltose	30/18	7	GH12/7	10	Gabo
maltose	30/18	7	GH12/7	12	Egret, Federation
maltose	30/18	7	GH13/9	8	Pavon-76
maltose	30/18	7	GH14/1	8	Hartog, WW15
maltose	30/18	7	GH14/1	9	WW15
maltose	30/18	7	GH17/8	8	Bencubbin, Bindawarra, Dagger, Federation, Insignia
maltose	30/18	7	GH2/11	8	Bencubbin, Bindawarra, Dagger, Egret, Gabo
maltose	30/18	7	GH26/7	12	Hartog
maltose	30/18	7	GH8/8	4	Warigal
maltose	30/18	8	GR8/8	4	Warigal

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	30/18	8	GH10/12	8	Bindawarra
maltose	30/18	8	GH17/8	8	Bencubbin, Dagger, Egret, Federation, WW15
maltose	30/18	8	GH28/7	3	Oxley
maltose	30/18	8	GH8/10	10	Bindawarra
maltose	30/18	8	GH8/8	3	Machete
maltose	30/18	8	GH8/8	7	Chinese Spring
maltose	30/18	8	GH8/8	8	Spear
maltose	30/18	8	GH8/8	9	Chinese Spring
maltose	30/18	9	GH8/8	9	Dagger
maltose	30/18	9	GR8/8	4	Warigal
maltose	30/18	9	GH2/11	10	Hartog
maltose	30/18	9	GH28/7	7	Oxley
maltose	30/18	9	GH8/8	3	Spear
maltose	30/18	9	GH8/8	4	Machete
maltose	30/18	9	GH8/8	8	Warigal
maltose	30/18	9	GH8/8	9	Spear
maltose	30/18	10	GH8/10	8	Bindawarra, Insignia
maltose	30/18	11	GH8/10	8	Insignia
maltose	30/18	12	GH1/4	10	Egret
maltose	30/18	12	GH8/10	10	Bencubbin
maltose	30/25	1	GR28/7	3	Halberd
maltose	30/25	1	GR8/8	6	Schomburgk
maltose	30/25	3	GR28/7	3	Halberd, Machete
maltose	30/25	3	GR28/7	5	Halberd
maltose	30/25	3	GH21/6	3	Halberd
maltose	30/25	3	GH28/7	3	Kite
maltose	30/25	3	GH8/8	6	Aroona
maltose	30/25	4	GR8/8	6	Schomburgk
maltose	30/25	4	GH21/6	3	Halberd, Warigal
maltose	30/25	4	GH28/7	3	Chinese Spring, Halberd
maltose	30/25	5	GR28/7	3	Chinese Spring, Halberd, Kite, Machete, Schomburgk, Spear
maltose	30/25	5	GR8/8	3	Halberd
maltose	30/25	5	GR8/8	6	Schomburgk
maltose	30/25	5	GH21/6	3	Aroona, Chinese Spring, Halberd, Kite, Oxley, Schomburgk, Warigal
maltose	30/25	5	GH21/6	4	Aroona
maltose	30/25	5	GH21/6	6	Aroona, Kite
maltose	30/25	5	GH28/7	3	Chinese Spring, Halberd, Kite, Schomburgk, Spear
maltose	30/25	5	GH28/7	5	Kite, Machete

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	30/25	5	GH8/8	3	Aroona, Kite
maltose	30/25	5	GR8/8	3	Aroona
maltose	30/25	6	GR28/7	3	Kite, Schomburgk, Warigal
maltose	30/25	6	GR28/7	5	Schomburgk
maltose	30/25	6	GR8/8	3	Schomburgk, Spear
maltose	30/25	6	GR8/8	4	Schomburgk
maltose	30/25	6	GH21/6	3	Chinese Spring, Machete, Oxley
maltose	30/25	6	GH28/7	3	Halberd, Machete
maltose	30/25	6	GH28/7	4	Kite
maltose	30/25	6	GR28/7	3	Aroona
maltose	30/25	7	GR28/7	3	Schomburgk, Spear
maltose	30/25	7	GR8/8	4	Kite
maltose	30/25	7	GH21/6	3	Aroona, Chinese Spring, Machete, Oxley, Schomburgk, Warigal
maltose	30/25	7	GH28/7	3	Kite
maltose	30/25	7	GH8/8	3	Aroona, Schomburgk
maltose	30/25	7	GR28/7	3	Aroona
maltose	30/25	7	GR28/7	5	Aroona
maltose	30/25	8	GR28/7	3	Kite, Machete, Schomburgk, Spear
maltose	30/25	8	GR8/8	3	Halberd
maltose	30/25	8	GR8/8	5	Kite, Warigal
maltose	30/25	8	GH21/6	3	Aroona, Halberd, Kite, Machete, Oxley, Schomburgk, Warigal
maltose	30/25	8	GH21/6	4	Schomburgk
maltose	30/25	8	GH28/7	3	Halberd, Kite, Machete, Schomburgk, Warigal
maltose	30/25	8	GH28/7	4	Chinese Spring, Machete
maltose	30/25	8	GH8/8	3	Aroona
maltose	30/25	8	GR28/7	3	Aroona
maltose	30/25	9	GR28/7	3	Kite, Spear, Warigal
maltose	30/25	9	GR8/8	3	Kite
maltose	30/25	9	GH21/6	3	Aroona, Chinese Spring, Kite, Machete, Oxley, Schomburgk, Warigal
maltose	30/25	9	GH21/6	4	Aroona, Schomburgk, Warigal
maltose	30/25	9	GH21/6	5	Kite
maltose	30/25	9	GH21/6	6	Halberd
maltose	30/25	9	GH28/7	3	Chinese Spring, Schomburgk, Spear
maltose	30/25	9	GH8/8	4	Aroona
maltose	30/25	9	GH8/8	6	Aroona
maltose	30/25	9	GR28/7	5	Aroona
maltose	30/25	10	GH21/6	3	Oxley

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
maltose	30/25	10	GH21/6	6	Schomburgk
maltose	30/25	11	GH28/7	3	Chinese Spring
sucrose	18	4	GH2/11	8	Chinese Spring
sucrose	18	5	GH1/4	12	Chinese Spring
sucrose	18	5	GH10/12	8	Oxley
sucrose	18	5	GH2/11	8	Oxley
sucrose	18	5	GH8/10	8	Atys
sucrose	18	6	GH1/4	8	Chinese Spring
sucrose	18	6	GH1/4	10	Chinese Spring
sucrose	18	6	GH1/4	11	Chinese Spring
sucrose	18	6	GH10/12	8	Chinese Spring
sucrose	18	6	GH17/8	8	Chinese Spring
sucrose	18	6	GH2/11	8	Chinese Spring, Oxley
sucrose	18	6	GH8/10	8	Chinese Spring
sucrose	18	6	GH8/10	11	Oxley
sucrose	18	7	GH1/4	8	Oxley
sucrose	18	7	GH16/4	7	Schomburgk
sucrose	18	7	GH17/8	8	Chinese Spring, Oxley
sucrose	18	7	GH17/8	9	Oxley
sucrose	18	7	GH2/11	8	Oxley
sucrose	18	8	GH10/12	8	Chinese Spring, Oxley
sucrose	18	8	GH17/8	8	Oxley
sucrose	18	8	GH2/11	8	Oxley
sucrose	18	8	GH8/10	9	Oxley
sucrose	18	9	GH10/12	9	Chinese Spring
sucrose	18	9	GH17/8	8	Oxley
sucrose	18	10	GH2/11	8	Chinese Spring
sucrose	18	10	GH8/10	8	Chinese Spring
sucrose	18	11	GH8/10	8	Chinese Spring
sucrose	30/18	1	GH1/4	9	Insignia
sucrose	30/18	1	GH10/12	8	Bencubbin
sucrose	30/18	1	GH10/12	9	Bindawarra, Dagger
sucrose	30/18	1	GH14/1	8	Gabo
sucrose	30/18	1	GH16/4	8	Bindawarra
sucrose	30/18	1	GH2/11	8	Insignia
sucrose	30/18	1	GH8/10	9	Hartog
sucrose	30/18	2	GH16/4	8	Egret
sucrose	30/18	2	GH2/11	11	Federation
sucrose	30/18	3	GH10/12	8	Egret
sucrose	30/18	3	GH16/4	9	Pavon-76

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
sucrose	30/18	3	GH18/6	8	Hartog
sucrose	30/18	3	GH2/11	8	Federation
sucrose	30/18	4	GH1/4	9	Bindawarra
sucrose	30/18	4	GH17/8	8	Egret
sucrose	30/18	4	GH2/11	8	Federation
sucrose	30/18	5	GH1/4	7	Hartog
sucrose	30/18	5	GH1/4	8	Egret
sucrose	30/18	5	GH10/12	8	Bencubbin, Dagger, Egret, Federation
sucrose	30/18	5	GH10/12	9	Egret
sucrose	30/18	5	GH13/9	8	Pavon-76
sucrose	30/18	5	GH16/4	8	Egret
sucrose	30/18	5	GH17/8	8	Bindawarra, Dagger, Egret, Federation, Gabo, Insignia
sucrose	30/18	5	GH17/8	9	Bindawarra
sucrose	30/18	5	GH2/11	8	Bencubbin, Bindawarra, Federation, Hartog, WW15
sucrose	30/18	5	GH2/11	9	Bencubbin
sucrose	30/18	5	GH2/11	10	Federation
sucrose	30/18	5	GH8/10	8	Dagger
sucrose	30/18	5	GH8/10	10	Insignia
sucrose	30/18	6	GH1/4	7	Pavon-76
sucrose	30/18	6	GH1/4	8	Bindawarra
sucrose	30/18	6	GH1/4	9	Dagger
sucrose	30/18	6	GH1/4	10	Bindawarra, Dagger, Federation
sucrose	30/18	6	GH1/4	11	Dagger
sucrose	30/18	6	GH10/12	8	Dagger, Egret, Federation, Gabo, WW15
sucrose	30/18	6	GH12/7	10	Hartog
sucrose	30/18	6	GH12/7	11	Pavon-76
sucrose	30/18	6	GH13/9	8	Pavon-76
sucrose	30/18	6	GH14/1	8	Pavon-76, WW15
sucrose	30/18	6	GH16/4	8	Gabo
sucrose	30/18	6	GH16/4	9	Hartog
sucrose	30/18	6	GH17/8	8	Bencubbin, Bindawarra, Dagger, Egret, Gabo, Hartog, Insignia, WW15
sucrose	30/18	6	GH17/8	9	Bencubbin, Bindawarra, Dagger
sucrose	30/18	6	GH18/6	9	Dagger
sucrose	30/18	6	GH2/11	8	Bencubbin, Dagger, Gabo, Insignia, WW15
sucrose	30/18	6	GH2/11	9	Dagger, Federation

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
sucrose	30/18	6	GH2/11	10	Federation, Hartog
sucrose	30/18	6	GH2/11	11	WW15
sucrose	30/18	7	GH10/12	8	Bencubbin, Dagger, Egret, Federation, Gabo
sucrose	30/18	7	GH10/12	9	Pavon-76
sucrose	30/18	7	GH12/7	12	Egret
sucrose	30/18	7	GH13/9	8	Pavon-76
sucrose	30/18	7	GH14/1	8	Pavon-76
sucrose	30/18	7	GH17/8	8	Bencubbin, Bindawarra, Dagger, Hartog, Insignia, WW15
sucrose	30/18	7	GH2/11	8	Bencubbin, Gabo
sucrose	30/18	8	GH1/4	12	Insignia
sucrose	30/18	8	GH10/12	8	Bencubbin, Federation, Pavon-76
sucrose	30/18	8	GH10/12	9	Bencubbin
sucrose	30/18	8	GH17/8	8	Bencubbin, Dagger, Federation
sucrose	30/18	8	GH8/10	10	Bindawarra
sucrose	30/18	9	GH13/9	8	Pavon-76
sucrose	30/18	9	GH2/11	10	Hartog
sucrose	30/18	11	GH13/9	8	Pavon-76
sucrose	30/18	11	GH17/8	8	Egret
sucrose	30/18	11	GH2/11	11	Pavon-76
sucrose	30/18	12	GH1/4	9	Egret
sucrose	30/18	12	GH1/4	11	Egret
sucrose	30/18	12	GH8/10	10	Bencubbin
sucrose	30/25	3	GR16/11	8	Halberd
sucrose	30/25	4	GR16/1	8	Halberd
sucrose	30/25	4	GH16/11	4	anther Orofen
sucrose	30/25	4	GH16/11	8	Halberd
sucrose	30/25	5	GR16/1	8	Halberd, Machete, Spear, Warigal
sucrose	30/25	5	GR16/1	9	Machete
sucrose	30/25	5	GR16/1	10	Atys, Machete
sucrose	30/25	5	GR16/1	11	Halberd
sucrose	30/25	5	GR16/11	3	Halberd
sucrose	30/25	5	GR16/11	4	Atys, Halberd, Machete, Spear
sucrose	30/25	5	GR16/11	8	Atys, Halberd, Kite, Machete, Spear
sucrose	30/25	5	GH16/1	8	anther Orofen
sucrose	30/25	5	GH16/11	3	Halberd
sucrose	30/25	5	GH16/11	8	Atys, Halberd
sucrose	30/25	6	GR16/1	8	Kite, Machete, Spear, Warigal
sucrose	30/25	6	GR16/1	9	Halberd
sucrose	30/25	6	GR16/1	10	Atys, Halberd, Machete

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
sucrose	30/25	6	GR16/1	11	Atys, Machete, Schomburgk, Warigal
sucrose	30/25	6	GR16/11	4	Halberd, Spear
sucrose	30/25	6	GR16/11	8	Atys, Halberd, Kite, Machete, Spear
sucrose	30/25	6	GR16/11	9	Schomburgk
sucrose	30/25	6	GH16/1	8	Warigal
sucrose	30/25	6	GH16/1	9	Warigal
sucrose	30/25	6	GH16/1	11	Warigal
sucrose	30/25	6	GH16/11	4	Atys, Machete
sucrose	30/25	6	GH16/11	8	Atys, Halberd, Warigal
sucrose	30/25	6	GH16/11	9	Atys
sucrose	30/25	6	GR16/1	10	Aroona
sucrose	30/25	6	GR16/11	3	Aroona
sucrose	30/25	6	GR16/11	8	Aroona
sucrose	30/25	7	GR16/1	8	Machete, Spear, Warigal
sucrose	30/25	7	GR16/1	9	Atys, Kite, Spear
sucrose	30/25	7	GR16/1	10	Halberd, Warigal
sucrose	30/25	7	GR16/1	11	Machete, Spear
sucrose	30/25	7	GR16/11	3	Schomburgk, Spear
sucrose	30/25	7	GR16/11	4	Machete, Schomburgk, Spear
sucrose	30/25	7	GR16/11	8	Machete, Schomburgk, Spear
sucrose	30/25	7	GR16/11	9	Schomburgk
sucrose	30/25	7	GR16/11	10	Schomburgk
sucrose	30/25	7	GH16/1	9	Warigal
sucrose	30/25	7	GH16/11	8	Atys, Spear
sucrose	30/25	7	GH16/11	9	Atys, Orofen
sucrose	30/25	7	GR16/1	8	Aroona
sucrose	30/25	7	GR16/11	4	Aroona
sucrose	30/25	8	GR16/1	8	Halberd, Spear, Warigal
sucrose	30/25	8	GR16/1	9	Warigal
sucrose	30/25	8	GR16/1	10	Halberd, Spear
sucrose	30/25	8	GR16/11	3	Atys
sucrose	30/25	8	GR16/11	4	Atys
sucrose	30/25	8	GR16/11	8	Kite, Orofen, Schomburgk
sucrose	30/25	8	GR16/11	9	Atys, Schomburgk
sucrose	30/25	8	GH16/11	4	Halberd, Machete, Spear
sucrose	30/25	8	GH16/11	8	Orofen
sucrose	30/25	9	GR16/11	3	Atys, Schomburgk
sucrose	30/25	9	GR16/11	4	Atys, Spear
sucrose	30/25	9	GR16/11	8	Kite, Machete, Spear
sucrose	30/25	9	GH16/11	3	anther Orofen
sucrose	30/25	9	GH16/11	8	anther Orofen, Halberd, Schomburgk, Spear

Sugar	Incubation temperature (°C)	Microspore stage	Donor plant environment	Cold pretreatment (days)	Genotype
sucrose	30/25	9	GR16/1	11	Aroona
sucrose	30/25	10	GR16/11	8	Kite
sucrose	30/25	10	GR16/11	10	Schomburgk
sucrose	30/25	10	GH16/11	8	Kite
sucrose	30/25	10	GH16/11	9	Atys
sucrose	30/25	10	GR16/11	8	Aroona
sucrose	30/25	12	GR16/11	9	Atys
sucrose	30/25	12	GR16/11	8	Aroona

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