PLANT REGENERATION FROM MICROSPORES OF BARLEY *Hordeum vulgare* L.

by

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Dedication

I dedicate this thesis to my wife Beverley and our daughters, Rebecca and Gabrielle. Their support has sustained me throughout the duration of this project. Should any good thing come out of this work it is thanks to their sacrifice.

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I wish to thank Dr. Robin S. Nelson for his friendship, guidance and encouragement, especially during the writing of this thesis. I should also like to thank Dr. Sinclair Mantell, my academic supervisor, and my Industrial supervisor, Dr. Rob L. Lyne of Shell Research Limited, for their continued interest and advice over the period of 1983-1987.

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Abstract

There have been a number of reports on the differentiation of plants from immature microspores of barley. However, the efficiencies of plant recovery reported were extremely low. The aim of this study was to develop the techniques of barley anther culture, with a view to improving the efficiency of plant production and to increase scientific understanding of some of the processes involved in embryogenesis.

Microspore embryogenesis (androgenesis) occurred only in anther lobes protected from direct contact with the culture medium. Any direct contact of these with the medium before day 21 of culture was fatal for androgenic structures formed. Mean green plant yields per 100 anthers cultured were increased from 9 to greater than 20 by orientating anthers "on-edge" at the start of culture. Improved efficiency of plant production was due to increased numbers of anthers responding rather than greater plant production per responding anther.

The morphology of androgenic structures varied from callus to well organised embryo-like structures. Plants only developed from the embryo-like structures.

The influence of media and their components on the development of immature zygotic embryos and androgenic structures in vitro was investigated. Sucrose, or a metabolite thereof, was found to inhibit embryogenesis. Replacing sucrose with alternative carbohydrate sources overcame the inhibition and permitted embryogenesis. As a result mean green plant yields per 100 anthers cultured were increased to greater than 120.

The density of anthers and/or isolated microspores in culture was found to affect pollen embryogenesis. Optimisation of anther density with other cultural factors have resulted in mean green plant yields of greater than 1750 per 100 anthers cultured.

In excess of 30% of the pollen in some anthers developed into embryos. However, the major drawback was the inability of the culture system to sustain development of the majority of embryos initiated to plants. The likely reasons for this are discussed.

The use of alternative carbohydrate sources permitted the efficient culture of microspores isolated from anthers. Up to 1% of the total donor anther pollen developed into plants; of these, more than 75 % were green.

One hundred microspore-derived barley plants were grown to maturity. Of these 70% were fertile and appeared normal when compared to plants of the same variety grown from seed. The remaining 30% were only distinguishable from seed-derived plants after tiller emergence when they were observed to be phenotypically haploid.

In conclusion, a model for explaining the observed effects of cultural conditions on barley pollen embryogenesis is put forward and discussed in the light of current knowledge of androgenesis in plants.

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Chapter 1

General introduction

1.1 Introduction

Man is heavily dependent upon plants for shelter, fuel and food. This dependence has led him to manipulate their development in an effort to produce varieties with characters which suited his needs more closely. Modern crop cultivars have been developed from wild types by a combination of careful plant selection, crop management and plant breeding. The earliest implementations of crop management are known to have occurred in southwestern Asia as long as 10 thousand years ago (Allard, 1960). It is likely that plant selection, in some form, predated this. By comparison, plant breeding is a more recent technique. Only with the rediscovery and verification of Mendel's work in 1900, did scientific breeding begin to evolve into the methods currently used. Such "conventional" methods are limited, to a large extent, by incompatibility barriers within and between plant groups. The advent of *in vitro* plant tissue culture techniques has permitted the development of many novel breeding methods to augment and to supplement the "conventional" arsenal of the breeder.

An example of one such technique, that could soon have an impact on the "conventional" breeding methods, is anther culture in cereal crops such as barley (*Hordeum vulgare* L.). Barley is a major cereal crop which is used widely in industry as a brewing substrate and in agriculture as an animal feed stock. It is naturally self-fertile, so that offspring can be derived which are homozygous for many obvious phenotypic characters (Briggs, 1978). Conventional "pedigree" breeding involves the generation of genetic variability by artificially crossing two selected parents to produce an F1 generation. Selection of individuals begins in the F2 generation and

this process may continue with selfing in order to attain homozygosity for the desired phenotypes well into the F8 or F10 generations. Breeding objectives that were either technically or economically unrealistic with such "pedigree" breeding methods may be attempted now with the aid of tissue culture techniques since homozygous breeding lines can be produced more rapidly.

The production of haploid plants, ie they contain only one set of unpaired chromosomes, and dihaploid plants (see below) using in vitro techniques is now feasible. Their potential as valuable breeding materials has been recognised since the early part of this century (Blakeslee et al, 1924). Haploid plants contain the gametophytic number of chromosomes in the sporophyte. (In true diploid species a single genome copy is present in uninucleate pollen before it enters the "S" phase of the cell cycle (Moment et al, 1973). It was hypothesised that doubling the chromosomes of the haploid lines would produce a plant with two identical sets of chromosomes (Blakeslee et al, 1924). Doubled haploids (dihaploids) would allow, therefore, the development of completely homozygous lines from heterozygous parents within a single generation. This has distinct advantages for dioecious species and out-breeding plants, the self-incompatibility mechanisms of which prevent inbreeding. Using dihaploids in inbreeding species such as barley will reduce the many generations normally needed to obtain homozygosity and will simplify genetic studies.

Occasionally, haploid plants occur spontaneously within normal plant populations, but these events are so rare, and are limited to so few species, that their value as research and/or breeding tools is

limited (Kimber et al, 1963). An early example of a higher plant haploid was described by Blakeslee et al (1922) in Datura stramonium. Since then, examples have been recorded in a variety of species (See Nitzsche et al, 1977, for examples).

There are several techniques currently available for the production of haploid and dihaploid plants. These range from methods involving specific genotypes, eg the hap gene in barley (Nitzsche et al, 1977), through chromosome elimination techniques (Kasha, 1974) to the regeneration of plants from cultures of immature ovaries (San Noeum, 1976), anthers and pollen (eg Guha et al). Despite considerable research in recent years, these techniques have had only limited success.

An ideal method for haploid/dihaploid production must be capable of consistently producing large numbers of homozygous individuals in which there should be no genotypic variation in response and regenerations from their hybrid populations should not be biased, ie the phenotypic variations within the population of regenerates should be similar to those observed in a typical F2 generation resulting from a conventional cross. Most importantly, however, the whole process must be efficient in terms of resource input (Snape, 1982).

The use of pollen as a source of haploid plants has several potential advantages over other techniques currently available. For example, pollen is abundant and each pollen cell is a discrete unit containing a single genome copy donated during meiosis (Sunderland, 1973). However, a major requirement for its use is a reproducible method for the consistent regeneration of plants from individual

pollen cells.

Induction of callus from cultures of mature pollen from the gymnosperm Ginkgo biloba was reported as early as 1953 (Tulecke, 1953). However, the earliest reports of plant regeneration from cultured anthers of angiosperms were in the Solanaceous species, Datura innoxia (Guha et al, 1964). Pollen grains within anthers were induced to divide and underwent direct embryogenesis; the formation of haploid plants was demonstrated subsequently (Guha et al, 1966).

Initially, high frequencies of plant regeneration from pollen could only be demonstrated from a few model species (of the families Graminae and Solanaceae). Many more species only responded weakly, if at all (Sunderland, 1971 and 1973). The reasons for such weak responses are unclear though much information has become available recently on several systems including anther and pollen culture of barley (Foroughi-Wehr et al, 1983,1984; Sunderland et al, 1982,b). The full potential of barley anther culture has yet to be realised. Over the last decade the importance of anther pretreatment prior to culture has been demonstrated and much fundamental research has been undertaken on the early stages of microspore morphogenesis (Sunderland, 1982).

1.2 Developmental pathways of pollen in vivo

Pollen is produced inside anthers in sacs enveloped by a layer of polyploid, nutritive cells (the tapetum). The pollen develops in a highly characteristic manner in the form of separate, relatively synchronous units (Figure 1.2.1).

"Normal" microsporogenesis can be divided into three phases

characterised by a specific series of cellular events. These are:- Phase 1 in which pollen tetrads form from pollen mother cells, Phase 2 in which individual microspores dissociate and in Phase 3 in which microspores mature into pollen grains. Phase 2 ends and 3 begins with first pollen mitosis (Sunderland, 1973).

Sunderland, (1973), described pollen development in the angiosperm Nicotiana tabacum. Tetrads of spores formed at meiosis dissociate from the surrounding callose matrix during Phase 1. At the beginning of Phase 2 they are thin-walled, spherical cells ca 15μm in diameter and have a high nuclear/cytoplasmic ratio, with the nucleus being ca 10μm in diameter. When released as mature pollen at the end of Phase 3 they are bicellular and are ca 40μm in diameter. This increase in diameter is mainly due to vacuolation during Phase 2 but, after the first division, the vacuole disappears and active cytoplasmic synthesis accompanies still further enlargement.

In Phase 2, the cell wall develops and sporopollenin is deposited on the external surface of the spores as they enlarge. Within this tough protective exine a typical cellulosic wall, the intine, forms (Sunderland, 1973). Vacuole formation pushes the nucleus to the side of the spore. This is followed by nuclear replication and the start of Phase 3, ie first pollen mitosis.

Asymmetric division (Figure 1.2.1) forms two unequal cells (Sax et al 1933; LaCour, 1949). These are the double-walled vegetative cell and, within it, the smaller generative cell which lacks a true cell wall.

Similarly in Beta, generative cell walls show no specific staining for carbohydrate or pectin substances (Hoefert, 1969). At maturity,

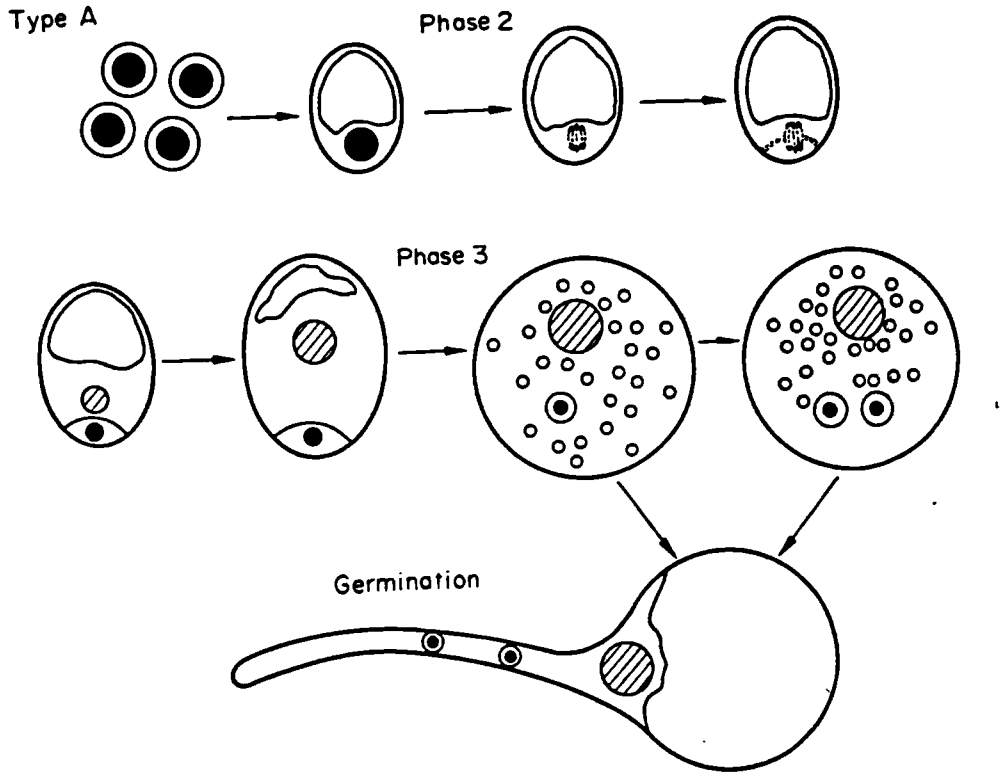


Figure 1.2.1 Normal developmental sequence of an angiosperm pollen grain following its release from the tetrad as a uninucleate microspore (Sunderland, 1973).

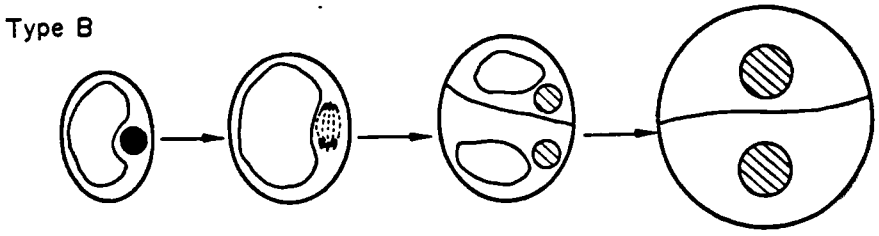


Figure 1.2.2. Anomalous development sequence in an angiosperm pollen grain following its release from the tetrad as a uninucleate microspore (Sunderland, 1973).

the vegetative cell of N. tabacum has a densely basophilic cytoplasm and diffuse nucleus, which stains lightly with acetocarmine stain. In contrast the generative cell has a lightly staining basophilic cytoplasm with a highly condensed, deeply staining nucleus.

The vacuole is reabsorbed and then, after the generative cell is released from the intine, the second mitosis occurs. Two sperm cells with highly condensed nuclei are produced as a result.

Apparently, errors in the process of normal pollen development are common. Two of the most frequent abnormalities are as follows: a) two equal-sized nuclei are formed at first pollen mitosis after which a cell wall is not always formed between the products (Figure 1.2.2), and b) the vegetative cell continues to divide into two equal cells. These abnormalities have been observed in many species of both Gramineae and Solanaceae (Sunderland, 1978).

The atypical mitotic patterns are often associated with abnormal synthesis activity after the first pollen mitosis. Subsequent gametophytic synthesis is restricted and a dimorphic population of pollen is formed as a result. Atypical pollen is smaller than normal and has a low affinity for acetocarmine stain, a reflection of low RNA and protein content (Dunwell, 1978). In mature anthers, these atypical pollen may have up to 4 cells and make up 0.4-13.5% of the total pollen (Dunwell, 1978).

In most species observed, irregular divisions occur at low and variable frequencies. However, various external agents (eg temperature changes and X-rays), if applied to the donor plant, have been shown to increase the frequency of these abnormalities

(Sunderland et al, 1977; Dunwell, 1978). Excision of anthers has also been shown to disrupt pollen development (Dunwell, 1978; 1981b). While in vivo, pollen has never been found with more than four cells.

1.3 In vitro development of immature microspores

Four developmental pathways may occur in immature pollen in culture (Dunwell, 1978). These are where, I) Embryo development occurs from repeated divisions of the vegetative cell, II) Embryos are derived from repeated symmetrical divisions of the microspore nucleus, III) the vegetative and generative nuclei may fuse and subsequently divide to form two symmetrical nuclei, and IV) Embryo development may occur from repeated divisions of the generative cell. The fourth pathway is less common but has been reported to occur in Hyoscyamus niger, (Raghavan, 1976a, 1977a, 1978).

According to Dunwell (1978), the first pollen mitosis in culture is less asymmetric than in vivo and consequently it is unwise to refer to the products as truly vegetative and generative nuclei. The only accurate method of analysing cell lineages in developing structures would be by genetically labelling the two pollen nuclei at culture initiation (Dunwell, 1978).

The efficiency of morphogenesis and subsequent development of microspore-derived structures is known to be influenced by both genetic and non-genetic factors. They may act throughout, or at specific stages in development. Microspores appear to be diverted from normal pollen development towards embryogenesis at some point between the early uninucleate and late binucleate stages. It is most likely that donor plant genotype, physiological condition and the

conditions used for culture influence the stage at which microspore development is diverted to embryogenesis. Information currently available on these factors is presented below.

1.4 Donor plants

Perhaps the most important influence throughout anther culture is the donor genotype (Sunderland, 1980; Genovesi et al, 1982). The genotype of the donor plant is the genetic base-line which dictates how the pollen develops initially, in vivo and subsequently how it will respond in vitro.

There are numerous reports which suggest that species within the same genus and cultivars within the same species respond differently in anther culture (reviewed by Dunwell, 1985).

The recombination of traits of high frequency response has been reported for several species (Misoo et al, 1978; Foroughi-Wehr et al, 1982; Maheshwari et al, 1983; Foroughi-Wehr et al, 1984; Dunwell, 1985). This suggests that specific genes may be associated with anther culture 'responsiveness'. Miah et al (1985) studied anther culture in rice (Oryza japonica and O.indica). Using two japonica and two indica cultivars as parents they carried out a four parent diallel analysis. Anther responsiveness was inherited as a recessive character determined by a single block of genes. Chowdhury (1985) used six genotypes of Nicotiana rustica to study interaction between genotype and environment and their effect on anther response. He found significant parental differences within and between crosses, which indicated that anther responsiveness was affected by additive gene action.

The development of the donor plant is also affected by non-genetic factors. The environment in which the donor plants are grown affects their physiological condition and in turn, affects the condition of their pollen and ultimately its potential to develop into multicellular structures. Dunwell, (1976) showed that both photoperiod and light intensity during donor plant growth affected subsequent microspore embryo and plant yields. Since that work, growth temperature has been shown to affect subsequent anther response (Keller et al, 1978a; Foroughi-Wehr et al, 1979; Lyne et al, 1986; Thurling et al, 1984), as has vernalisation time (Keller et al, 1983; Lazar et al 1984).

Sunderland (1978) and Tsay (1981; 1982) examined the effect of some nutritional aspects of donor plants of tobacco, in particular nitrogen status, on anther culture response. Sunderland found that plants starved of nitrogen responded best. Contrary to this Tsay, who looked at a range of five different nitrogen concentrations (0, 1.5, 4.5, 15.0, 45.0 mM), found that donor plants grown in liquid culture containing 15 mM. nitrogen responded best in culture. The nitrogen sources used by Tsay varied between $Ca(NO_3).4H_2O$, NH_4NO_3 and KNO_3, the combinations of which varied between treatments. However, as Dunwell (1986a) points out the donor plants were grown in the glasshouse and interpretation of the results may have been confounded by environmental variation during the flowering period of different plant batches.

The age of the donor plant is also known to affect anther culture response of several species including tobacco (Dunwell, 1976); rape (Thurling et al, 1984); rice (Lupotto, 1982); wheat (Jing et al,

1982); triticale (Orlikowska, 1977). The first flowers harvested always gave higher yields of regenerated plants than those produced later. This apparent fall-off of anther culture response may be due to degeneration of microspore quality. However, it may be the result of inconsistencies in the selection of microspores for culture.

1.5 Optimal microspore stage for best response in cultures

It is widely recognised that the stage of microspore development at the time of flower harvest is critical (Dunwell, 1986a,b). The stage of pollen development which gives optimal androgenesis varies between species (Table 1.5), and even varieties of the same species (Foroughi-Wehr et al, 1984). The relative developmental stages are set out in Figure 1.5.

Table 1.5.

Examples of the optimal pollen stages for androgenesis observed in four different anther culture systems.

Species	Pollen Stage	Reference
Nicotiana tabacum	First Pollen Grain Mitosis	Sunderland et al (1971)
Hyoscyamus niger	Uninucleate Microspore	Raghavan (1975)
Nicotiana knightiana	Post Mitotic Pollen	Sunderland (1979)
Hordeum vulgare	Miduninucleate	Sunderland et al (1980)

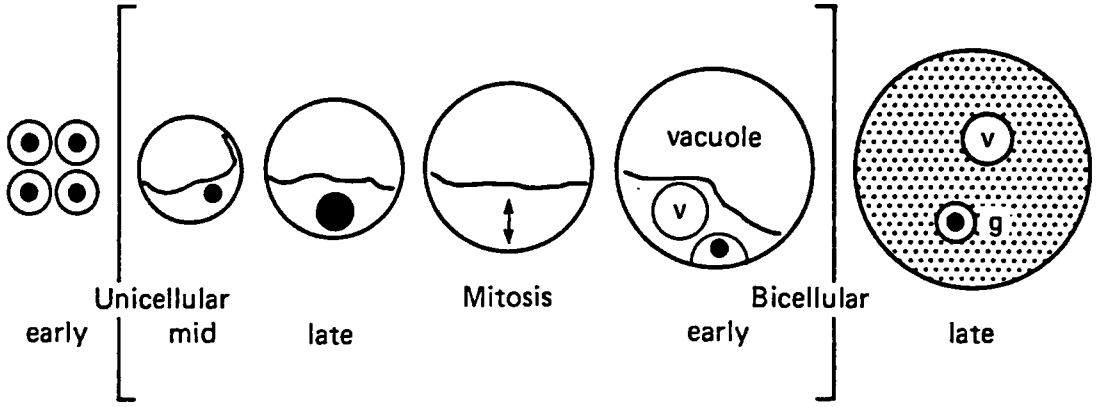


Figure 1.5.1 Diagram indicating the nomenclature for developmental stages in pollen ontogeny (Sunderland, 1973).
The term "microspore" (or spore) is used to denote the haploid cells formed by meiosis. After release from the pollen meiocytes, the spores enlarge and undergo an unequal division to give the generative (g) and the vegetative (v) cells. These two cells comprise the male gametophyte or pollen grain.

1.6 Pollen pretreatment

If the anthers or pollen are cultured directly after removal of the flowers from the plant, their induction frequencies are almost invariably low (Sunderland 1978; 1979; Sunderland et al, 1979b,c; Huang et al, 1982). In most cases, only a small proportion of the induced pollen survives to emerge from the anther. Induction frequencies can be dramatically enhanced by suitable pretreatment of excised flowers or anthers before culture (Nitsch et al, 1973; Keller et al, 1979; Genovesi et al 1979; Imamura et al, 1981; Klimazewska et al, 1983). The physical environment of the pollen, ie whether it is in an intact excised tiller, flower, spike or anther, and the type of culture vessel used, ie plastic/glass, petri dish/flask with or without water, has been shown to influence the effect of any pretreatments on anther responses (Huang et al, 1982).

A variety of pretreatments have been shown to influence anther culture response (viz Section 3.2. for definition of anther responsiveness). These include low and high temperature (Nitsch et al, 1973; Rashid et al, 1980; Huang et al, 1982;), reduced atmospheric pressure (Nitsch et al, 1973; Imamura et al, 1981), anaerobiosis (Imamura et al, 1981), water stress, saturated atmosphere (Dunwell, 1981b) and centrifugation (Sangwan-Noreel, 1977).

Almost all of these pretreatments appear to impose stress on the pollen in some way. Some authors include flower and anther excision and/or surface sterilisation as stress pretreatments (Sangwan-Noreel, 1977). This is quite feasible but difficult, if not impossible, to quantify. However, there is evidence that embryogenic induction in

tobacco pollen is independent of external media stimuli (Dunwell, 1981b). Dunwell (1981b) suggested that anther dissection is the trigger which changes pollen development. He also found beneficial effects on anther responses by pretreating excised tobacco anthers in a water-saturated atmosphere for 2-3 days. Anther response and especially plant production were greatly enhanced, the former being almost doubled and the latter more than quadrupled. This pretreatment was thought to be stressful (Dunwell, 1981b), but it may actually reduce the water stress which may occur when anthers are placed into culture by decreasing any dehydration effects. The pollen utilises nutrients from the anther until it can no longer meet the nutritional requirements of the developing structures. This may be after the fourth day after excision since response falls-off dramatically if pretreatment continues beyond this point (Dunwell, 1981b).

A number of workers have used centrifugation as a pretreatment (Sangwan-Noreel, 1977; Dunbar, 1979; Zhu De Yao, 1982). Sangwan-Noreel (1977) claims to have improved androgenic response in Datura innoxia by centrifugation in conjunction with temperature pretreatment. More recently, Zhu De Yao (1982) reported an improvement in androgenesis following centrifugation.

Centrifugation has been shown to stimulate other tissues in culture (Bonga et al, 1975; Kawase, 1964), but how centrifugation enhances anther culture response remains unclear. Cytological examination of anthers of several angiosperms revealed displacement of cell organelles (Dunbar, 1979) which, as may be expected, varies with the centrifugal force applied and time of treatment. Thirty minute's centrifugation at 1.0-1.2x10g of Lilium henryi pollen is thought to

affect pollen wall formation (Heslop-Harrison, 1971). With Pisum sativum pollen, most cell organelles are redistributed (ie displaced to the side of the cell) after two hours centrifugation (Bouck, 1963). Exactly how organelle redistribution might affect anther culture response is unclear. Other short-term pretreatments involving reduced atmospheric pressure and/or anaerobiosis have been reported to stimulate pollen embryogenesis in tobacco (Imamura et al, 1981). Both types of pretreatment may have the same mode of action, eg by affecting oxygen levels within the tissue systems. It has been suggested that pretreatment at reduced pressures for 30 minutes or "quasi-anaerobic respiration", ie 95% nitrogen + 5% oxygen, reduced the dissolved oxygen within the system leading to anaerobiosis which stimulated pollen embryogenesis. Early respiration activity during the first 15 minutes of pretreatment was thought to be via the Pentose-phosphate pathway. This subsequently shifted to the Embden-Meyerhof pathway (Roberts, 1969). This was supported by evidence of KCN sensitivity (Imamura et al, 1981). They suggested that this shift may, in some way, be comparable to the shift in metabolic pathways often present at seed germination (Imamura et al, 1981).

1.7 Temperature pretreatment

Temperature pretreatment is, perhaps, the most widely used pretreament (eg Huang et al, 1982). Both high and low temperatures are used routinely. High temperature pretreatment, 30-35C for 1-2 days, is an absolute requirement for obtaining anther response in Brassica napus (Dunwell, 1985a,b; Keller et al, 1978a) and B.campestris (Keller et al, 1978b). Temperature pretreatment is known to enhance anther culture response (Dunwell, 1986a). Nitsch et al

(1973) first demonstrated the importance of cold pretreatment with Datura innoxia. Since then, examples with other Solanaceae (Sunderland et al, 1979b,c; Chowdhury, 1985) and Graminae (Dunwell, 1986a) have been reported. Fouletier (1974) advocated the use of cold pretreatment with anther cultures of rice. Now cold pretreatment is commonly used to stimulate high levels of response in anther and pollen cultures of the Graminae species studied to date (Zeng et al, 1980; Sozinov et al, 1981; Huang et al, 1982; Qu et al, 1982; Fitch et al, 1983).

1.8 Local environment during pollen development

The mechanism(s) by which microspores develop into multicellular structures and then into plants remains unexplained. However, it is known that the biochemical environment of pollen during pretreatment differs from normal in vivo pollen development. The biochemical constituents of anthers vary between anthers prior to culture, at different stages of maturity in vivo, and during microsporogenesis (Hinchee et al, 1984). Dramatic changes in endogenous levels of substances known to influence morphogenesis such as hormones (Norstog, 1970; Johansson et al, 1977; Umbeck et al, 1979; Daie et al, 1981; Tanimoto et al, 1984), polyamines (Bagni et al, 1984; Galston, 1983; Altman et al, 1981; Apelbaum et al, 1981; Bradley et al, 1984), sugars (Hinchee et al, 1984; van den Ende et al, 1984; Tran Thanh Van et al, 1985) and amino acids (Dunwell, 1978; Ito, 1978a,b) have been reported. The cumulative effect of the above influences throughout pollen development ,in vivo and in vitro, will control the biochemical environment and, possibly, anther culture response.

Foroughi-Wehr's group has researched and identified some responsive genotypes of importance to breeding programmes (Foroughi-Wehr, et al, 1983; Foroughi-Wehr, et al, 1984). However, their efforts so far have focused on the production of callus from immature microspores and its subsequent redifferentiation into plants. With this approach they were able to produce two green plants per one hundred anthers cultured. More recently there has been a report of direct embryogenesis and plant regeneration from immature microspores of barley (Lyne,et al, 1986). Using this method, it has been possible to produce an average of nine green plants per 100 anthers cultured.

1.9 Regeneration of albino plants from pollen

A common problem in anther culture systems is the regeneration of albino plants. This is especially so in cereals where, in some cases, the proportion of albinos can be as high as 100% (Foroughi-Wehr et al, 1984, barley; Karim et al, 1985, rice; Datta, et al, 1987, wheat). Little work has been carried out to explain the cause of albinism, but when the DNA of proplastids from seven callus-derived albino plants of barley variety Sabarlis was analysed (Day et al, 1985) portions of this genome were shown to be deleted. When and how this deletion took place remains a subject of speculation (Lyne, 1985).

Some varieties of barley, eg. Igri and Sabarlis, produce a high proportion of green plants. These were used to develop the technique to an efficient level so that a high proportion of albino plants could be tolerated.

1.10 Aims of this study

The results of experiments in which the effects of various cultural factors on plant regeneration from barley anthers and isolated microspores are presented in this thesis.

Previous work had shown that plants could be regenerated from barley microspores when the anthers were cultured under suitable conditions, (see Section 2.10). In all these previous reports (Section 1.7) the efficiency of the process was extremely low (a maximum of eight green plants developing per 100 anthers cultured, Lyne et al, 1986), and to achieve this it was necessary to culture the pollen within the anther. Very little success had been achieved from attempts to culture microspores isolated from the anther.

The influence of genotype on anther response in barley has been demonstrated previously (Foroughi-Wehr et al, 1984). Two varieties of barley, the spring variety Sabarlis and the winter variety Igri, both of which respond relatively well in culture (Clapham, 1973; Foroughi-Wehr et al, 1984), were used in this study.

The aim of this work was to develop techniques for barley anther culture, with the view to improving efficiency and to increasing our understanding of the processes involved in pollen embryogenesis. Such improved techniques may be commercially important as they would be applicable to barley breeding programmes.

Chapter 2

Methods and materials

2.1 Introduction

The experimental work reported in this thesis was carried out in two laboratories between October 1983 and October 1986. The majority of the work was undertaken in the "Plant Tissue Culture" facilities of my employers,"Shell Research Limited", at their Sittingbourne Research Centre (SRC). (This was in addition to my contractual duties as a research technician in the same unit). The rest of the work was in the "Unit of Advanced Propagation Systems" at London University's Wye College.

The Plant Tissue Culture group at SRC was set up in 1980 to develop novel plant breeding tools for crops of commercial interest to Shell-group companies. A cereal anther culture programme was initiated in 1983 from which the present project developed.

Wye College is approximately 25 miles south-east of Sittingbourne. The UAPS has a much longer tradition than SRC and has an international reputation, not least, for its work on the development of micropropagation systems for oil and coconut palms.

2.2 Equipment

Conviron S10h: Controlled environment cabinets.

Vindon Scientific: Refrigerated incubators

Phillips : 501B series Scanning Electron Microscope.

EMScope: SP2000 Sputtercryo

Hitachi: S-430 Scanning Electron Microscope

Hexland and Wantage: Sputter coater

Heraeus Chrieste: Digifuge, bench-top centrifuge

Schott: Model A osmette freezing point osmometer

Plastic disposables:		Supplier
500 ml and 125 ml sterile filtration units.		BDH
Petri dishes:	14cm diameter	Sterilin
	9cm "	"
	5cm " deep	"
	9cm " two-compartment	"
	60cm Specimen containers	"
	3.5cm diameter petri dishes	Costar
	24x1cm diameter Multi-welled dishes	"
Pipettes:	1.0 ml graduated	Sterilin
	5.0 ml "	"
	10.0 ml "	"
	25.0 ml "	"
Plant pots:	12.5 cm diameter plastic	
	7 cm square Opti pots size 7k	

Supplied by Fyffes-Monrow of Maidstone.

Special Glassware:

Gas tight culture vessels were custom made in the glassblowing department at SRC.

Other Consumables:

"Nescofilm" sealing film. Supplied by Baird and Tatlock

2.3 Chemical reagents

All chemicals were of analytical grade or similar, and were obtained through the usual channels. Some of the more important or hard to acquire items are listed below. The formulation of all the media used in this study are listed in Appendix 1.

Murashige and Skoog Powder, without sugar, agar or hormones: (Murashige et al, 1962) — Flow Laboratories, Irvine Scotland.

Hormones:

ABA (Abscisic acid)

BAP (6-Benzylaminopurine)

Kinetin

Zeatin

Zeatin riboside

2iP ([2-Isopentenyl]Adenine)

2,4-D (2,4-Dichlorophenoxyacetic acid)

IAA (Indole-3-acetic acid)

IBA (Indol-3-butyric acid)

NAA (1-Naphthaleneacetic acid)

— Sigma London Chemical Co. Poole, Dorset

Other Chemicals:

FMC Sea Plaque Agarose — Miles Scientific, Stoke Poges, Slough.

Ficoll 400 — Pharmacia

$U^{14}C$ Sucrose

$U^{14}C$ Glucose

$U^{14}C$ Fructose

$U^{14}C$ Maltose

— Amersham International Buckinghamshire, England.

Scintillation fluid: LKB:Optiphase Safe — Fisons Scientific Equipment Limited, Loughbrough, LE11ORG, England

Plant fertiliser:	Chempak I Chempak II	Prepacked Fertilisers Ltd Bingley Road, Hoddesdon, Herts, EN110LR.
Potting media:		
	Terra-green	J. McLauchlan, Horticulture limited, Brook House, Ingreengate, Thirst North Yorkshire.
	Perlite	Silvaperl Products Ltd. P.O. Box Dept. 38, Harrogate, N. Yorkshire HG28JW
	Fisons Levington potting compost	Fisons plc, Ipswich.

2.4 Aseptic technique

Aseptic techniques were similar to the standard methods published by Thomas et al, (1975). Some modifications were necessary for fine instruments used to manipulate delicate plant material. Instruments that required frequent sterilisation were dipped in absolute ethanol and those that were heat resistant were flamed. However, delicate instruments that required repeated sterilisation were immersed in absolute ethanol and left to dry in a laminar flow hood before use, since these instruments were damaged by flaming. Tissue culture media also support the growth of many micro-organisms, especially saprophytic bacteria and fungi. All nutrient media, culture vessels and instruments used in aseptic handling of plant tissues were sterilised before use. Plant material was also surface sterilised before use. Plastic petri dishes and culture vessels were obtained in pre-sterilised packs. Glassware and instruments were sterilised by autoclaving at 121C (103 KPa) for 20 minutes. Open glass containers

were autoclaved in a double layer of aluminium foil. Nutrient media components were autoclave sterilised at 115C for 15 minutes. Thermolabile media components, and those components suspected of being thermolabile, were filter sterilised through a 0.22μm cellulose nitrate membrane, held in disposable "Nalgene"filtration units of 125ml or 500ml capacity. The sterile-filtered thermolabile media components were added to agar containing media just before dispensing at ca 40C.

Plant material was surface sterilised by spraying exposed surfaces with absolute ethanol. All manipulations involving aseptic plant material and sterile media were conducted in a laminar air-flow cabinet.

2.5 Standard media preparation

All media were prepared, filter sterilised using a 0.22μm filter, and stored at 4C as x2 concentrates with the omission of the support component of the medium. Before use, the media were diluted with an equal quantity of double strength support phase. The support phase, either Sea Plaque Agarose or $Ficoll_{400}$, was autoclave-sterilised at 121C for 15 minutes and allowed to cool to ca 50C before mixing with the x2 concentrate medium. All agarose-containing media were poured when the molten media had cooled to ca 40C. Plates were stacked six-high with an empty plate on top to minimise subsequent formation of condensation on petri dish lids. Plates were left unsealed in a lamina flow hood overnight before use. If any condensation had formed the plates were left open at the back of the laminar flow hood to dry the surface of the media before use.

2.6 Seed storage

All seeds were stored in a 100ml glass screw-top jar at 4C.

2.7 Sources of mother plants

Initial experiments were conducted with the two-rowed spring barley cv Sabarlis. The seed was supplied originally by Dr.N.Sunderland of the John Innes Institute,Norwich. Stocks were multiplied subsequently by "selfing" in the glasshouse at Sittingbourne.

The supply of Sabarlis spring barley was superceded by the two-rowed winter barley cv Igri. Experiments using Igri are specified. Seed of this variety was supplied by Nickerson RPB, Lincoln.

2.8 Conditions for the growth of mother plants

Sabarlis plants were grown according to the method described by Lyne et al, (1986). Sabarlis seeds were sown, one to a 12.5cm diameter pot in "J.Arthur Bowers Ericacious Compost". They were germinated at ca 20C and transferred to a controlled environment cabinet (Section 2.2.) when the seedlings were ca 10cm tall. Plants were grown under the following regime:

Temperature	:	12 C
Day/Night	:	16/8 hours
Light (at pot height)	:	300 PAR ($\mu E.m^{-2}.s^{-1}$)
Relative humidity	:	60-80%

Plant nutrition was as described in section 2.9. Plants of Igri were grown as for Sabarlis but with the following modifications:- seeds of Igri winter barley were sown, one to a 7cm square pot, containing the following compost mix in a ratio of 2:2:1:-

Fisons Levington potting compost: Perlite: Terra-green.

They were germinated in a growth room at 25C under ca 150 $\mu E.m^{-2}.s^{-1}$.

Germination was usually completed 5 days after sowing. Seedlings were grown under the same conditions for a further 2 days before being transferred to 4C for 8 weeks to vernalise. During vernalisation, the seedlings were kept under low light with a 10-hr photoperiod and daylight intensity of 120-150 $\mu E.m^{-2}.s^{-1}$, and night-time light intensity of 7-10 $\mu E.m^{-2}s^{-1}$.

The vernalised seedlings were transferred to 12.5cm diameter pots, with the same soil mixture. The seedlings were placed into Conviron S10h growth cabinets and grown in the same regime as the Sabarlis, with the exception of fertiliser application which was as set out in the Table 2.9.

2.9 Plant nutrition

For Sabarlis:-

Chempak no.1 33:10:10

Chempak no.2 25:15:15

One hundred gm of each Chempak preparation were dissolved in 9 litres of tap water. This was transferred to a Cameron automatic diluter which diluted the concentrate 1:1000 as it was dispensed. Each plant received approximately 2 litres per week.

For Igri:-

Chempak I: 22.5g

Chempak II: 45.0g

Dissolved in 10.0 litres of tap water

Table 2.9
Plant nutrition protocol for Igri winter barley after vernalisation

Weeks after vernalisation	Volume (ml) of fertiliser per pot per week	Application volumes and frequencies
1	0	0
2	5	1x 5 ml
3	10	2x 5 ml
4	25	2x 10 ml + 1x 5 ml
5	35	3x 10 ml + 1x 5 ml

Aliquots of fertiliser were applied at set intervals throughout each week, ie during the third week of plant growth in the controlled environment chamber, 2x 5ml aliquots were given to each pot with the first on Monday and the second on Friday. This increased as in Table 2.9 until the fifth week when 3 x 10ml and 1x 5ml were given in 10ml aliquots on Monday, Wednesday and Friday and then the 5ml aliquot on Sunday. After week 5, each plant received 35ml per week in the same way.

2.10 Spike selection and excision

Spike excision and selection was according to the method described by Lyne et al (1986). Spikes containing anthers at the mid-uninucleate stage were selected and pretreated for 28 days at 4C according to the method of Huang et al (1982). At the end of pretreatment microspore viability was assessed by acetocarmine staining and spikes containing less than 40% viable pollen were discarded.

2.11 Anther culture

The anther culture method used initially was according to Lyne *et al* (1986). Anthers were dissected from suitable spikes and placed onto 10ml of medium in a 5cm diameter x 18mm deep plastic Petri dish. Each dish was sealed with "Nescofilm" sealing film. Four 5cm diameter Petri dishes were placed into a 14cm diameter plastic Petri dish which, in turn, was also sealed with Nescofilm. These dishes were then wrapped with aluminium foil and incubated at 25C for 28 days. The foil cover was removed after 28 days and the cultures transferred to an illuminated incubator at 20C receiving *ca* 70$\mu E.m^{-2}.s^{-1}$. In earlier experiments, any structures > 1.5mm across were transferred to FD redifferentiation medium (Appendix 1.A), in 5cm diameter Petri dishes, to develop into plants. Once the plantlets had grown to approximately 2cm tall, they were transferred to M and S medium (Murashige *et al*, 1962) containing 2% sucrose and 0.8% Sea Plaque Agarose. They were subsequently cultured as described above before being potted out into a peat-based compost. For later experiments, plant regeneration occurred on the anther culture medium. Plantlets were transferred directly onto M and S medium, omitting the step on redifferentiation medium.

2.12 Embryo culture

The method used for embryo culture was modified from Dunwell (1981,a). Immature caryopses were removed from spikes of barley plants grown in controlled environments at 12C under 16-hr. photoperiod at a light level of *ca* 300 $\mu E.m^{-2}.s^{-1}$. They were dehusked and surface sterilised in 15% (v/v) "Domestos", for 10 minutes. This was followed by four x 5-minute washings in sterile distilled water

(Dunwell, 1981,a). The surface-sterilised caryopses were stored in sterile water until all of the embryos were dissected out. Embryos were cultured on 10 ml of medium solidified with Sea Plaque Agarose. The culture regime was similar to those described for anther culture by Lyne et al (1986). The dishes were sealed with "Nescofilm" and placed into 140mm diameter plastic Petri dishes which were themselves sealed with "Nescofilm". Cultures were then transferred to illuminated incubators at 25C under constant low light (ca 30-40 $\mu E.m^{-2}.s^{-1}$), produced by six Thorn minature 525mm fluorscent tubes.

2.13 Design of experiments

The developmental stage of pollen at harvest is crucial for its successful response in culture. However, the development of pollen in a barley spike is not synchronous (Sunderland, 1973; Dale, 1975). Anthers in the middle of the spike contain developmentally more advanced pollen than those towards the spike-tips. Even pollen within a single anther is not totally synchronous (Dale, 1975). The pollen asynchrony contributes to the variability of anther response in culture (Kao, 1981). This causes considerable problems when planning experiments to compare different treatments as it can confound the results and confuse their interpretation. Therefore, experiments were planned to compensate for anther variability. Anthers were randomised between experimental treatments according to a randomised block design (Clarke, 1976). Randomisation for experiments are given in Appendix 3.

2.14 Statistical analysis of data

All statistical analyses were carried out using the Genstat IV Analysis programme (1984) on the Vax "cluster" at Shell Research.

Unless otherwise stated the levels of significance quoted are at the 95% level ($p<0.05$). Least significant difference (LSD) bars (Clarke, 1976) are given on relevant figures.

2.15 Scanning electron microscopy

a) At Shell Research:

Sample preparation and examination were after the methods of Robards et al (1979). They were mounted in "Celltech" and frozen in slushed nitrogen. Anthers were fractured under vacuum. All samples were immediately sputter-coated with gold in an EM scope SP 2000 Sputtercryo. They were then transferred, still under vacuum, into the cold stage of a Phillips 501B series Scanning Electron microscope and examined at 30kv.

b) At Wye college:

Once again, samples were set in "Celltech". They were frozen in liquid nitrogen and, while still submerged, covered with a gas tight cap. This was to prevent water from the atmosphere condensing on the sample during transfer to the cold-stage of the sputter coater. The cryo unit and sputter coater were manufactured by Hexland of Wantage. The scanning electron microscope used was an S-430 manufactured by Hitachi (Robards et al, 1979).

2.16 Osmolarity, and osmolality determinations

A molal solution contains one mole of solute dissolved in one kilogram of solvent, resulting in more than a litre of solution and is therefore slightly more dilute than a molar solution.

Osmotic pressure, vapour pressure, freezing point and boiling point of a solution are termed "colligative" properties as they are all

Figure 2.16.1.

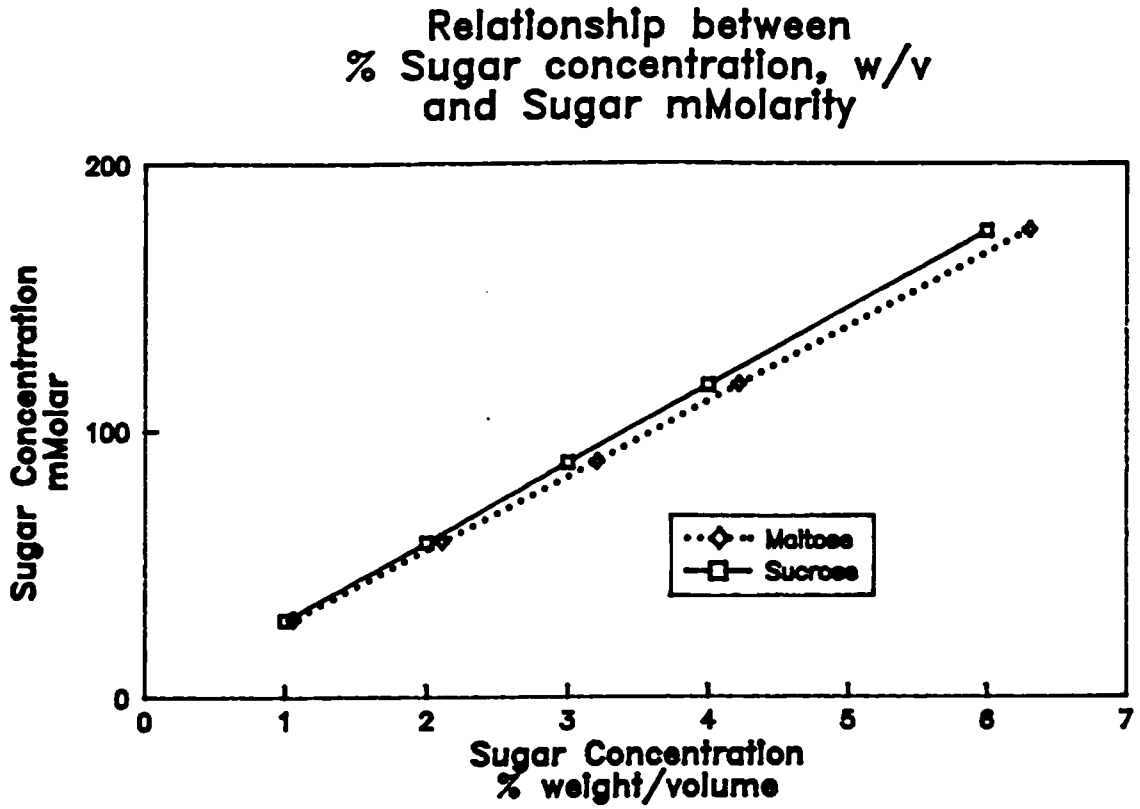
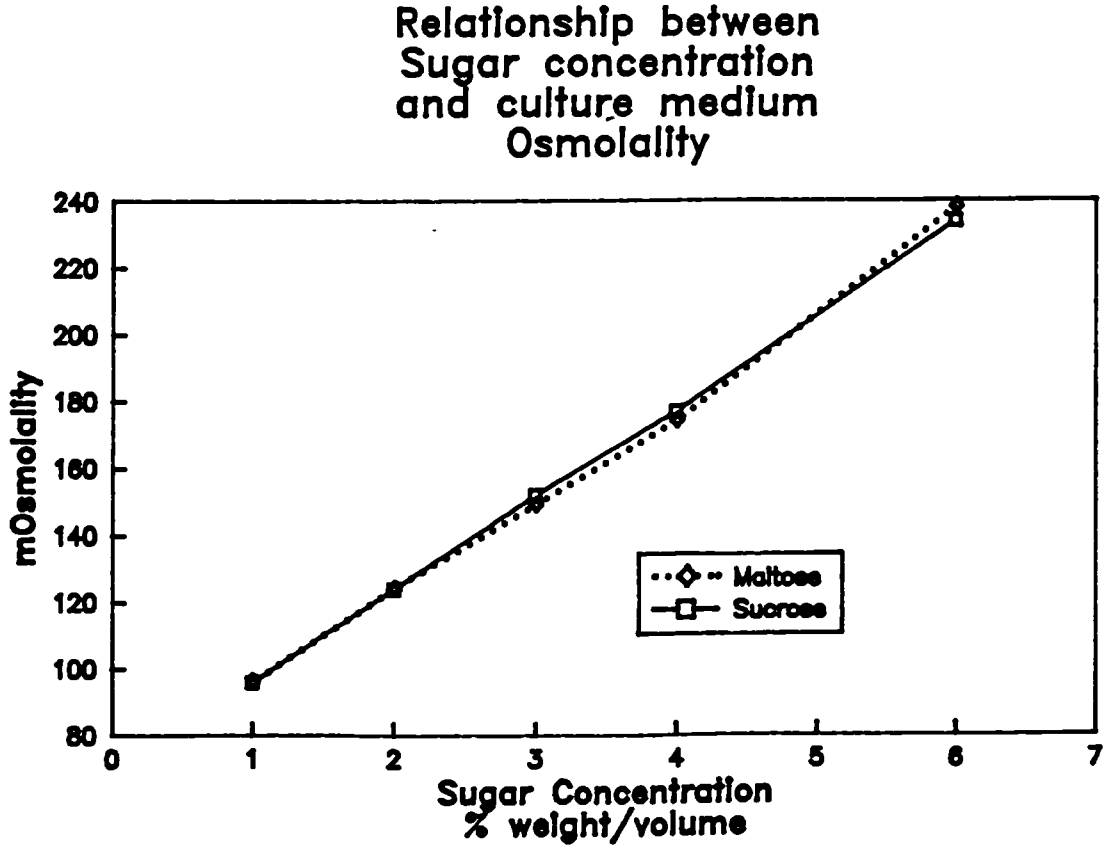


Figure 2.16.2.



directly related to the concentration of the solution. As the concentration of the solution increases, its freezing point is lowered. The amount by which it is lowered is a simple measurement which can be used to determine the concentration of the solution or any of the other colligative properties. This is the principle on which a freezing point osmometer operates.

For every additional particle dissolved in a solvent the freezing point will be lowered by a uniform amount. One mole of particles dissolved in a kilogram of water will lower the freezing point by 1.858C. The measurement obtained using a freezing point osmometer is expressed in milliosmoles (mOsm) where one mOsm per kilogram change in concentration will cause a change of 1.858×10^{-3}C in the freezing point (Nelson, 1983).

Figure 2.16.1 shows the relationship between the % w/v, of sucrose and maltose, and molarity. Figure 2.16.2 shows the relationship between sugar concentration, % w/v, and the osmolality of the culture media.

2.17 Microscopy and photography

Routine cytological and photomicroscopy were carried out using either Zeiss Universal "R" or Leitz EB20 "DiaLux" photomicroscopes. A Wild M3 dissecting microscope was used for sample dissection and manipulation.

Most of the important photographs were taken by Shell's professional photographer, Mr.Colin Nicholson, using professional equipment.

2.18 Radiochemical experiments

All the sugars used in these experiments were uniformly ^{14}C labelled.

Table 2.18
Properties of radiolabelled sugars used

Sugar	Molecular Weight Unlabelled	Molecular Weight Labelled	Radiochemical Purity, %	Radioactive Concn. Ci/ml	Specific Activity Ci/ mole
Sucrose	342	360	99	200	560
Glucose	180	189	98	200	270
Fructose	180	189	97-98	200	274
Maltose	360	380	98-99	200	501

2.18.1 Radiolabelled sugar uptake into the anther

The experiments were carried out aseptically using 24-"welled" culture dishes. One dish was used for the four sugar treatments at each time point in the time-course experiments (Experiment 5.5). Each experiment was replicated five times and results presented as mean values +/- standard deviations.

Anthers were excised from selected pretreated spikes and allocated to each treatment and time point at random (Appendix A.2.11). At time=0, one ml of the appropriate culture medium containing 2 μCi (10μl) of labelled sugar was pipetted into the corresponding well. The plates were sealed with "Nescofilm" and wrapped with aluminium foil then placed into an incubator at 25C. At the end of the incubation period, anthers were washed sequentially in four aliquots of chilled medium without labelled sugar. This was to remove the majority of labelled sugar which had accumulated in the anther-freespace (Chapter5.5.1). After washing, the anthers were plunged into boiling 80%

ethanol:water and extracted for two minutes. This was to prevent further metabolism and to extract soluble sugars. Radioactivity in the extracts was determined by liquid scintillation spectrometry using LKB Phase Safe scintillation fluid (Long, 1976,a,b).

2.18.2 $^{14}CO_2$ Evolution determination

Experiments were carried out aseptically in gas-tight reaction vessels (150ml glass desiccators).

Anthers were cultured in small culture vessels 1cm in diameter and 2cm deep. A similar vessel was used to hold the CO_2 absorber, 10% KOH. Both vessels were placed into the gas-tight culture vessels. The absorber was replaced at each time point and ^{14}C determined by liquid scintillation spectrometry.

A problem encountered with the scintillation spectrometry methods employed initially was that of chemiluminescence. This was caused by using KOH as the CO_2 absorber. An alternative absorber, "Carbosorb", marketed by Packard plc was used to reduce the problem. Unfortunately, under the conditions required for the experiment Carbosorb vapourised and was absorbed by the sample and eventually killed it.

The problem of chemiluminescence was eventually overcome by using LKB's Optiphase Safe scintillation fluid since it is capable of mixing with up to 0.25ml of 10% KOH without significant chemiluminescence. KOH was replaced regularly to prevent saturation by CO_2.

2.18.3 Radiolabelled sugar determination by TLC

Solvent:	Formic acid	15%
	Methyl ethyl ketone	30%
	2-Methyl-2-propanol	40%
	Water	15%
TLC plate:	Camlab Macherey-Nagel Cel 300-25	
	precoated TLC plate 0.25mm cellulose MN300	
Standards:	5mg/ml Sucrose (does not stain).	
	Glucose	
	Fructose	
	Maltose	

Elution time: Two runs of two hours, making sure that the plate was dried between runs.

Spray reagent: Anisidine 1.2% (w/v). TOXIC!

Phthalic acid 1.6% (w/v).

dissolved in ethanol.

Spot development: Spray the plate with spray reagent and develop at 100C for five minutes.

The sucrose spot was detected using a Geiger-Muller counter.

Sample preparation:- Anthers were cultured, as described above (Section 2.9), in aliquots of the media containing the appropriate radiolabelled sugar sample. At culture initiation 0.5ml of the appropriate medium was sampled from each of the treatments. This was placed into 0.95ml of ethanol to precipitate the $Ficoll_{400}$, which would otherwise affect the TLC of the sugars. The samples were then centrifuged at high speed in an airfuge for five minutes to pellet the Ficoll precipitate. The supernatant was removed and stored at

-20C before TLC analysis. This procedure was repeated after seven, 28 and 40 days post culture initiation.

The TLC plate was dried and sugar spots were located, initially by spraying to identify the reducing sugars, but then by autoradiography (Lark, 1968) to identify all the labelled sugars. Single-track scanning was carried out, using an "Isomess IM3000" RadioTLC Analyser, on tracks of samples taken at culture initiation (controls) and after seven and 40 days of culture. The peaks were integrated and a quantitative estimate of the relative amounts of labelled sugar made. Twenty µl aliquots from these samples were spotted onto a TLC plate and chromatographed as described above.

Chapter 3

Anther culture

3.1. Introduction

Earlier reports on the culture of barley anthers describe the regeneration of plants from immature microspores via callus (Sunderland, 1982; Foroughi-Wehr *et al*, 1984). These produced two and five green plants, respectively, per 100 anthers cultured. More recently, a method allowing direct embryogenesis from immature microspores was described (Lyne *et al*, 1986). This method gave a yield of nine green plants per 100 anthers cultured. When expressed as a proportion of the number of microspores cultured the androgenic efficiency is very low, *ie* 0.0035%. Clearly, there was considerable scope for improvement.

The techniques used for barley anther culture may be divided into three steps: donor plant growth, spike and/or anther pretreatment and anther culture. The importance of the condition of the donor plant has been established (Section 1.5). In particular, the effect of growth temperature on barley source plants has been examined (Lyne *et al*, 1986).

The pretreatment of spikes at reduced temperature, at 4C for 28 days (Section 1.6), was found to enhance microspore development giving rise to callus from barley anthers (Huang *et al*, 1982). A similar enhancement was reported for direct embryoid formation from immature barley microspores (Lyne *et al*, 1986).

The effects of cultural conditions on callus production and subsequent plant production have been described by Lyne *et al* (1986). However, until now little has been done to investigate the cultural requirements for direct embryogenesis, as the phenomenon of direct

embryogenesis has only recently been reported (Lyne et al, 1986).

In this chapter, the methods of Lyne et al (1986), were used as a baseline against which all other experiments were evaluated. A reprint of this paper is submitted in support of this thesis and is attached inside the back cover.

3.2 Anther culture

Anthers were selected from six pretreated spikes, (Figure 3.2.1), as described in section 2.10. The anthers were cultured on 10ml of FC medium (Appendix 1.1), (Figure 3.2.2). Cultures were incubated at 25C in the dark for 28 days. The proportion of responding anthers, ie any anther producing macroscopically visible structures emerging from it, was assessed (Table 3.2. and Figure 3.2.3). The cultures were then transferred to a continuously illuminated incubator ($70\mu Em^{-2}.s^{-1}$) at 25C. Any callus or embryoid larger than 1.2mm that developed during the next four weeks was transferred to FD regeneration medium (Appendix 1.1). Those plants equal or greater than 0.5 cm long were removed from the culture dishes and recorded (Table 3.2.. and Figure 3.2.4).

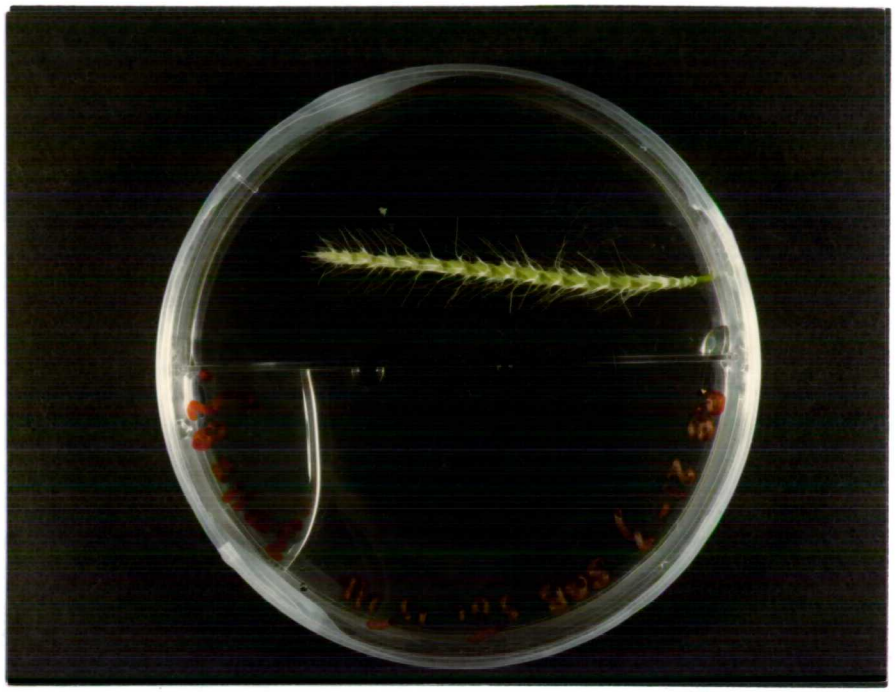


Figure 3.2.1. Barley spike in a two-compartment petri dish ready for cold pretreatment. Sterile water maintains humidity throughout pretreatment, but the pollen is protected from the lethal effects of direct contact with the water.



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Figure 3.2.2. Pretreated barley anthers on solid medium at the start of culture.

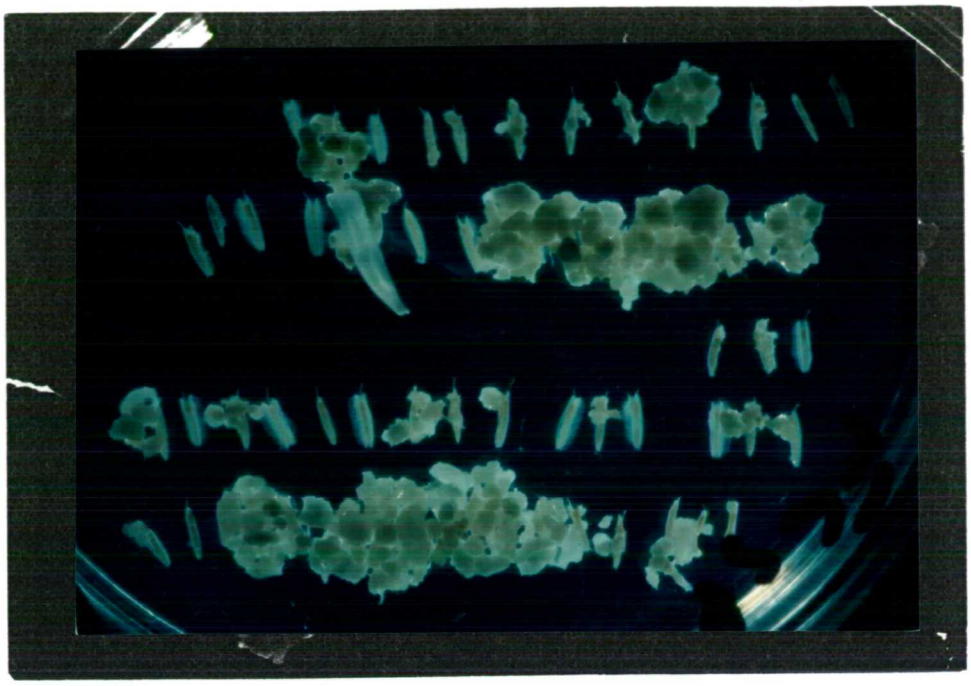


Figure 3.2.3. Responding cultures after 28 days incubation in the dark at 25C.



Figure 3.2.4. A green microspore-derived barley plant 40 days after the initiation of anther culture on FC medium.

Table 3.2.
Levels of anther response and plant regeneration in an anther culture experiment after Lyne et al (1986)

	Total	Mean per spike	Mean per 100 anthers cultured	Mean per 100 responding anthers
Anthers	a			
- Cultured	375	62.5	-	-
- Responding	99	16.5	26.4	-
Plants				
- Green	32	5.3	8.3	32.3
- Albino	33	5.5	8.8	33.3

a) Total excised from six spikes.

3.3. Anther orientation

In previous experiments, anthers were plated in a random fashion (as described in section 2.10). Three thousand anthers were plated, 26% of these responded. Barley anthers are asymmetrical, (Figure 3.3.1), and could be cultured in one of two orientations, flat or on-edge (Figure 3.3.2).

Anther orientation on the culture medium appeared to influence their response. The effect of anther orientation was quantified by comparing the results of two experiments carried out simultaneously . In the first experiment pretreated anthers were cultured randomly orientated on solid medium, ie. as they settled on the medium. In the second experiment anthers were orientated "on-edge" at the start of culture. Anther response, the number of macroscopically visible multicellular structures and plant yield were scored as described in Section 3.2.

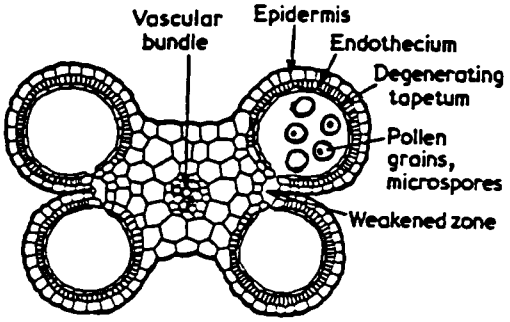
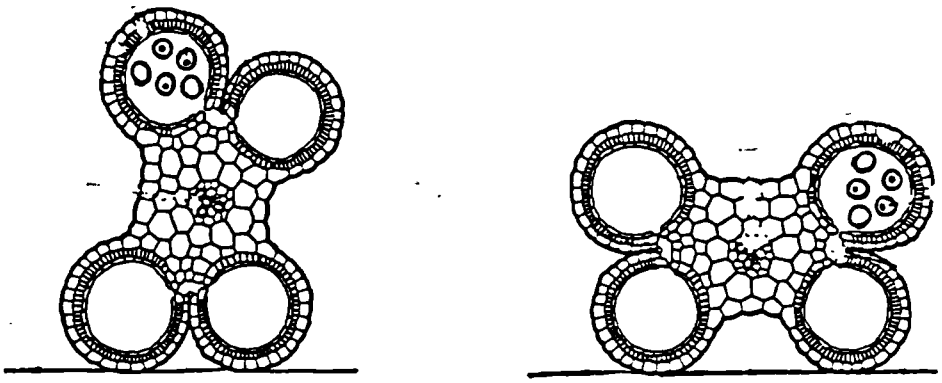


Figure 3.3.1. The anther of a barley stamen, near maturity, in transverse section after Lermer et al, (Briggs, 1978)



"On-edge" "Flat"

Figure 3.3.2. Transverse sections through barley anthers to illustrate the orientation of anthers on the surface of the media. After Lermer et al, (Briggs, 1978).

Embryoid development was only observed in the upper lobes of anthers placed on-edge, ie. with a single lobe in contact with the culture medium (Figure 3.3.3). No development was observed in the lower lobe. When anthers were lying flat, ie. both lobes were in contact with the medium, the microspores were generally shed onto the medium and did not develop further (Figure 3.3.4).

Table 3.3.1 summarises results from two experiments carried out to examine the relationship between anther orientation and plant regeneration. As some anthers placed "on-edge" fell into the "flat" position, orientation was checked after 28 days incubation. The proportions of anthers remaining "on-edge" were 28% and 71%, respectively. The data show clearly that little or no response was observed in anthers lying flat on the medium. However, a high proportion of anthers lying "on-edge" responded, producing green and albino plants.

Mean yields of five and 20 green plants per 100 anthers cultured were obtained in Experiments 1 and 2 respectively. Most of this difference could be attributed to the higher proportion of anthers remaining "on-edge", and therefore capable of response, in the second experiment. Anthers that did respond produced plants at a similar frequency in both experiments. This emphasises the importance of maximising the numbers of anthers responding when seeking to improve the overall yields from anther culture.



Figure 3.3.3. Anthers orientated "on-edge" on the surface of the medium at the start of culture. Embryoid development has occurred in the upper lobe of many of the "on-edge"anthers. No multicellular structures were observed in the anther lobes which contacted the medium.

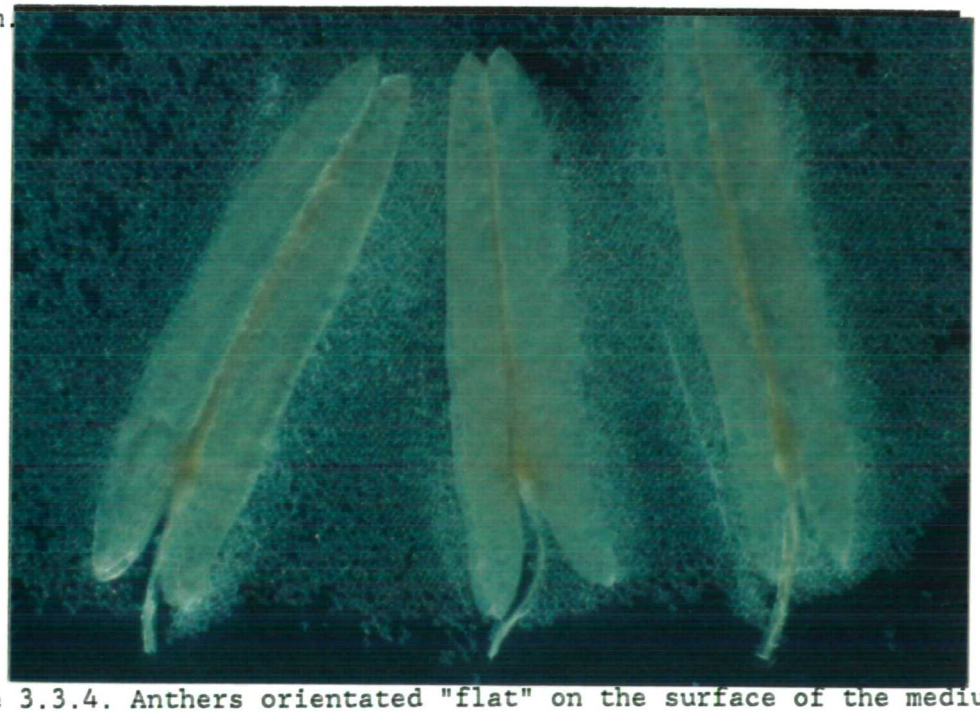
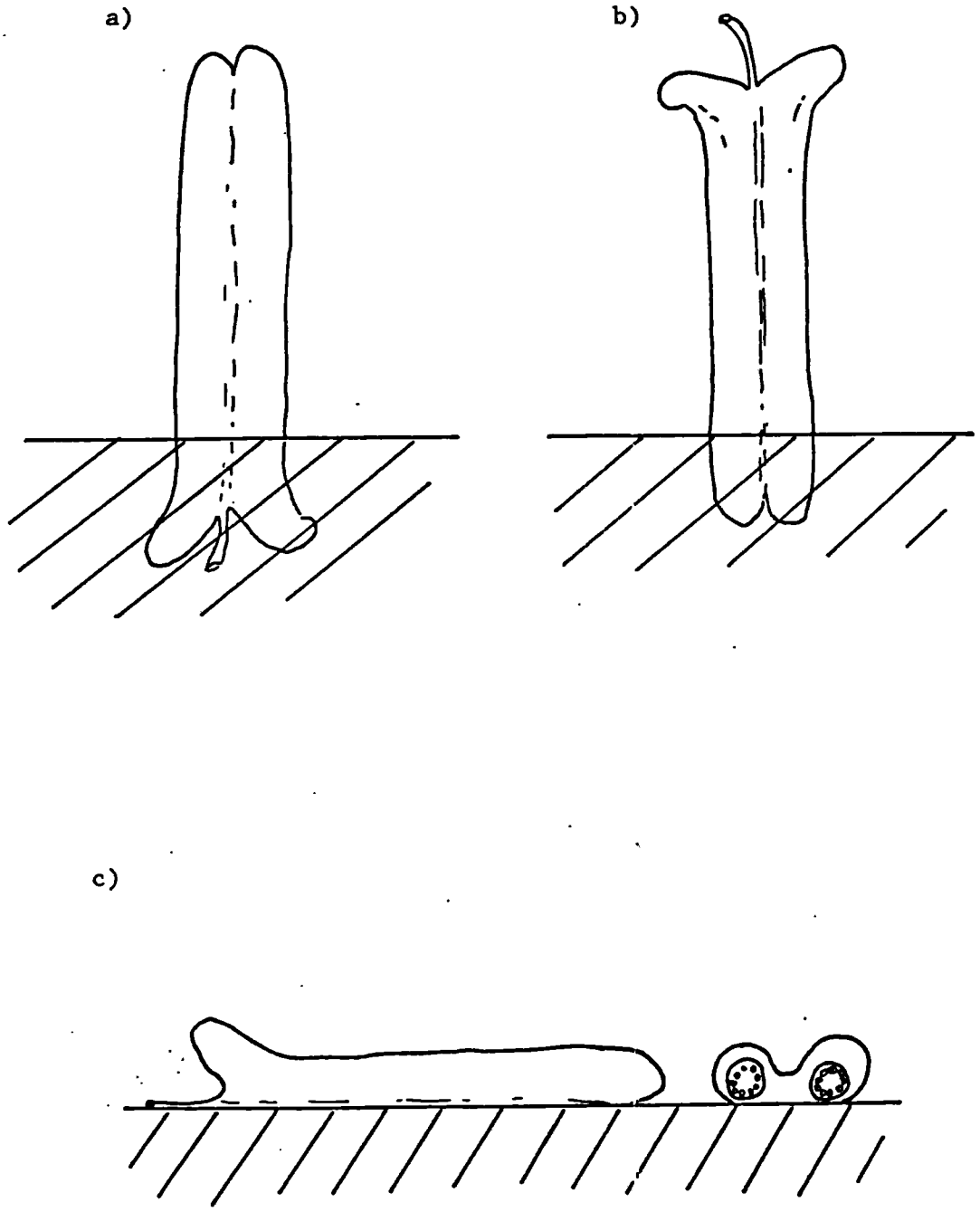


Figure 3.3.4. Anthers orientated "flat" on the surface of the medium. Both lobes of the anthers were in contact with the medium from the start of culture. Microspores appear to be shed onto the medium. No further development was observed.

Figure 3.3.5. Alternative orientations investigated for anthers cultured on solid media.



a) Anther orientated with basal end and filament embedded in the medium.

b) Anther orientated with top embedded in the medium.

c) Anther bisected along the filament axis and cultured as two halves.

Table 3.3.1.
Effect of orientation on anther response and plant regeneration

	a) Experiment 1		b) Experiment 2	
Orientation after 28 days culture	On-edge	Flat	On-edge	Flat
Number of anthers	99	252	553	230
Number of structures transferred	401	1	3462	0
Anthers responding	60	1	480	0
Plants regenerated -green	19	0	160	0
-albino	23	0	408	0
Plants regenerated per 100 anthers responding -green	32	0	33	-
-albino	38	0	85	-

a) Anthers from 5 spikes, random orientation.
b) Anthers from 12 spikes, on-edge orientation.

Various other orientations were tried, (See Figure 3.3.5), but none of these gave any response.

The results of this experiment have been published. A reprint of the paper is attached to the back cover of this thesis.

3.4 Determination of multicellular structure survival after contact with the medium at timed intervals

It was apparent from earlier observations that microspore-derived multicellular structures could survive contact with the medium. The precise size and developmental stage, ie. timing, after which they could survive contact with the medium was unclear. The anthers

orientated in the on-edge position may have been protecting the pollen or actually inducing microspore morphogenesis into multicellular structures.

Anther response varied between different spikes and different spikelets within individual spikes. Anthers were randomised between different treatments within an experiment to minimise any compounding effect which may have been caused by anther variability (Appendix 2).

Anthers obtained from pretreated spikes were dissected and randomly assigned to a treatment (Appendix 2.1). There were seven treatments, each containing 48 anthers. All anthers were cultured on the standard FC medium (Appendix 1.1), but orientated as described in the Table 3.4. Anthers were cultured in the "on-edge" position and subsequently reorientated in the flat position after selected intervals.

Table 3.4.
Survival of microspore-derived multicellular structures following reorientation of anthers after different periods of isolation from the medium

Treatment No.	Orientation "On-edge" Period (days)	Orientation Flat Period (days)	Anther Response a) 1	2	3	4	5	Mean
1	0	28	0	0	0	0	0	0
2	1	27	0	0	1	0	0	0.2
3	3	25	1	3	0	0	0	0.8
4	7	21	0	0	0	1	0	0.2
5	14	14	5	6	4	0	0	3.0
6	21	7	37	34	41	37	41	38.0
7	28	0	38	43	37	29	39	37.2

a) Replicates were placed into culture and scored after 28 days.

Little, if any, response was observed in anthers that were reorientated into the flat position before three weeks (Table 3.4.).

In treatments 6 and 7 only larger, well organised microspore-derived multicellular structures survived to produce plants. Those anthers that did respond in treatments 1-5 showed a common feature: in each case, the anther had opened along the stromium before reorientation. Thus, when it was placed flat on the medium, structures in the upper half of the loculus escaped contact with the medium and, therefore, survived. In a subsequent experiment, anthers were carefully reorientated ensuring medium contact with the whole anther and associated structures. There was no development in cases where the anthers had been flattened before three weeks.

The microspore-derived structures seemed to acquire some degree of tolerance to the medium at some time between weeks 2 and 3.

3.5 Androgenic structures

The androgenic structures arising from cultured barley anthers showed varying degrees of organisation, from callus to well-formed embryoids (Figures 3.5.1-5). In previous experiments, not all the structures produced developed into plants. Before the efficiency of the anther culture technique could be improved further, it was necessary to determine whether plants developed from all of the structures or only specific types. This experiment monitored the development of different structural types to see which were able to develop into plants.

Structures from several anther culture experiments were allocated to classes according to their degree of organisation (Figures 3.5.1-5). About 1250 samples from each class were transferred to regeneration medium (FD Appendix 1.1), solidified with 0.5% Sea Plaque Agarose,

and their subsequent development recorded (Table 3.5.).

Table 3.5.
The number of plants evolving from structures allocated to each class group

Structure type[a]	Number of structures transferred to regeneration medium	Number of plants produced	
		green	albino
I	1290	a) 105(8.0)	255(20.0)
II	1250	20(2.0)	164(13.0)
III	1250	0	0
IV	1250	0	0

Values in parentheses = % of total.

a: See figures 3.5.1-3.5.5 for description.

Plants only developed from structures that have been classified as type I or II. These varied from well organised structures which have similar morphology to normal zygotic embryos through to densely organised clumps of cells with smooth surfaces but not well-developed enough to be classified as embryoids (viz stages B-E Figure 3.2.1). The less well-organised structures were similar to the embryoidal callus produced by somatic embryogenesis from Pennisetum americanum described by Vasil et al (1982,a,b).

Those type II structures that did develop into plants went through an organisation stage which was indistinguishable from type I structures. The remaining structures either continued to develop into large calluses and died, or died immediately. Only those structures which developed directly into embryoids formed plants.

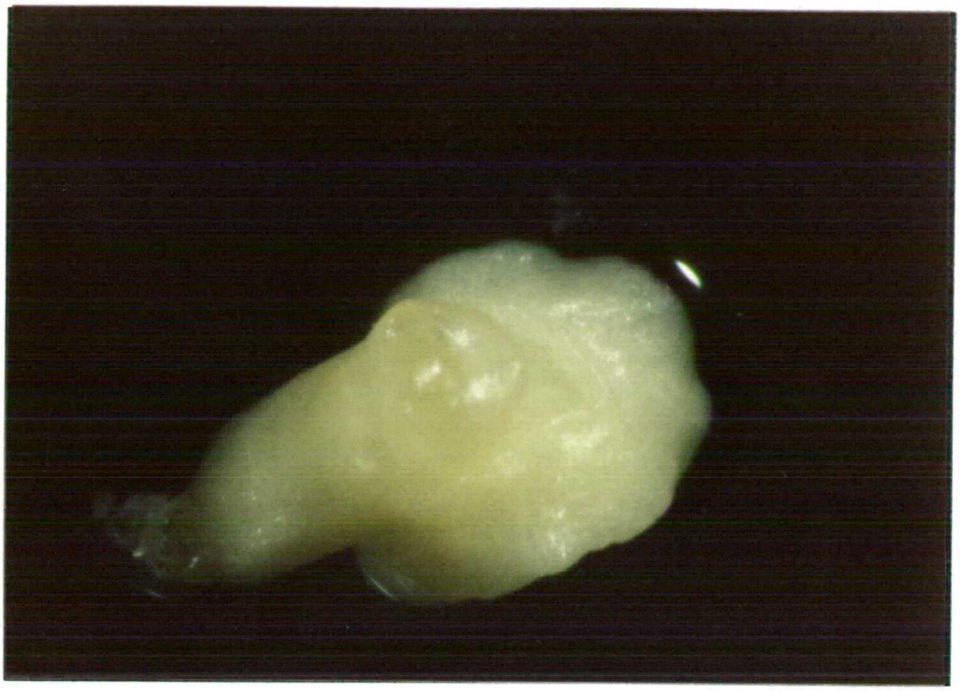


Figure 3.5.1. Type I microspore-derived structure. Note that its morphology is similar to that of a normal zygotic embryo of barley.

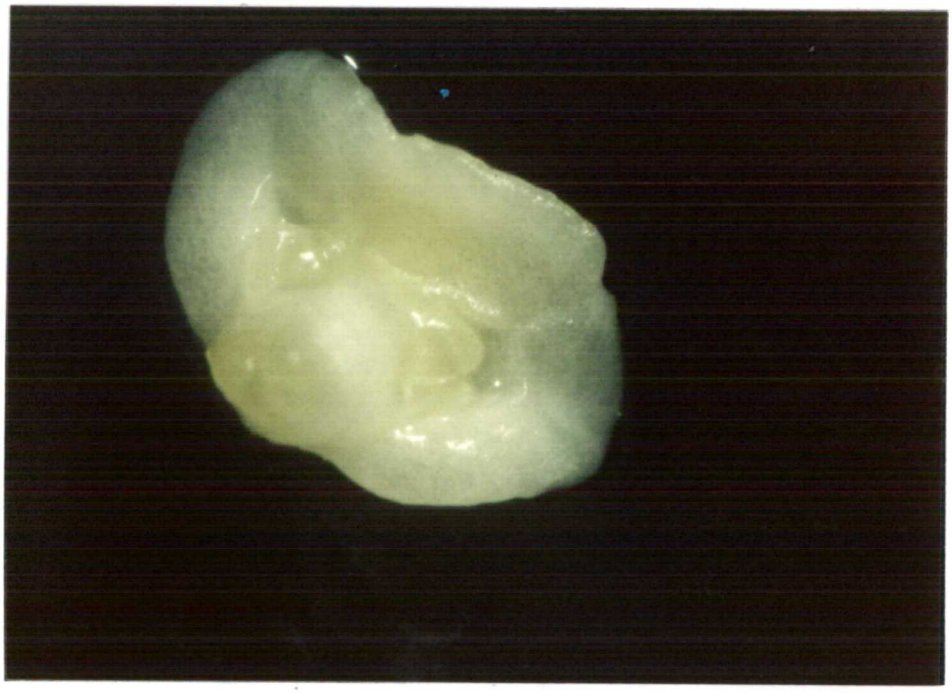


Figure 3.5.2. Type I microspore-derived structure. Note that although similar to a normal zygotic barley embryo it has two cotyledons. This is apparently not uncommon in zygotic embryos of barley (Gymer, 1985, pers. comm.; Barley breeder Nickersons Seed Company).

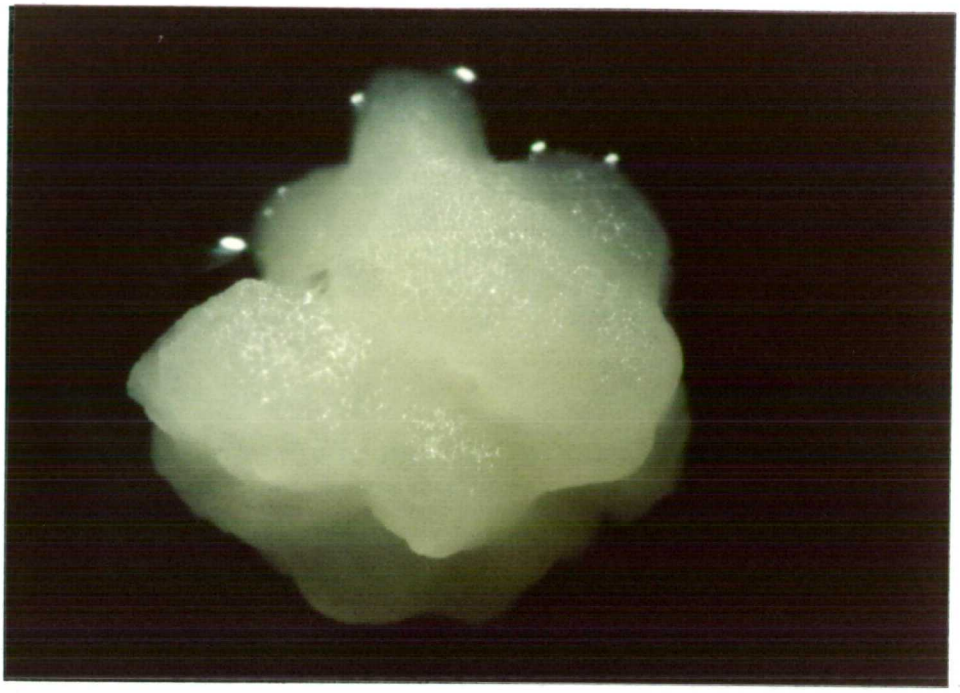


Figure 3.5.3. Type II microspore-derived structure. Similar to embryoidal callus produced by Pennisetum americanum, Vasil et al (1982a,b).



Figure 3.5.4. Type III microspore-derived structure. Nodular callus, but still showing a degree of organisation.

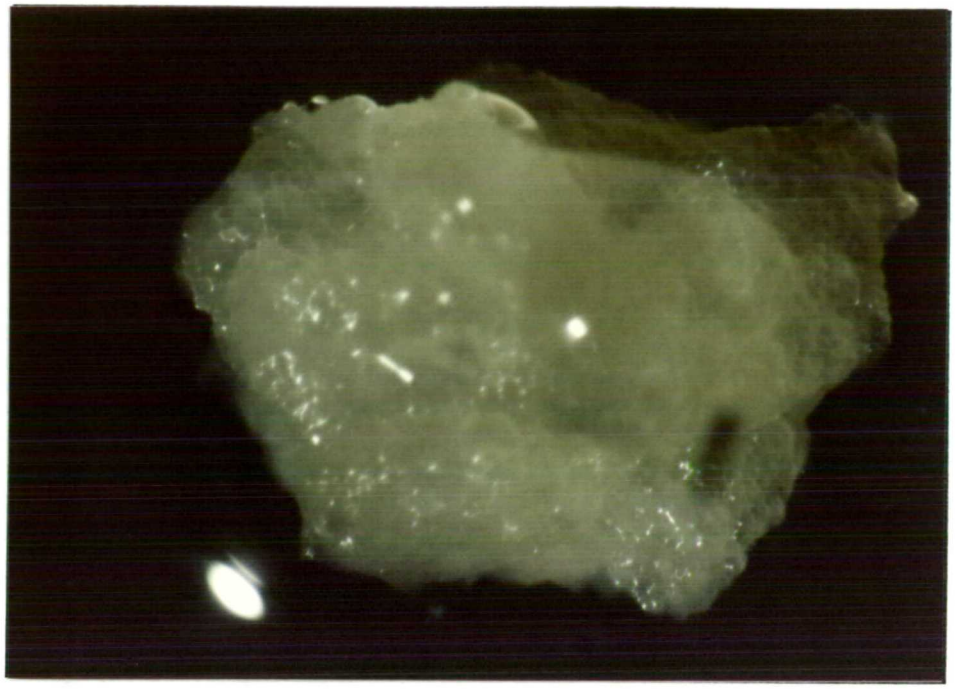


Figure 3.5.5. Type IV microspore-derived structure. Friable callus with little sign of organisation.

3.6 Discussion

Both embryo development and levels of plant regeneration in the first experiment (3.2) were similar to those reported by Lyne et al (1986). Nine green plants per 100 cultured anthers were obtained. However, culture response was not uniform; firstly, not all anthers responded in the same way - the orientation of the anthers on the medium affected their response; secondly, not all structures survived the earlier stages of culture (the first 28 days), (see Section 3.5) and thirdly, the morphology of those that did survive ranged from callus to well formed embryos. Not all of these developed into plants. Some died prematurely while others continued to grow into large calluses and then died.

Effects of anther orientation on anther response in culture have been reported previously for Datura (Sopory et al, 1976) and Nicotiana (Misoo et al, 1981). Anthers of Datura responded better when cultured "flat" rather than "on-edge" (Sopory et al, 1976). The experiments with Nicotiana indicated that anthers responded best when cultured vertically, with one end partially embedded in the medium (Misoo et al, 1981). This contrasts with the observations in this study with barley in which the best response was consistently found in anthers orientated "on-edge".

The orientation of barley anthers in culture dramatically affected their response. No development was observed in anther lobes in contact with the medium. Microspores in "flat" anthers, or the bottom lobes of "on-edge" anthers responded poorly or not at all. However, they did respond in a high proportion of the top lobes of "on-edge" anthers. By orientating anthers in the "on-edge" position at the

beginning of culture a mean of 20 green plants per 100 anthers cultured was obtained. Comparative levels of anther response, plant yield per 100 anthers cultured and per 100 anthers responding are given for two spring barley varieties (Table 3.6.).

The data shown have been obtained in work carried out at 3 different laboratories. The levels of plant yields per responding anther are remarkably similar and emphasise the importance of optimising numbers of anthers that respond in culture.

Table 3.6.
Comparative levels of anther response and plant production from varieties of spring barley and from experiments carried out in three different laboratories.

Barley variety	Reference	% Anther response.	Plant yield per 100 anthers cultured.	Plant yield per 100 anthers responding.
Sabarlis	Hunter and Lyne (Unpublished)	23	9	30
Sabarlis	Hunter This study (section 3.2)	26	8	32
Sabarlis	Hunter This study (section 3.3 expt.1)	17	5	32
Sabarlis	Hunter This study (section 3.3 expt.2)	61	20	33
Sabarlis	Sunderland and Huang (1982)	13	5	38
Dissa	Hunter and Lyne (Unpublished)	10	3	30
Dissa	Foroughi-Wehr et al (1981)	11	2	23

The effects of barley anther orientation on response have recently been confirmed by Shannon et al (1985).

An explanation of the effect of anther orientation on response can, at this point, only be speculated upon:-

The anther may simply act as a physical barrier between the pollen and the medium thus allowing the pollen to develop into androgenic structures in isolation from media influences. Alternatively, the anther may take an active role in embryogenic pollen development, perhaps detoxifying media components to products that the androgenic structures can use or at least tolerate. Or, the anther may act as a semipermeable membrane; setting up a gradient (or gradients) which allow regulated nutrient supply to the developing androgenic structures from the medium.

Anther orientation may act in a similar manner to "nurse" culture systems referred to by Bajaj (1983). Isolated microspores of tomato were cultured on top of a filter paper disc which was isolated from the medium by resting on top of clusters of intact anthers. Green parenchymous cells formed from 60% of the microspores cultured in this way within two weeks. However, no development was recorded in control cultures where the filter paper was in direct contact with the medium (Sharp, 1972). Later Pelletier (1973) successfully induced androgenesis in microspores of Nicotiana tabacum using Petunia callus as a nurse tissue. With both techniques direct contact with the culture medium was avoided.

In this study the level and type of response varied within and between anthers, as did the survival of androgenic structures.

Contact with the medium and/or isolation from the anther appeared to be detrimental to microspores and young androgens. However, larger structures could survive both. This effect was confirmed and quantified by artificially reorientating "on-edge" anthers to the flat position after periods of culture. Once reorientated, a liquid film infiltrated and formed over all structures within the anther. Structures that contacted the medium before 21 days died, including those that remained in contact with the anther. This could explain the death of young androgenic structures in anthers that had developed apparently normally but which suddenly died. Development of large numbers of androgenic structures in the top lobe of "on-edge" anthers may lead to instability and make the anthers susceptible to toppling. The androgenic structures may subsequently contact the medium before they can tolerate direct contact and thus die. In earlier experiments reorientation was not complete and those androgens that remained isolated from the liquid medium continued to develop (Figure 3.6.).

The androgenic structures that survive to be >0.5mm diameter were transferred to regeneration medium. The morphology of these structures varied from callus to embryo. The morphogenic fate of transferred androgens was followed by classifying them into four groups according to their level of organisation (See Section 3.5). Their subsequent development was monitored following their transfer to regeneration medium.

Only type I and II structures developed into plants. All other classes of structures remained disorganised and eventually died.

Liquid film of medium over the microspore-derived structure. Resulting in death.

The microspore-derived structure appears to remain dry during culture. It continues to develop and may form a plant.

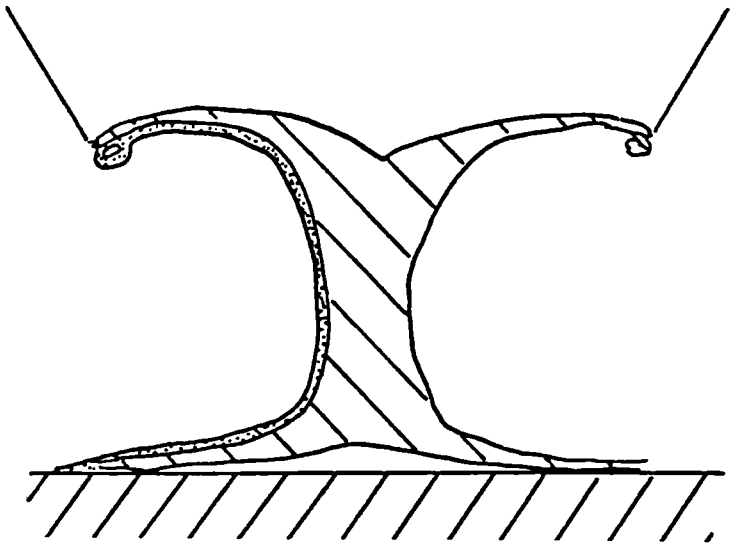


Figure 3.6. Transverse section through a cultured anther illustrating a possible explanation for the survival of small numbers of microspore-derived structures in anthers cultured "flat" on the surface of the medium.

In previous reports, plants regenerated from immature microspores of barley were via redifferentiation from callus, eg, Xu et al (1981); Kao (1981); Foroughi-Wehr et al (1983). The genetic variability of plants derived from callus cultures has been reported widely, (eg Powell et al, 1984). There are conflicting reports on the degree of phenotypic variation of microspore callus-derived plants. Androgenic lines of barley were significantly different from the parental line Berenice, (San Noeum et al, 1982) and "Considerable phenotypic variation" was found in lines of barley cv Sabarlis, (Powell et al, 1984). While Foroughi-Wehr et al, (1983) reported variation in microspore callus-derived rye plants, they found microspore callus-derived barley plants to be "true-breeding" (Foroughi-Wehr et al, 1983). In addition, no phenotypic variation was observed in microspore callus derived lines of rice (Chen et al, 1982).

Plant regeneration obtained in the anther cultures described in this study appear to be via direct embryogenesis. This route of plant regeneration may avoid the potential problems with genetic instability often associated with callus cultures (as discussed above). The advantages of direct embryogenesis for dihaploid barley production have yet to be confirmed.

3.7 Conclusion

Orientation of anthers at the beginning of culture gave a four-fold increase in the proportion of anthers responding in culture. The apparent increase in green plant yield could be explained by the increased numbers of responding anthers. However, green plant yield per responding anther remained constant.

Only well organised structures, ie. embryoids, developed into plants. In chapter 4, experiments to study zygotic embryo development in vitro and to investigate barley anther culture using a variety of media are described.

Chapter 4

Effect of media composition on the development of immature zygotic embryos and androgenic structures

4.1 Introduction

The efficiency of barley anther culture was improved by increasing the proportion of anthers that responded in culture (Chapter 3). This was a result of orientating the anthers "on-edge" at the beginning of culture. However, plant yields per responding anther remained unchanged. Further improvements in these yields would be achieved if a greater proportion of microspores within each anther became androgenic and developed, via direct embryogenesis, into plants. By optimising direct embryogenesis, the problems of genetic instability encountered with callus-derived plants (Powell et al, 1984, Section 3.5) may also be overcome.

Wilson (1977) reported that, in whole spike cultures of barley, up to 70% of the microspores within some anthers developed into calli. Unfortunately only a small fraction of these redifferentiated into plants. This report is of great significance since it suggests that a much higher proportion of barley microspores may be capable of embryogenesis than has been obtained to-date (Hunter, 1985).

In the methods used in this thesis, plants developed via direct embryogenesis from immature microspores. Almost 30% of the embryoids transferred to regeneration medium developed into plants (Section 3.1).

The barley anther culture media used by Sunderland et al (1982b), Foroughi-Wehr et al (1979) and Kao (1981) were all intended to induce callus from immature microspores. It may be possible to enhance induction of embryogenesis and subsequent plant development by using a medium formulated for the culture of immature zygotic embryos of

barley.

In this chapter the influences of several media components on the development of immature zygotic embryos and anther culture-derived structures were investigated to select a suitable candidate medium with which to optimise microspore-derived embryo production.

4.2 Embryo orientation

Two hundred immature caryopses were removed from 10 spikes of cv Sabarlis grown under standard controlled conditions (Section 2.7). These were surface sterilised as described earlier (2.10). Caryopses were taken at random and 100 embryos, 0.2-1.0mm long, were dissected and cultured at a density of 10 per plate on 'E'medium (Norstog, 1973); (Appendix 1.1) supplemented with 20g/l sucrose, 400mg/l alanine and solidified with 5.0g/l Sea Plaque Agarose. Ten embryos were cultured in each plate. They were randomly assigned into two groups of five. One group of five embryos were orientated scutellum-up (ie with their shoot and coleorhyza in contact with the medium) and the other scutellum-down (ie with their scutellum in contact with the medium).

The growth characteristics of all the embryos were recorded weekly. Larger embryos germinated first and were removed from the plates when their shoots were ca 25mm long.

Ninety-six percent of the embryos cultured scutellum-down developed and germinated normally. The remaining 4% degenerated to form globular callus which did not differentiate under these culture conditions. These embryos were all smaller than 0.25mm long.

Embryos cultured scutellum-up did not develop in the same way. Only

54% germinated to form normal-looking plants. Watery callus formed on the edge of the scutella of the larger embryos. Smaller embryos degenerated into globular or watery callus that did not redifferentiate under these conditions. All embryos developed necrotic regions where they contacted the medium.

Three phases in immature zygotic embryo development in vitro were identified in these experiments (Section 4.1) and the response of these to culture are summarised in Table 4.2.

Table 4.2

Effect of embryo orientation on development of zygotic embryos of different sizes in vitro

Approximate Size of Embryo (mm)	Orientation on medium	Survival rates
< 0.25	S.U.	Embryos degenerated into callus and died.
"	S.D.	"
0.25-0.7	S.U.	"
"	S.D.	Embryos continued to enlarge and germinated to produce normal-looking plants.
>0.7	S.U.	"
"	S.D.	"

SU= Scutellum-Up SD= Scutellum-Down

4.3 The effect of different basal media on immature zygotic embryo development in vitro

Immature zygotic embryos were dissected and cultured scutellum-down as described previously (Sections 2.10, 4.2). Twelve embryos were dissected and pooled before being randomly assigned to one of four basal medium treatments each with three hormone levels, (a total of

12 treatments). Each treatment consisted of 10 plates each with 10 embryos, all of which were cultured scutellum-down (section 4.2). The basal media selected for this experiment were:

1) F medium (Appendix 1.1): the medium used for earlier barley anther culture experiments in this thesis.

2) E medium (Appendix 1.1): modified from the immature embryo culture medium of Norstog (1973).

3) E0.01 medium (Appendix 1.1): E medium (2) with the addition of 0.01mg/l of ABA.

4) C medium (Appendix 1.1): modified from the minimal medium of Caboche (1980), formulated for the low density culture of tobacco protoplasts.

For this experiment all media contained 60g/l sucrose, and 5.0g/l Sea Plaque Agarose. Media with the suffix "O" had no auxin or cytokinin supplement. Those media with the suffix "D" contained 0.4mg/l IAA and 0.4mg/l BAP and those with the suffix "C" contained 1.0mg/l IAA and 1.0mg/l BAP (Appendix 1.1).

Embryo development was monitored weekly for six weeks after culture initiation. The number of germinating embryos and any developmental characteristics were recorded. The data were analysed statistically (according to methods described in Section 2.14).

The percentage of embryos that germinated, for each treatment, are given in Figures 4.3.1- 4.3.4.

Figure 4.3.

The Influence of Basal Media and Hormone Concentration on Immature Zygotic Embryo Development

Figure 4.3.1. **F Medium**

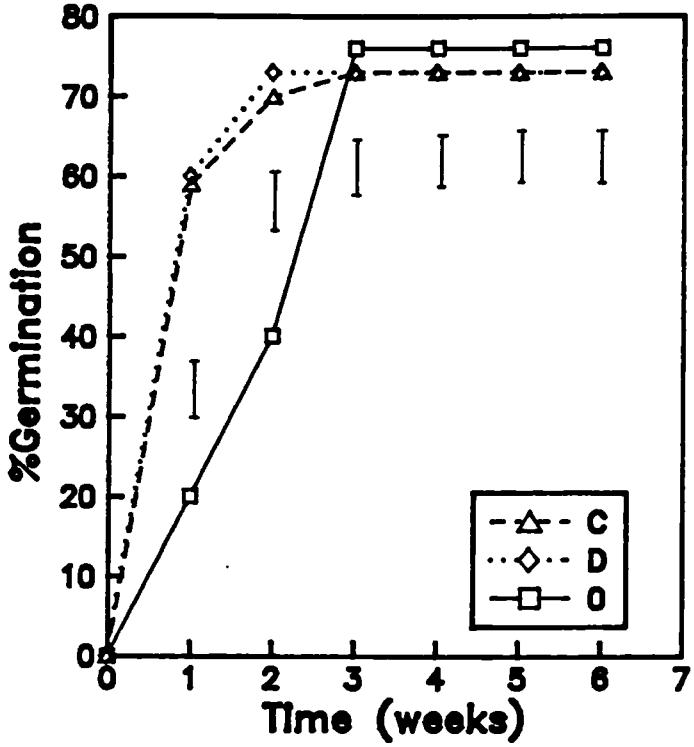


Figure 4.3.2. **C Medium**

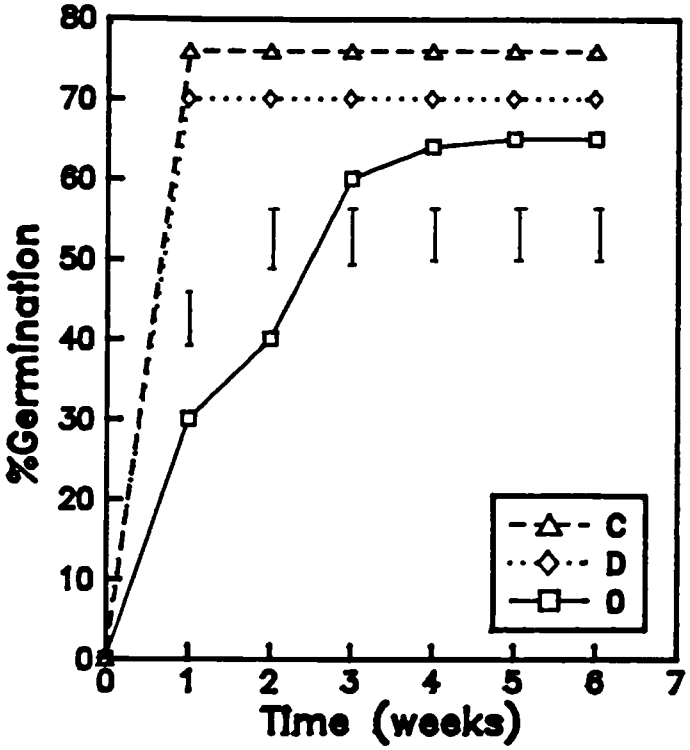


Figure 4.3.3. **Medium E**

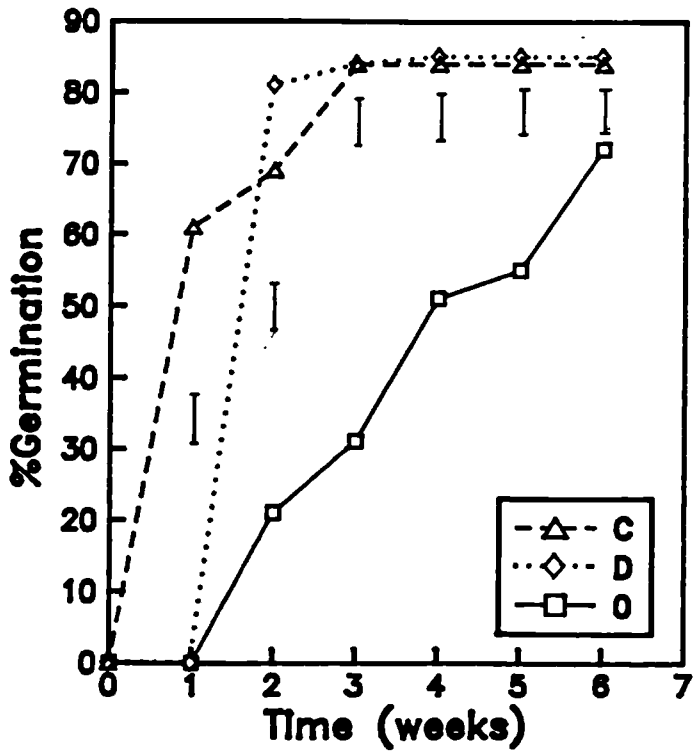


Figure 4.3.4. **E0.01 Medium**

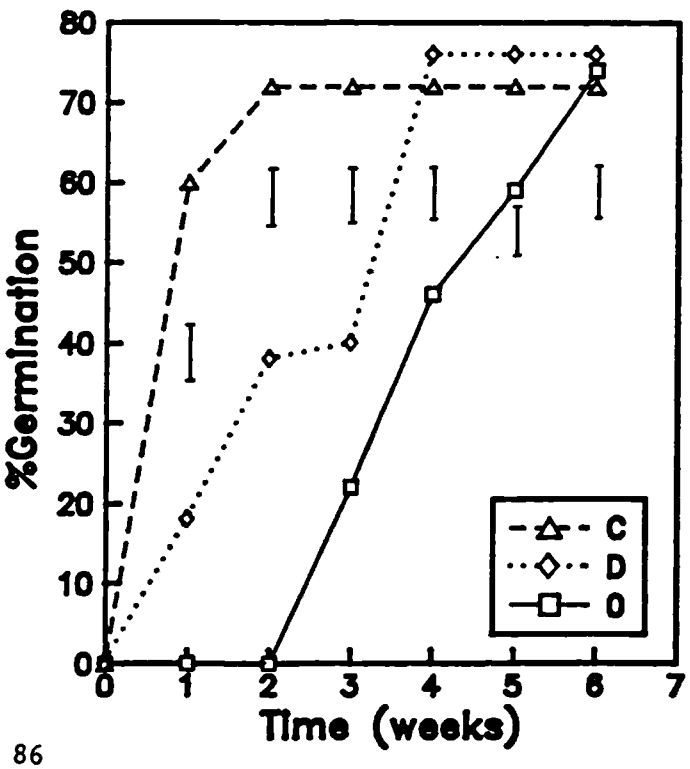
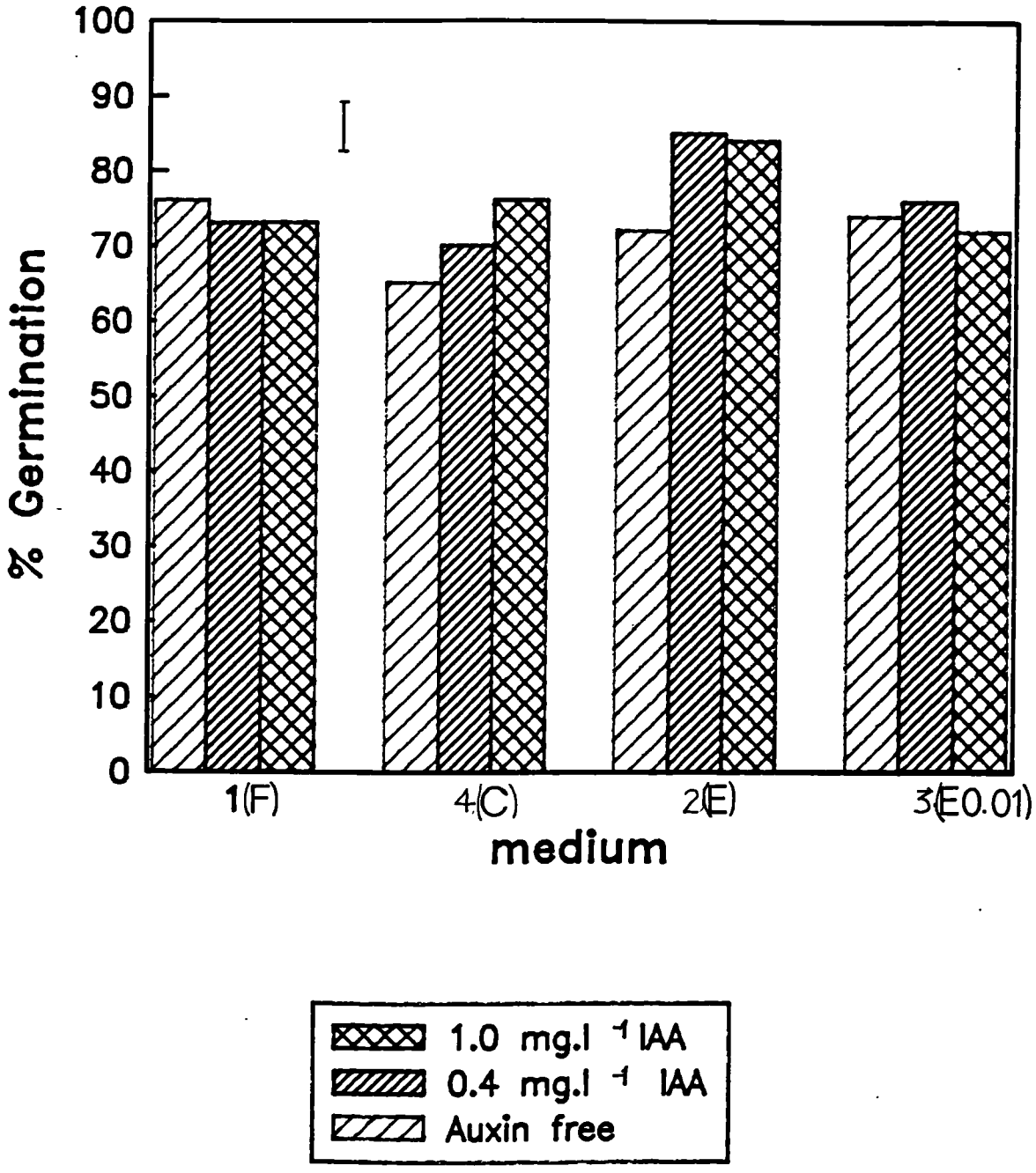


Figure 4.3.5. % Embryo germination after six weeks of culture



Both basal medium and hormone levels affected the rate of embryo development and germination. Embryos on all media with auxin and cytokinin generally developed more quickly than on those without. Embryos on C and F basal media generally germinated more quickly than those on E or EO.01. However, after 6 weeks, germination of embryos on E media with hormone supplements (ie EC and ED) was significantly better than in all other treatments. The addition of 0.01mg/l ABA to the E media; EO(0.01), ED(0.01) and EC(0.01) retarded embryo development (Figure 4.3.5).

Two embryo characteristics seemed to be common in all cultures, regardless of basal medium or hormone concentration. 1) Embryo size seemed to affect development, those greater than 0.25mm long generally developed normally but smaller ones degenerated into globular callus and died. 2) More than 80% of the surviving embryos developed necrotic regions on their scutellum after 3 weeks of culture. These regions were always at the point where they contacted the medium.

4.4 The effect of basal medium on anther culture response and plant production

Several basal media were examined to assess their affect on the initiation and subsequent morphological development of androgenic structures. All other media components were constant for the treatments tested.

The media were supplemented with 60g/l sucrose, 1.0 mg/l IAA and BAP (Foroughi-Wehr et al, 1979; Lyne et al, 1986). The gelling agent was 5g/l Sea Plaque Agarose, (Lyne et al 1986).

In the first of two preliminary experiments the media used were identical to those used in the immature zygotic embryo culture experiment (Section 4.3).

Anthers were harvested from pretreated spikes (Section 2.9; 2.10) of cv Sabarlis and assigned to treatments according to a "randomised block" design (Appendix 2.1). This was so that variation in response between anthers (viz Sunderland 1982) would not complicate data analysis unduly. Each treatment received 60 anthers and was replicated 10 times.

Anther response, numbers of structures in each class and number of green and albino plants were recorded for each trial.

Green plant yields per 100 cultured anthers were best on medium F. However, medium E without ABA supported the greatest number of androgenic structures.

Treatments F and E were repeated as reference points in a second experiment. In addition, medium H, prepared by mixing F and E media in a 1:1 ratio, and medium J, which was another of the media developed by Norstog (1967) for the culture of immature zygotic barley embryo culture, were also evaluated. The results of these two preliminary experiments are summarised in Appendix 3, (Figures A.3.1-6). Anthers did not respond on medium J. Average green plant yield per 100 cultured anthers was best on medium H. Results from media F and H did not differ significantly when analysed statistically.

Treatments on media; F, E, and H were repeated in the experiment described here to confirm the media effects observed in the earlier

trials (Appendix 3). Medium G was chosen as the fourth treatment since this treatment was a modified F medium in which alanine was deleted as a result of discussions with J.Dunwell (1983) who revealed that Foroughi-Wehr did not use alanine in current barley anther culture media.

In the main experiment described here, anther response on medium E was significantly better than that found on medium H or F (Figure 4.4.1) . There were no significant differences between the responses of anthers cultured on media E and G or between G and the remaining treatments (Figure 4.4.1). There was no significant difference between green plant yields per 100 cultured anthers (Figure 4.4.2) on either F, H or G media, but these yields were all significantly higher than those obtained on medium E. Anthers cultured on medium E, F and H responded in this experiment as observed in the preliminary experiments (Appendix 3).

Again, one observation common to all treatments was that androgenesis only occurred in the top lobe of "on-edge" anthers. As before, androgenic structures did not survive direct contact with the media before ca day 21 after culture initiation.

4.5.The effects of different cytokinins on androgenesis in barley anther cultures

Three naturally occurring and two synthetic cytokinins were chosen for assessment. All five had been used routinely to promote embryogenesis in mono- and dicotyledonous tissue culture systems (Raghavan, 1986) (Table 4.5.).

The cytokinin treatments and the cytokinin-free control all contained

Figure 4.4. The effects of different basal media on barley anther culture.

Figure 4.4.1. ANTHER RESPONSE

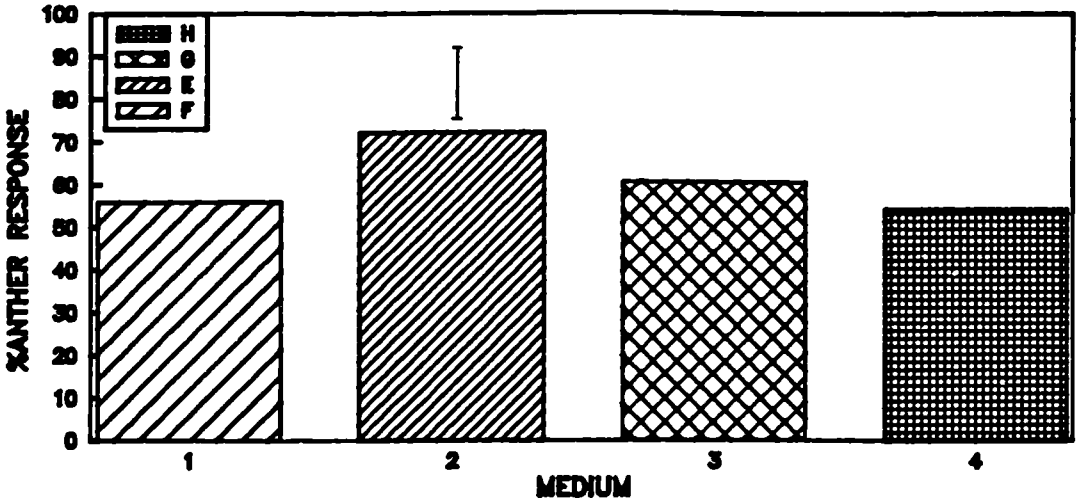


Figure 4.4.2. GREEN PLANTS

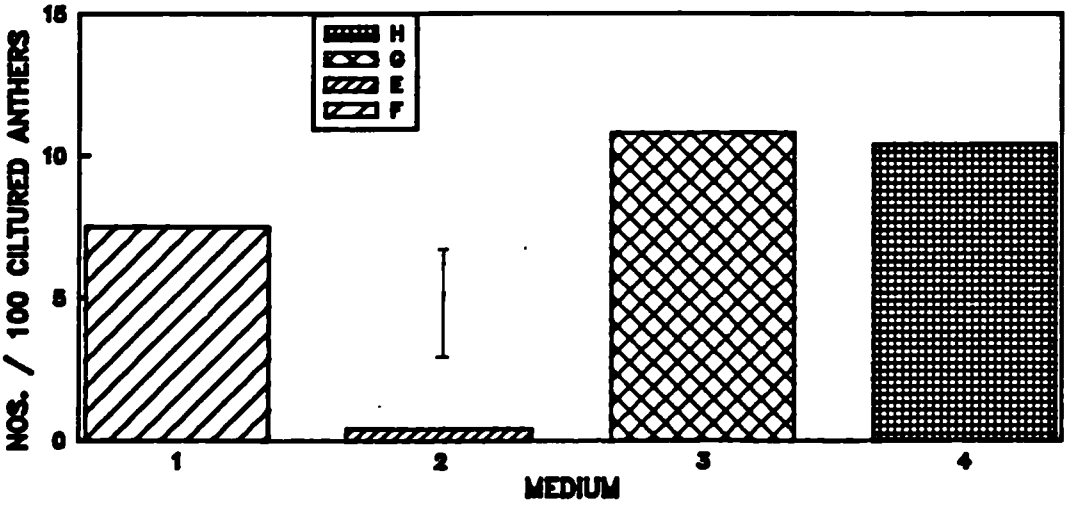
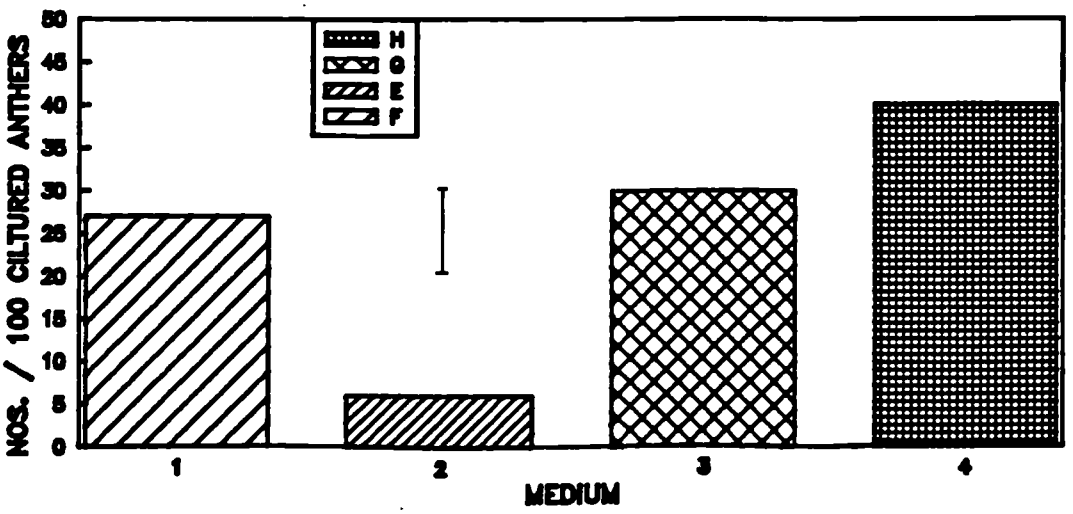


Figure 4.4.3. TOTAL PLANTS



1.0mg/l IAA (all media used for anther culture in this study up to this point had included this level of IAA (Lyne et al, 1986; Clapham, 1973).

Table 4.5.
Natural and synthetic cytokinin treatments used in this study

Cytokinin	Source	Mol.Wt	Concn. mg/l	Molarity $mMx10^{-3}$
BAP	Synthetic	215.2	1.0	4.44
Kinetin	Synthetic	225.3	1.0	4.65
Zeatin	Natural	219.2	1.0	4.56
Zeatin Riboside	Natural	351.4	1.6	4.55
2iP	Natural	203.2	1.0	4.93

Anthers from pretreated spikes were dissected and randomly assigned to treatments (Appendix 2.3). There were six treatments which were replicated five times. Each treatment received 48 anthers from each of six spikes used for each replicate.

Anthers were cultured "on-edge" using G medium (Appendix 1.1). All other culture conditions were as described earlier (Section 2.10.). Anther response (Figure 4.5.1) did not differ significantly between the treatments although as in previous experiments, androgenesis only occurred in the top lobe of anthers remaining "on-edge". There was no significant difference between treatments with respect to the production of less organised structures, ie. type III and IV structures as described earlier (Section 3.5.).

With the exception of the kinetin treatment, the BAP treatment was

Figure 4.5. The effects of different cytokinins on barley anther culture.

Figure 4.5.1 ANTHER RESPONSE

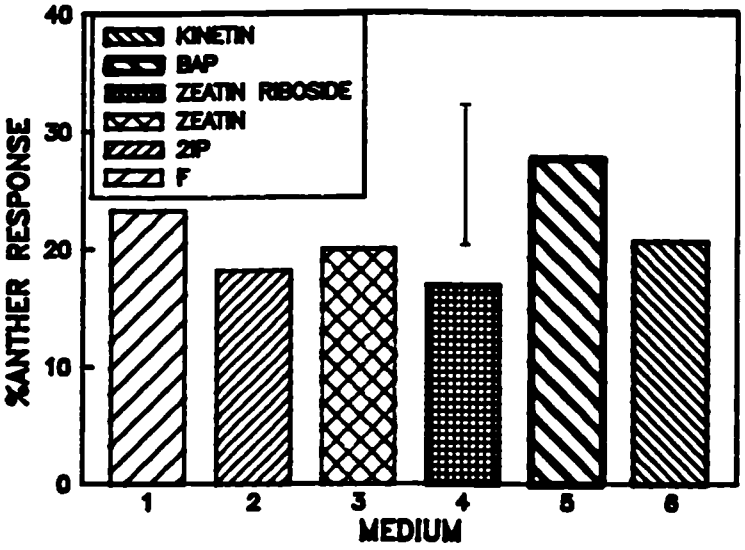


Figure 4.5.2. GREEN PLANTS

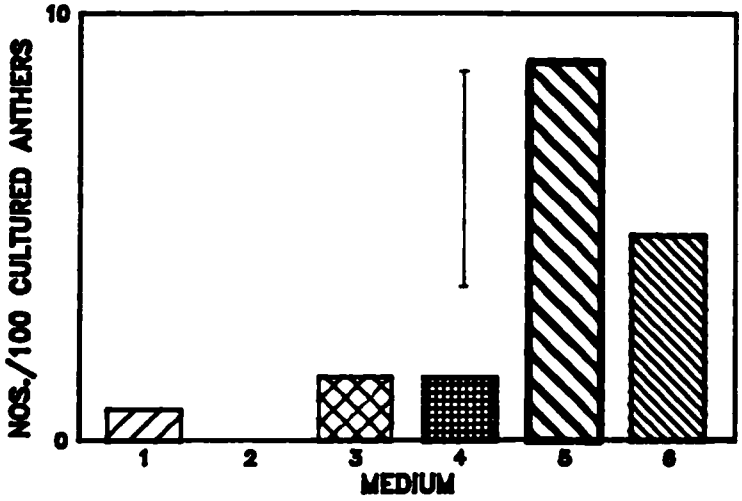
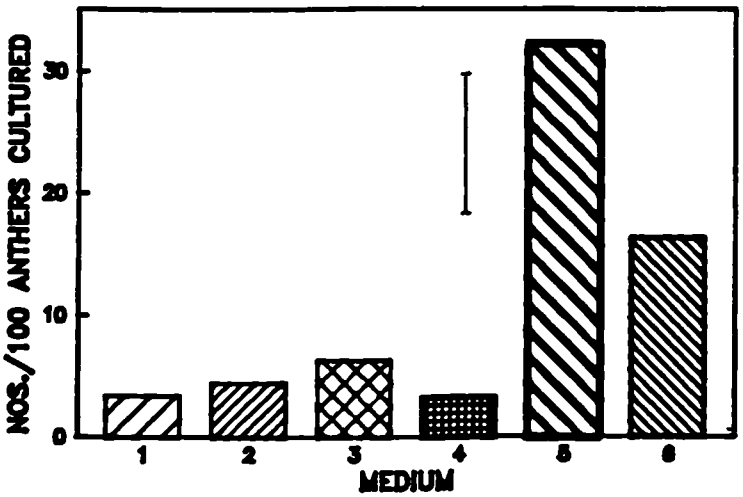


Figure 4.5.3. TOTAL PLANTS



significantly better than the other treatments with respect to both embryoid and green plant production (Figure 4.5.2) and total plant production (Figure 4.5.3). The kinetin treatment was significantly better than the remaining treatments for total plant production, but was not significantly better for green plant production. Significantly more green and total plants per responding anther were obtained on BAP medium than on the other treatments. There was still no response in anther lobes that were in direct contact with the culture media.

4.6. Effect of auxins on androgenesis in barley anther cultures.

Preliminary experiments were undertaken to select a suitable auxin for further investigation. Medium G (Appendix 1.1) containing 1.0mg/l BAP was used as a basal medium to compare the naturally occurring auxin IAA with the synthetic auxins IBA, NAA and 2,4-D (treatments 1 to 4) and two auxin-free controls (treatments 5 and 6) (Table 4.6.).

Table 4.6.
Auxin treatments investigated for barley anther culture

Treatment	Auxin	Source	Molecular weight	Concentration mg/litre	Molarity ($mM \times 10^{-3}$)
1	IAA	Natural	175.2	1.0	5.7
2	IBA	Synthetic	203.2	1.0	4.9
3	NAA	Synthetic	186.2	1.0	5.4
4	2,4-D	Synthetic	221.0	1.0	4.5

Anthers from six pretreated spikes were dissected and randomly assigned to each of the treatments (Appendix 2.4). Each treatment received 48 anthers and was replicated twice.

Androgenesis occurred in all treatments. On-edge anther orientation was not an absolute requirement when anthers were cultured on media containing synthetic auxins since androgenesis occurred in both lobes of several anthers on these media independent of orientation. However, direct embryogenesis and plant production only occurred in the control and IAA treatments. The remaining treatments produced large numbers of calli but no embryos. IAA was selected for further investigation with the aim of optimising embryogenesis and improving plant yields.

4.7. The effect of IAA concentration on androgenesis in barley anther culture

A randomised block experiment was used to confirm the advantageous influence of IAA and to determine the optimum concentration of this auxin for maximum green plant production.

Anthers from pretreated spikes were dissected and randomly assigned (Appendix 2.5) to treatments 1-6 (Table 4.7.1). The anthers were placed on-edge at the start of culture.

Table 4.7.
IAA treatments examined

Treatment	Sterilisation	IAA concentration mg/litre
a) 1	Autoclaved **	1.0
2	Filter-sterilised *	1.0
3	" "	0.3
4	" "	0.1
5	" "	0.05
6	" "	0

a) The basal medium was medium G (Appendix 1.1) with 1.0mg/l BAP.
* = Filter-sterilised through a 0.2µm filter membrane
**= Autoclave-sterilised at 115C for 15 minutes.

Each treatment received 48 anthers and was replicated seven times.

The results are summarised in Figures 4.7.1; 4.7.2 and 4.7.3. No significant difference was seen between the treatments for any of the parameters investigated, with the exception of green plant production per 100 anthers cultured. In this case, there was a trend of increased yields with reduced concentrations of IAA. IAA is known to be light-labile (Yamakawa et al, 1979). The experiment was repeated using freshly prepared medium to confirm that the light-labile IAA had not degenerated before or during the early stages of the initial experiment (Section 4.7). Each treatment was replicated five times. The results of the repeated experiment were consistent with those obtained in the initial experiment. Therefore exogenous IAA was deleted from the media used in subsequent experiments described in this study.

Figure 4.7. The effect of IAA concentration on barley anther culture.

Figure 4.7.1. ANTHER RESPONSE

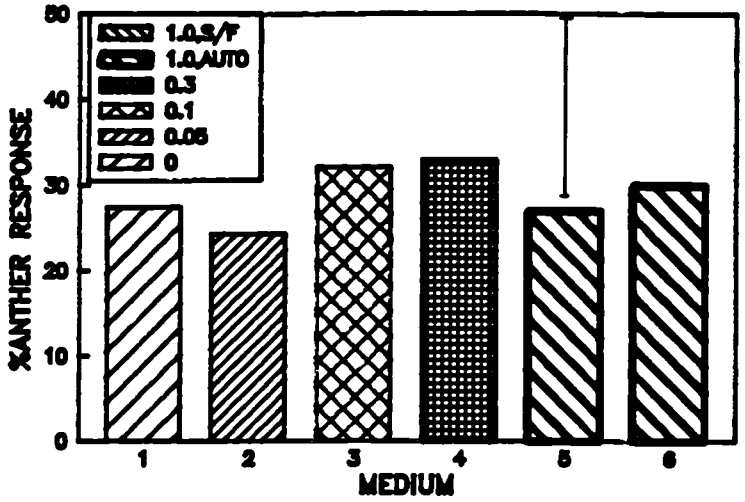


Figure 4.7.2. GREEN PLANTS

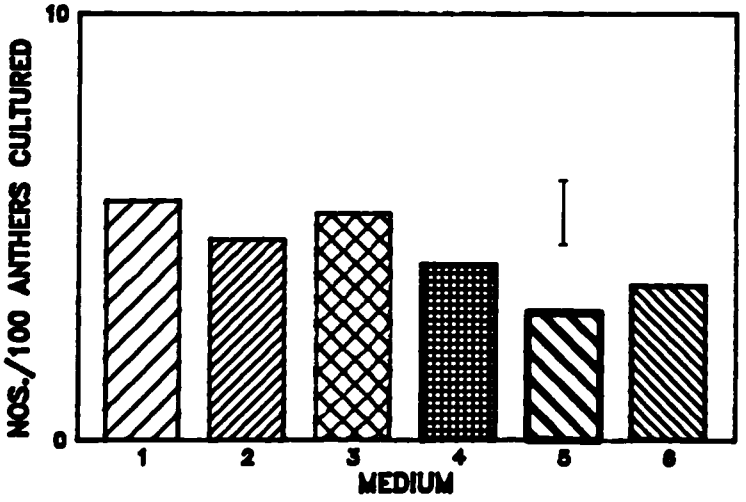
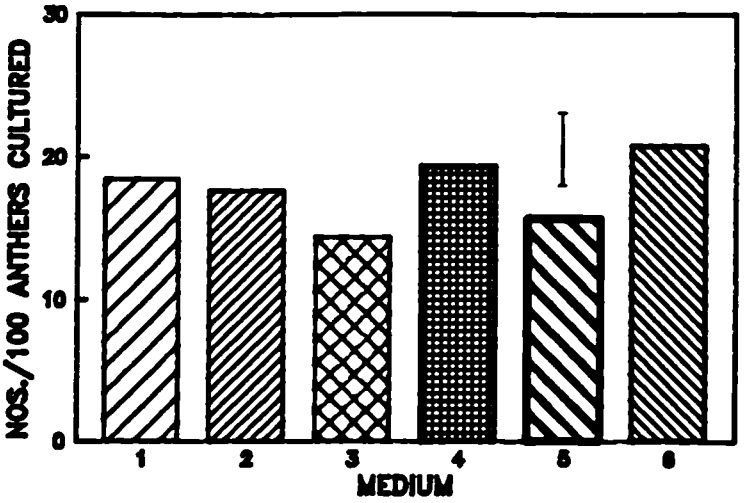


Figure 4.7.3. TOTAL PLANTS



4.8 The effect of amino acids on barley anther culture

The influence of amino acids on the development of plant tissue cultures has been reported widely (Raghavan, 1986 for a review). Both glutamine and proline had been found to enhance callus production in anther cultures of barley (Huang, 1982). In this experiment the effect of six different amino acid treatments on barley anther culture were assessed. Several amino acid treatments were considered and these are listed in Table 4.8.

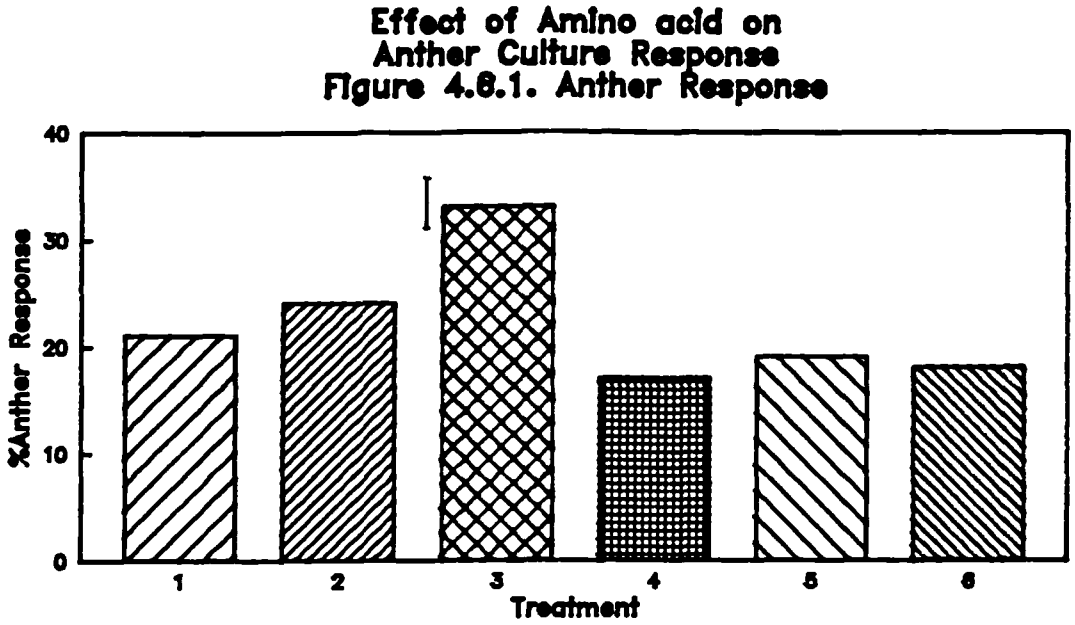
Table 4.8.
Amino acid treatments assessed

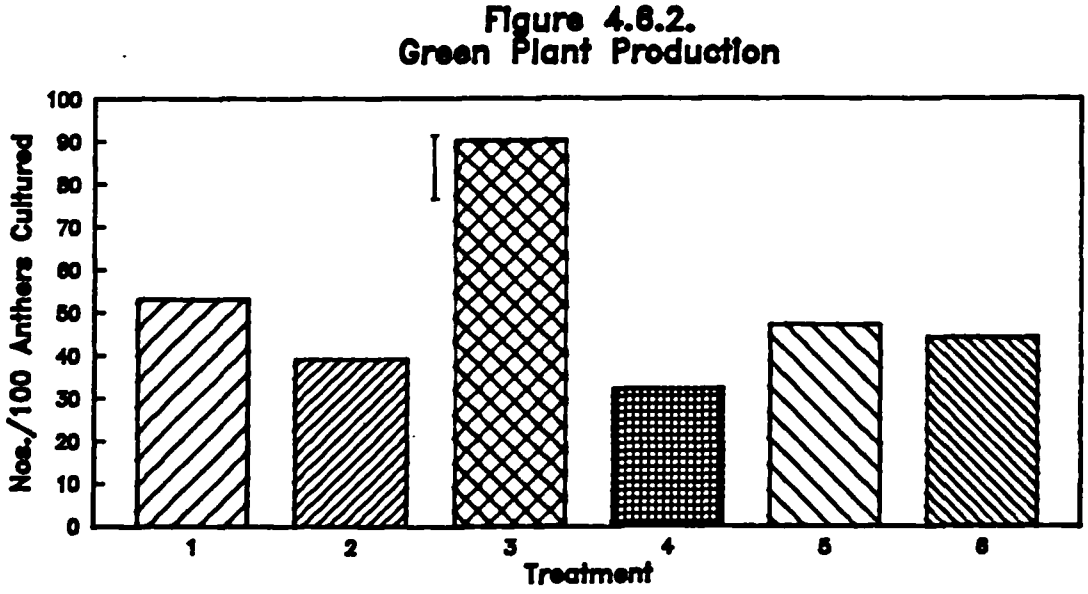
Treatment	Amino Acid	Concentration
1	Amino Acid Free	-
2	Alanine	4.5mM (400mg/l)
3	Glutamine	5mM
4	Glutamine	50mM
5	Proline	5mM
6	Proline	50mM

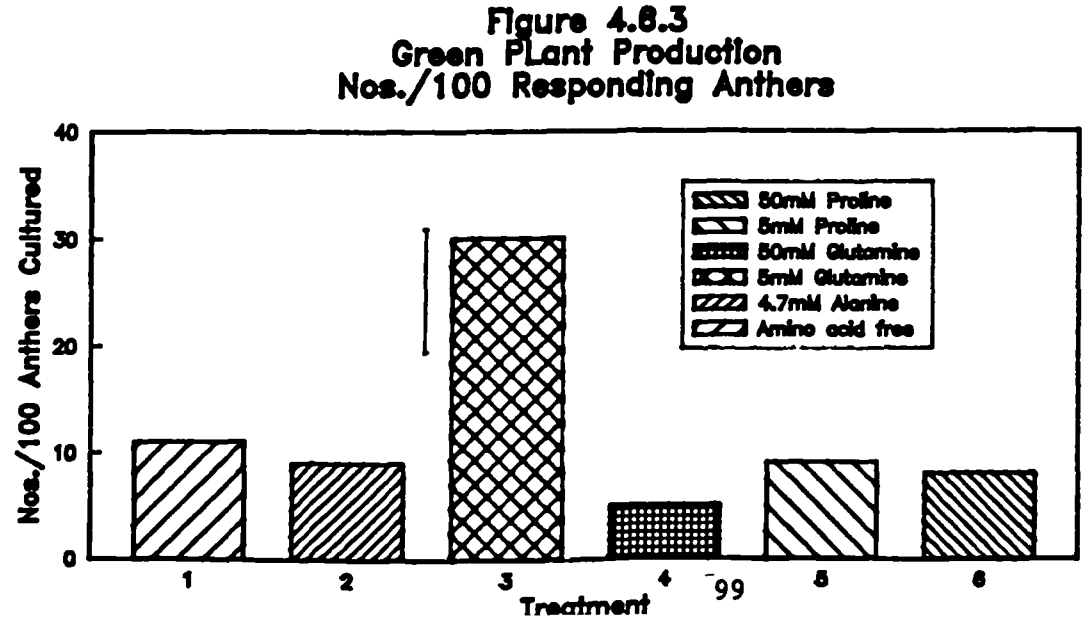
Treatment 1 was an amino acid-free control. Treatment 2 was the amino acid treatment that had been used in earlier experiments (Lyne et al 1986). The concentrations of amino acids used in this experiment were similar to those found to promote embryogenesis and growth in cultures of a variety of crops, eg barley (Norstog,1962; Xu et al, 1981; Sunderland et al, 1981) and alfalfa (Stewart et al, 1984,a,b).

Anthers from six pretreated spikes of the winter barley cv Igri were assigned to each treatment according to a randomised plot design (Appendix 2.6). Each treatment received 48 anthers and was replicated five times. The results are summarised diagramatically in Figures 4.8.1-3.

Figure 4.8. The effect of amino acid on barley anther culture.







Anther response was significantly greater on the 5mM glutamine treatment than on the others. The alanine treatment was next, significantly greater than the remaining treatments. There were no significant differences between the remaining treatments.

Green and total plant production per 100 anthers cultured and per 100 anthers responding were significantly greater on the 5mM glutamine treatment than all the others.

4.9. Effects of sugar on anther response and plant production

In this experiment, sucrose was compared with maltose as the primary carbohydrate source (See Section 4.10 for rationale behind maltose selection. Page 113-115).

Anthers from pretreated spikes of cv Sabarlis (Section 2.9) were cultured on-edge on the medium of Foroughi-Wehr et al, (1978). This medium did not contain exogenous amino acids. The primary carbohydrate source was either sucrose or maltose at 175mM. The experiment was replicated 10 times. Two spikes were used for each replicate. Twenty four anthers were assigned , at random, to each treatment and the results were analysed statistically (Section 2.14.).

The results are illustrated in figures 4.9.1-3. Anther response was significantly greater on the maltose medium. Green plant production was highly significant. The mean green plant yield was ten times greater on maltose than on sucrose-containing media. Part of this difference could be ascribed to the increased anther response. When the data were expressed as green plants per 100 responding anthers the anthers on maltose were twice as productive as those on sucrose.

Figure 4.9. The effect of sugar on barley anther culture.

Figure 4.9.1. **Anther Response**

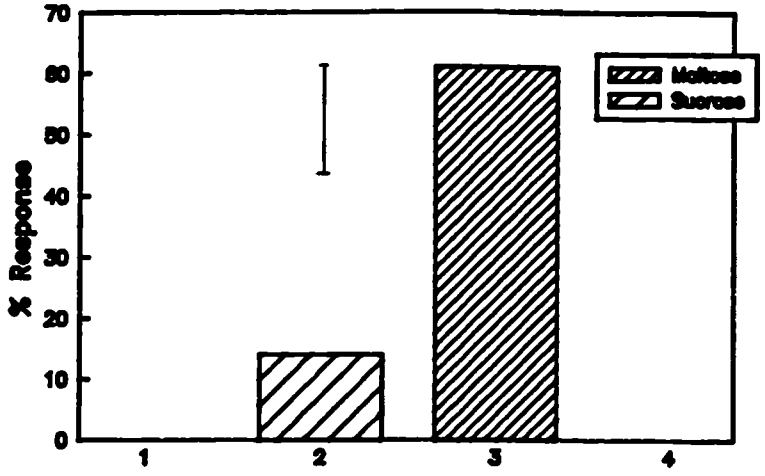


Figure 4.9.2. **Green Plants**

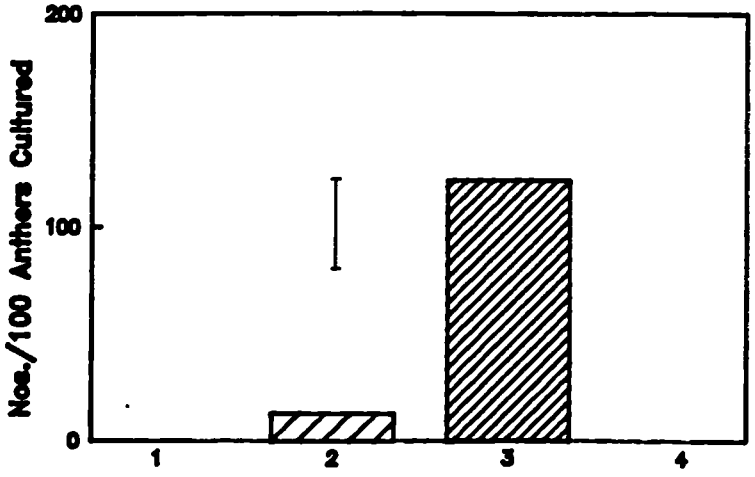


Figure 4.9.3. **Green Plants**

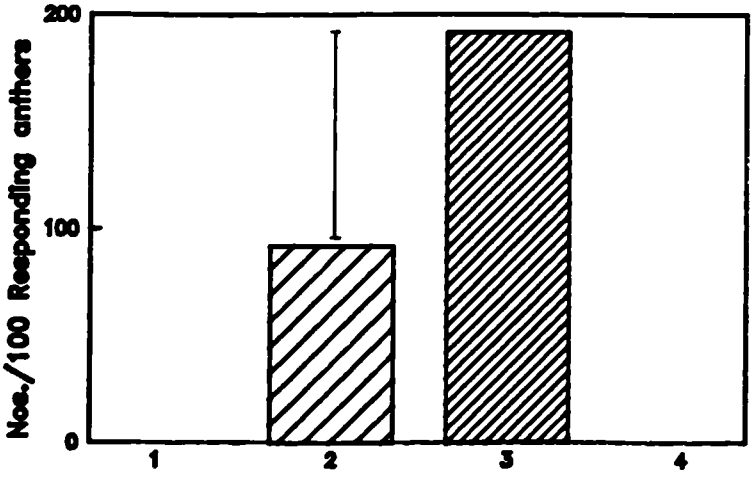




Figure 4.9.4. Barley anthers after 28 days culture on solid medium with sucrose as the primary carbohydrate source. Note only "on-edge" anthers have responded.

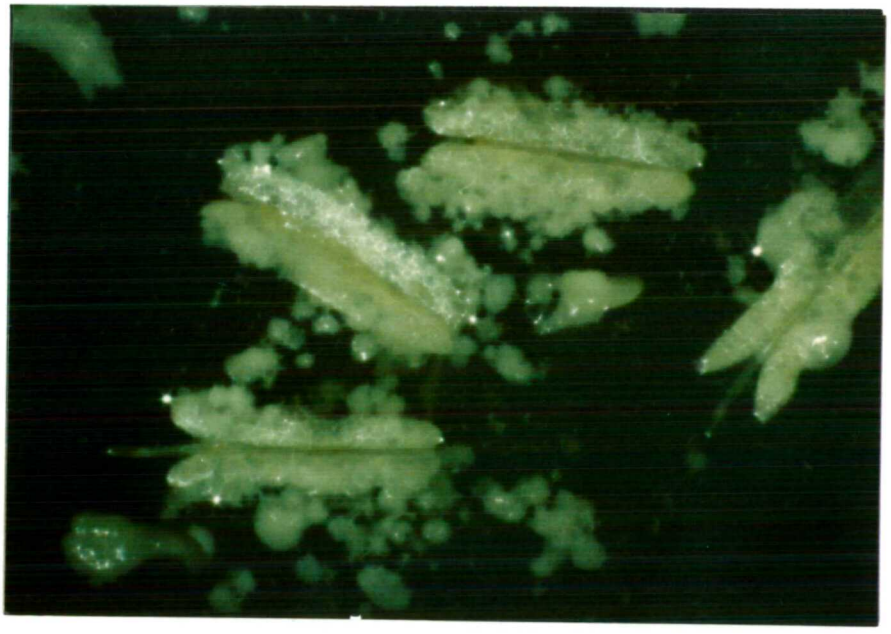


Figure 4.9.5. Anther response after 28 days culture on medium containing maltose as the primary carbohydrate source. Note response in both lobes of anthers cultured "flat" on the surface of the medium.

This is probably due to the fact that both lobes of the anthers responded on maltose medium, but only one lobe on sucrose medium.

Differences in albino plant yields were not significantly different in this experiment. Maltose was also capable of supporting the "normal" development of small (<200μm) zygotic embryos *in vitro* (data not shown).

4.10 Discussion

Barley anther cultures and excised immature zygotic embryos were used to study the effect of several defined media on their response and development.

Published methods for the culture of excised immature zygotic embryos varied according to author (*eg* Norstog, 1961; Cameron-Mills *et al*, 1977; Dunwell, 1981,a,b,c;1983). Excised embryos of barley were cultured scutellum-down (Norstog, 1961) or scutellum-up (Dunwell, 1981a, 1981b, 1983). Norstog reported that culturing immature embryos scutellum-up was detrimental to their development while Dunwell cultured embryos scutellum-up throughout a series of experiments on embryo development (1981a, 1981b, 1983). Dunwell did not comment on any differential orientation effect.

The apparent requirement for scutellum-down orientation of small immature zygotic embryos on the culture medium described by Norstog (1961) was also confirmed in the current study (Section 4.1). Orientation of larger embryos, *ie* 0.7mm and larger, on the medium was found to be less critical. Since Dunwell (1981,a,b; 1983) used larger embryos perhaps this explains why he did not record any adverse effects due to scutellum-up embryo orientation.

Norstog (1967) observed a differential survival which was dependent upon embryo size. He reported that "Survival of the smaller embryos, 200-300um, was not completely satisfactory....". Norstog referred to embryos which were 200-400μm long. Unfortunately, no information about embryo size distribution was given for individual or collective experiments. From the results shown in Sections 4.1 and 4.2, it would be expected that embryos of ca 0.25mm and smaller would die in the culture medium employed by Norstog et al (1963) and subsequently by Norstog (1965; 1967). Larger embryos would be expected to survive. If the embryo size distribution varies between experiments and between replicates within the same experiment, problems with reproducibility of results would be likley to be encountered.

Embryo survival in vitro appears to be related to some size or developmental factor. This is apparently true for both zygotic and androgenic embryos. Although there is no absolute size correlation available between zygotic and androgenic embryos, nevertheless there are striking similarities between their developmental and survival characteristics (Sections 3.4, 4.2, 4.3). The inability of small embryos to survive when in culture on the surface of the media can, at this stage, not be explained. However, the proposals put forward to explain the requirement of barley anther orientation to permit embryogenesis from immature microspores (Section 3.6) may also apply to the effect of embryo orientation on the survival of "intermediate-sized" embryos. Another explanation might be that the scutellum tissues may regulate nutrition and/or hormone supply to the rest of the embryo. They may modifiy the medium itself or regulate the polarity of nutrient supply to the embryo.

Since the cultural conditions were suboptimal for embryo development, and indeed apparently fatal to embryos during the early stages of development, the possiblity that a medium component(s) that was toxic or inhibitory to developing young embryos was investigated.

Androgenic structures have been obtained by culturing anthers of N.tabacum on water agar (Matsubayashi et al, 1977; Vagera et al, 1982); indeed, water may be beneficial at the start of culture (Imamura et al 1982) however, the continued survival of the microspores was dependent on the addition of a dilute mineral solution and some carbon source (usually sucrose). Dunwell (1986) recommended the use of the N6 basal medium of Chu (1978) with a sucrose concentration of 6-15% for cereals.

The media used for barley anther culture prior to this thesis were formulated to enhance callus formation from immature microspores (Kao, 1981; Huang et al, 1982; Foroughi-Wehr et al, 1982). The basal media selected for assessment were all defined media which had been formulated for plant tissue cultures as diverse as tobacco protoplast culture, barley anther culture and the culture of immature zygotic embryos of barley (Section 4.2, 4.3).

Immature zygotic embryos developed and germinated at different rates depending upon the type of basal medium used (Figure 4.3.). Although E medium was found to give highest embryo development and germination the differences between the media were small, and none of the media were able to support the development of the smallest immature embryos.

The differential effect of basal media on anther culture response and androgenic structure morphology was striking. All of the media were adjusted to pH 5.6 initially and, therefore, the macroelements would be expected to have dissociated into their ionic components (Appendix 1.2). The ionic constituents of the basal media were similar. Because of this, it is difficult to explain the lack of response on medium J.

Of the media that supported anther cultures the most striking differences were between medium G and medium E (Section 4.4, Appendix 1). There were differences in the concentration of several ionic constituents of these two media that may account for the varied responses. A major difference between the two media, G and E, was the nitrogen source (Appendix 1.2), the concentration and ionic form of which varied. These parameters are known to influence embryo development of cereals (Norstog, 1967, 1976, 1979; Amemiya et al, 1956), and androgenesis (Clapham, 1971, 1973, 1977). A 1:1 mixture of these two media supported an intermediate response (Section 4.4).

On-edge anther orientation remained a prerequisite for androgenesis. This was unaffected by any of the media tested in this experiment. A closer study of individual ionic medium components would be beneficial, but as the different basal media did not greatly enhance green plant production it was decided to use medium G as the basal medium for further studies since it was comparatively easy to prepare and did not differ significantly from the most productive medium, H, in its ability to support green plant production.

Media formulated specifically to culture immature zygotic embryos of barley did not significantly improve embryogenesis or green plant

yields in barley anther cultures.

The effects of plant hormones on the development and morphology of plant cells has been widely reported (eg, Bajaj, 1983; Ammirato, 1983; Ernst et al, 1984,a,b; Marsolais, 1985). In addition, both auxins and cytokinins were shown to influence optimal growth response in anther cultures of Gramineae (Clapham, 1977; Kao, 1981; Chein et al, 1983) and Solanaceae (Nitsch et al, 1969; Raghavan, 1975).

The plant hormone components of the media were investigated to optimise androgenesis and direct embryogenesis. Ideally a factorial experiment design would be used to investigate auxin/cytokinin interactions and select the optimal hormone regime. Unfortunately the availability of experimental material restricted the size and number of experiments.

When compared with the cytokinin-free control the three naturally occurring cytokinin treatments had no significant effects on the parameters scored (Section 4.5). The synthetic cytokinin treatments, however, enhanced androgenesis and influenced the morphology of the structures produced, with the BAP treatment producing a greater proportion of embryo-like structures than did the kinetin treatment. A differential effect between synthetic and naturally occuring cytokinins on callus induction in barley anther cultures has been reported previously (Kao et al, 1983). The synthetic cytokinins produced significantly higher numbers of calli per cultured anther than did the natural ones. BAP was twice as productive as kinetin. These results are consistent with the general view that BAP is a more potent cytokinin than kinetin which in turn is more potent than the

naturally occuring cytokinins (Wareing et al, 1978).

The influence of cytokinin on embryo morphology is more controversial. Al-Abta et al (1979) reported a correlation between endogenous cytokinin levels and embryo development in cultures of cytokinin-dependent celery cells. More recently, Ernst et al (1984a,b) have reported that cytokinins are important for cell growth and division, but they found no correlation between endogenous cytokinin concentrations and the events of embryo differentiation in cultures of cytokinin-independent Pimpinella anisum.

Additions of 1.0 mg/l BAP to the anther culture medium enhanced green plant yields more than the other cytokinin treatments. With the exception of the kinetin treatment this difference was statistically significant. The requirement of on-edge anther orientation for immature microspore embryogenesis was not affected by the presence or omission of cytokinin from the culture media.

In a preliminary experiment, synthetic auxins were able to partially overcome the requirement of on-edge anther orientation for androgenesis (Section 4.6). Unfortunately the structures produced were exclusively wet calluses.

One of the objectives in this thesis was to enhance direct embryogenesis to assess the homozygosity of the plants derived from the method developed here. Because of the reports that synthetic auxins tend to inhibit embryogenesis and induce callus which then becomes embryogenic (Raghavan, 1986), and the reports that progeny of a number of graminaceous species regenerated from microspore-derived

callus show segregation (Deambiogio et al, 1980; Huang et al, 1982; Ye et al, 1985; Raghavan,1986) only the influences of the natural auxin IAA were investigated further (Section 4.6.).

Of the culture parameters scored only Type I structures (embryoids) and green plant yields per anther cultured, were affected by IAA concentration. The optimum concentration of auxin was shown to be less than 0.3mg/l. At concentration of 0.3mg/l and above the yields of both products were reduced. These results are, none-the-less, in line with the general opinion that auxins tend to inhibit embryogenesis at higher concentrations (Fujimura,et al 1979; Raghavan, 1986).

Additions of IAA to immature zygotic embryo cultures promoted germination of embryos larger than 0.25mm long (Section 4.3), confirming the findings for older immature and mature embryos (Norstog,1972; Dunwell, 1981,a,b). The effect of IAA on anther and embryo cultures appears to be dependent upon the developmental stage of the embryo.

The influence of amino acids on the development and morphology of embryos from zygotic, somatic and androgenic origins of several species has been widely reported (eg. Norstog, 1961; Xu et al, 1981; Huang, 1982; Sangwan, 1983; Stuart et al, 1984a,b; Harada,et al, 1983) and reviewed by Raghavan (1986). In these reports, their effects on quantitative and qualitative embryogenesis vary between species and depend upon the type and concentration of amino acid used.

None of the amino acid treatments affected the requirement for anthers to be orientated on-edge to support androgenesis (Section

4.8). Some treatments did affect the quality and quantity of embryos which developed in culture.

The results confirmed the earlier finding that the addition of 4.7mM alanine to the culture medium did not significantly affect the efficiency of this anther culture method (Section 4.3). However, alanine at concentrations of 0.1-5mM stimulated androgenesis (Harada et al, 1983) and somatic embryogenesis (Stuart et al, 1984a,b).

The addition of 5mM glutamine to the anther culture medium enhanced anther response, embryogenesis and almost doubled green plant yields per cultured anther. This contrasts with the reports by Xu et al (1981) that barley anther cultures were unaffected or inhibited by the 0.68-5.47mM glutamine additions. However, Harada et al (1983) reported the stimulation of embryogenesis in anther cultures of N.tabacum by the addition of 0.1-5mM glutamine to the culture medium. Concentrations of 5-300mM glutamine have also been reported to stimulate embryogenesis in Medicago sativa (Stuart et al, 1984,a). Stuart et al (1984a) also reported that when these levels of glutamine were used in combination with 12.6mM NH_4^+, (as ammonium dihydrogen orthophosphate), they inhibited embryogenesis in the same system. The addition of 50mM glutamine to the barley anther culture medium inhibited anther response, embryogenesis and green plant production (Section 4.8). Concentrations of 10mM and above were supraoptimal for androgenesis in N. tabacum (Harada et al, 1983). At a concentration between 30-100mM, glutamine becomes inhibitory to somatic embryogenesis in Medicago sativa (Stuart et al, 1984,a).

Somatic embryogenesis in Medicago is stimulated by concentrations of

between 5-300 mM proline (Stuart et al, 1984,a). In the same report it was suggested that proline may have a general application in numerous species. However, the barley anther culture system was unaffected by the addition of 5mM proline to the culture medium. Huang (1982) reported that 0.7-4mM hydroxy-proline had no affect on barley microspore-derived callus induction. At 50mM, proline actually inhibited response and green plant yields. This contrasts markedly with the reports of enhanced somatic embryogenesis in Medicago (Stuart, 1984a,b).

The amino acid treatments investigated did not influence the survival of pollen in the bottom lobes of on-edge anthers or either lobe of flat anthers. They did, however, affect the induction and development of microspore-derived embryos (Section 4.8). A more detailed study of the influence of amino acids on direct embryogenesis from barley microspores is reported later (Section 6.2; 6.3; 6.4).

The media used so far could not sustain the growth of small embryos (<0.25mm), whether zygotic or androgenic isolated from the anther before the 21st day of culture. After degenerating into small globular callus they turned brown and developed no further. These events appeared to be related to embryo size and independent of origin since smallest embryos degenerated and turned brown more quickly than relatively larger embryos. In this case, the cause of death may be the same for both zygote and pollen-derived embryos.

One possible explanation for the ability of "on-edge" orientated anthers to support the development of very small embryos was that a medium component was toxic and that "on-edge" anther orientation

prevented this component from reaching inhibitory concentrations within the top lobe of the anther.

The biochemical environment of the young androgenic and zygotic embryos *in vitro* (Section 4.1-4.8) was compared with the information available in the literature about the *in vivo* biochemical environment during early zygotic embryogenesis of barley.

Several components of the culture media had already been investigated individually and were found to have either no effect on the requirement for anther orientation (Sections 4.5; 4.7; 4.8) or to be detrimental to microspore embryogenesis (Section 4.6). Other components of the media used in the earlier stages of this study (Sections 4.1-4.8) were consistent throughout. These included *eg* the type of sugar, vitamins, inositol and pH. In addition, other components had varied but had not been investigated individually, *eg* macro and micro elements and osmolality.

Sucrose was the primary carbohydrate source used in all of the media described. Although other sugars and sugar oligomers have also been used to culture immature zygotic cereal embryos (Raghavan, 1976,b), sucrose has always proved the most successful (Brown *et al*, 1890; Amemiya *et al*, 1956). In a more recent review, Raghavan (1986) states "Sucrose is unsurpassed, amongst others, for embryos of *Datura stramonium* (van Overbeek, *et al*, 1944), *Capsella bursa-pastoris* (Rijven, 1952), or *Hordeum distichum* (Cameron-Mills *et al*, 1977)". Assumptions about carbohydrate metabolism *in vivo* during early embryogenesis appear to have been almost exclusively drawn from data obtained in experiments on older embryos and this then extrapolated.

None of the media used in the experiments for cereal embryo culture were able to support early development of immature embryos.

Sucrose is the end-product of photosynthesis and considered to be the main carbohydrate transport material in plants (Duffus, 1979). In cereals, sucrose is transported to the seed where grain development is characterised by net synthesis and deposition of starch.

It was recognised that during barley grain development, in addition to the synthesis and deposition of starch, catabolic processes were active in starch hydrolysis and degradation (Duffus, 1979). During grain development energy was required for a wide variety of synthetic processes (Duffus, 1979). It remains unclear whether incoming sucrose or granular starch provides the direct substrate for energy production (Duffus, 1979).

Starch is present in developing pollen, tapetal tissues and seeds (Stanley et al, 1974; Heslop-Harrison, 1970; Duffus, 1979). In barley, β-amylase activity has been reported to be present throughout seed development (Williams, 1976). β-amylase activity has also been reported in secretions produced by pollen of Gramineous species just prior to pollen tube germination (Stanley et al, 1974). Maltose was selected to substitute sucrose because it is the major product of the β-amylolytic catabolism of starch (Briggs, 1978; Akazawa et al, 1980; Priess et al, 1980; Manners, 1985). In addition, maltose had been reported to be synthesised de novo from activated glucosyl residues during photosynthesis and it had been suggested that, in some species, maltose may have replaced sucrose as the primary carbohydrate source in young leaves of Acer species and

spinach (Kandler et al, 1980).

In maize, up to 22% of the dry weight of maturing pollen was starch (Stanley et al 1974). In maturing pollen grains the amount of starch, as a proportion of the pollen dry weight, varies according to the growth environment of the plants and, more importantly, if dehiscence is delayed the starch content in pollen falls (Stanley et al 1974). This may suggest the presence of active mechanisms in pollen for the metabolism of starch similar to those present in developing seeds.

When maltose was compared with sucrose as the primary carbohydrate source, the maltose treatment supported greatly improved green plant production and overcame the need for on-edge anther orientation.

The data are consistent with the hypothesis that sucrose, or a metabolite thereof, is toxic to microspores or developing embryos. By replacing sucrose with maltose, embryoid development occured in both anther lobes in any orientation, and also on the surface of the medium. These phenomena had not been observed on the sucrose-containing media in this study.

There has been one previous report of the use of maltose for anther culture (Raquin, 1983). Embryoid production from anthers of petunia on media containing a number of different sugars was compared. Maltose was the best of the sugars tested giving 22% more embryos than the sucrose treatment. No mention was made of anther orientation in this paper. The use of maltose for barley permits anthers to be cultured in any orientation. This reduces the time taken to set up cultures and significantly increased green plant yields. The observation that structures which had become isolated from the anther

were able to develop may suggest that this culture system will be suitable for the culture of microspores/pollen isolated from anthers at the beginning of culture.

4.11 Conclusion

Androgenesis and embryo development were affected by several media components, notably nitrogen and cytokinin. None of these affected the need for on-edge anther orientation in barley anther culture.

Sucrose, or a metabolite thereof, was found to inhibit androgenesis and early zygotic embryo development (<200μm) in this system. By using maltose the requirement for anther orientation was overcome, androgenesis was enhanced, small zygotic embryos and very small androgenic structures could develop into embryos and thence to plants even when isolated on the medium (Section 4.9).

Chapter 5
Investigations on the effects of sugars on the development of microspores in anther culture

5.1 Introduction

Experiments described in Section 4 led to the discovery that sucrose in the culture medium was detrimental to the development of both androgenic structures and zygotic embryos during their early growth. By replacing sucrose with maltose it was possible to sustain development even when the androgenic structures contacted the medium from the beginning of culture (Section 4.9).

In this chapter the influences of several sugars on this culture system were investigated in an effort to understand and, possibly, to explain the differential effects of sucrose and maltose observed earlier (Chapter 4.9). The results of these experiments are presented and their implications discussed.

5.2. The effect of different sugars on barley anther culture

The effects of sucrose and maltose on flat and on-edge anthers were compared with their component monosaccharides and a number of structurally related disaccharides and one trisaccharide (Table 5.2.1). Nine different sugar treatments were used for anthers cultured flat or on-edge.

Table 5.2.1
Sugar Treatments

Sugar	Monomeric structure	Concentration (mM)
Fructose	-	175
Glucose	-	175
Glucose	-	350
Sucrose	1-0-α-D-glucopyranosyl-β-D-fructofuranoside	175
Raffinose	α-D-Galactosyl-sucrose	175
Lactose	4-0-β-D-galactosyl-D-glucopyranose	175
Cellobiose	4-0-β-D-glucopyranosyl-D-glucopyranose	175
Trehalose	1-0-α-D-glucopyranosyl-α-D-glucopyranoside	175
Maltose	4-0-α-D-glucopyranosyl-D-glucopyranose	175

Anthers were excised from pretreated spikes and assigned to each of 18 treatments according to a randomised block experiment design (Appendix 2.8). Each treatment was replicated five times.

The data were analysed statistically (Section 2.14). The effects of carbohydrates on the responses of anthers orientated flat or on-edge were analysed separately for individual sugars (Table 5.2.2). The comparative effects of the sugar treatments were analysed first for flat anthers (Figures 5.2.1-3) and then for on-edge anthers (Figures 5.2.4-6).

Figure 5.2. The effect of sugars on barley anther culture. Anthers cultured "flat"

NB. The fructose treatment was omitted from Figures 5.2.1-6 because it inhibited androgenesis completely.

Figure 5.2.1. **Anther response**

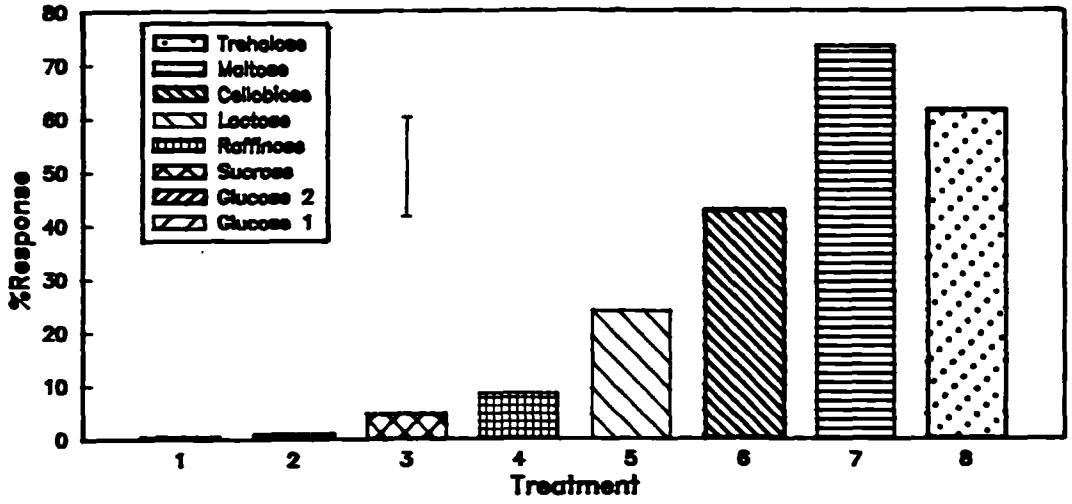


Figure 5.2.2. **Green Plants**

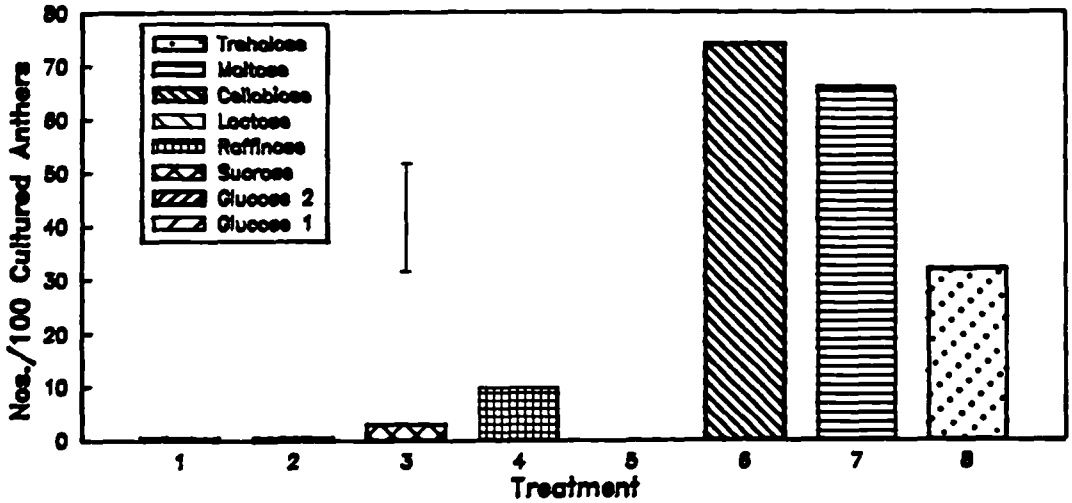


Figure 5.2.3. **Green Plants**

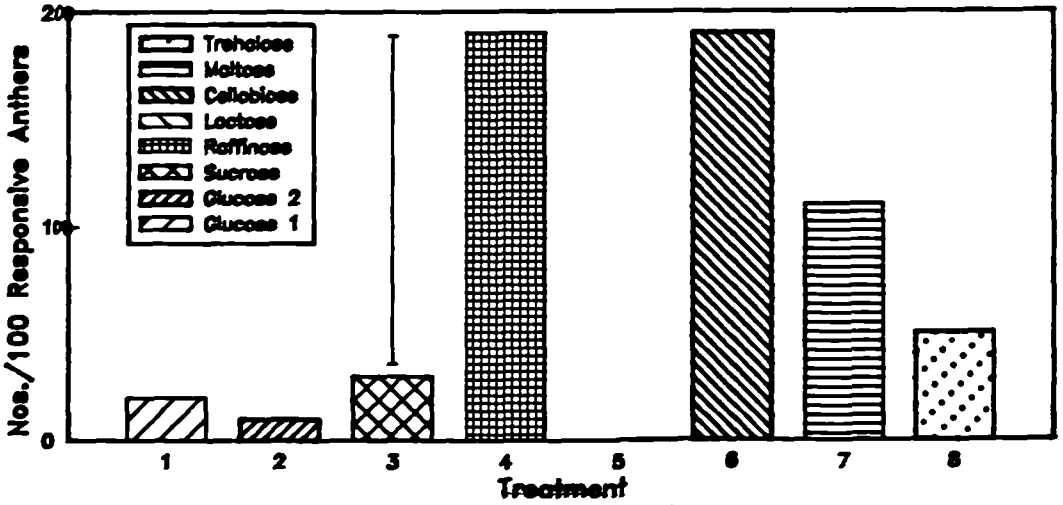


Figure 5.2. continued.
The effect of sugars on barley anther culture.
Anthers cultured "on-edge".

Figure 5.2.4. Anther response

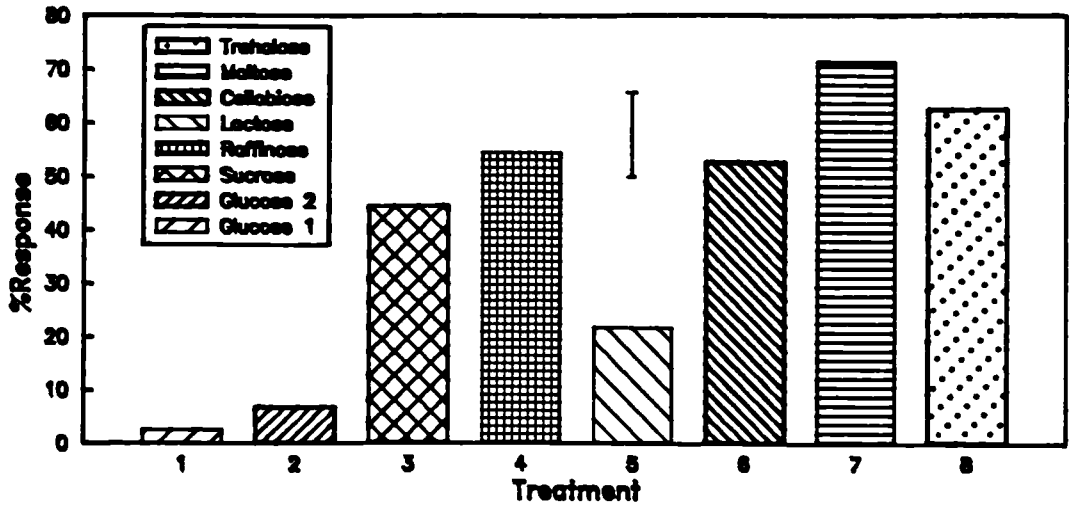


Figure 5.2.5. Green Plants

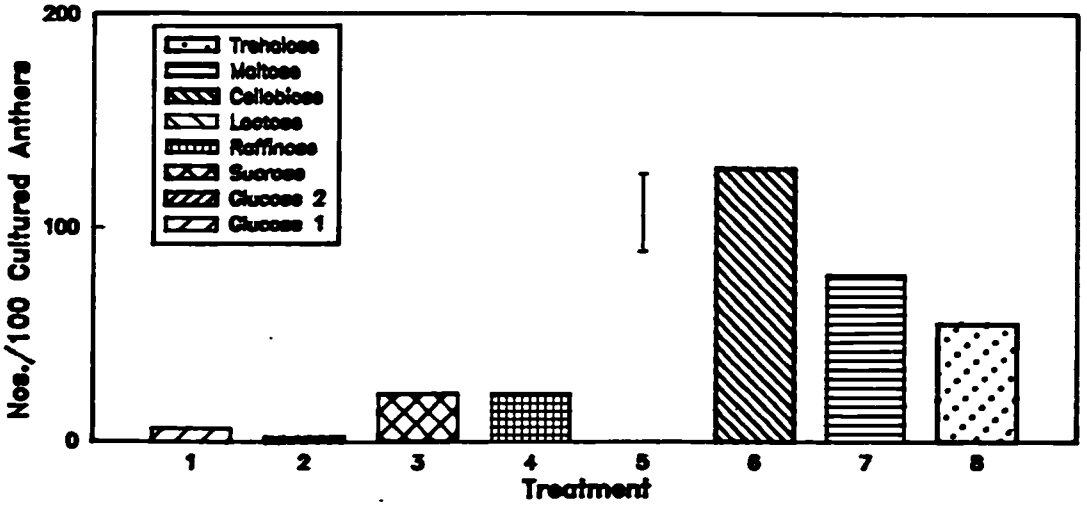


Figure 5.2.6. Green Plants

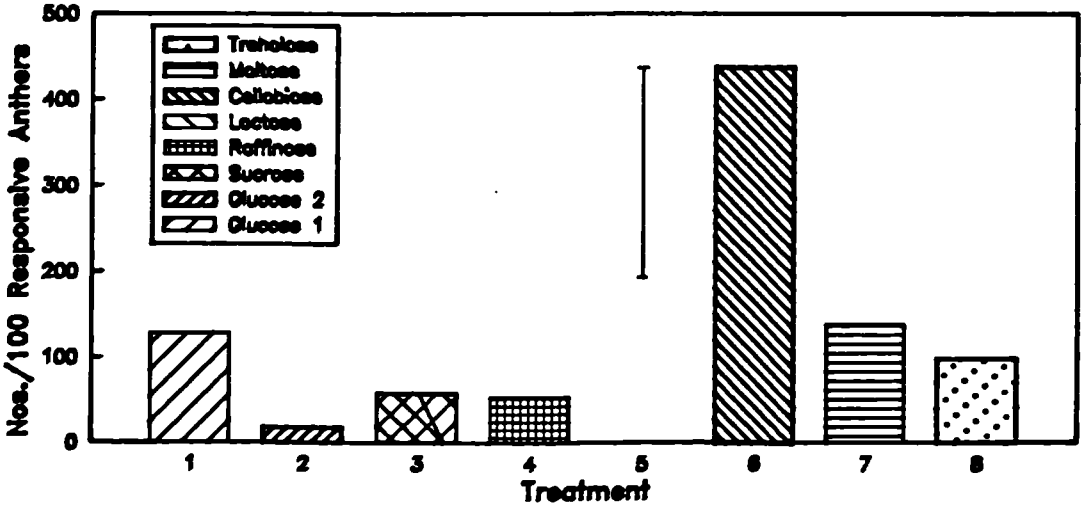


Table 5.2.2
The effect of different sugars on barley anther response
Summary of results

Group	Sugar	Result
1	Fructose Glucose*	Inhibit androgenesis independent of anther orientation.
2	Lactose	No effect of anther orientation on anther response but only calli produced, no plants.
3a	Sucrose	Anther orientation significantly affects initial response and final plant yields.
3b	Raffinose	Anther orientation significantly affected initial anther response, but, although mean green plant yields differed considerably, this difference was not significant when analysed statistically. This was due to the wide variation in the replicated results.
4	Trehalose Cellobiose Maltose	Anther orientation on the media in this group did not affect response or green plant production. Embryogenesis occurred in both lobes of the majority of anthers.

* Equimolal and equihexose concentrations.

The diglucose treatments, trehalose, cellobiose, and maltose, all produced significantly more green plants per anther cultured than did any of the other treatments. This effect was independent of anther orientation. The cellobiose treatment supported high levels of embryogenesis (Figure 5.2.7) and highest green plant production, although this difference was only statistically significant when the anthers were cultured on-edge.

Figure 5.2. continued.

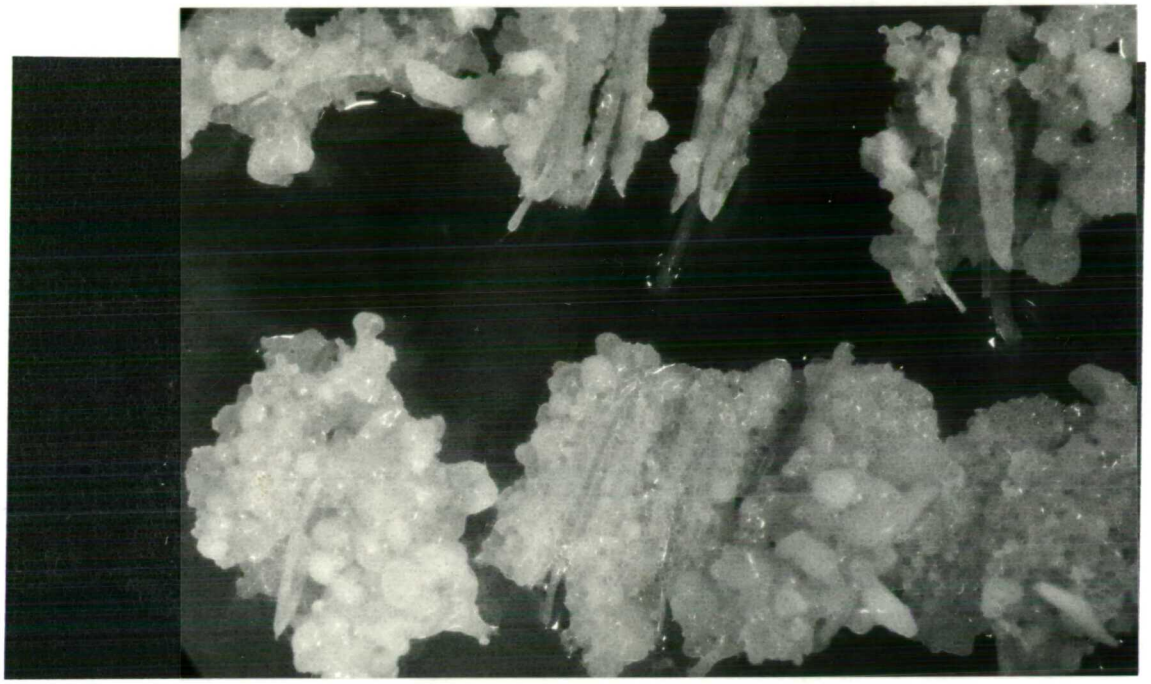


Figure 5.2.7.
Responding barley anthers after 28 days culture on medium containing cellobiose as the primary carbohydrate source.

5.3 The effects of sugar type and sugar concentration on anther culture response

Sugar concentration affects both the osmolarity of the culture medium and the availability of carbohydrate source. Both these parameters are known to influence androgenic response (Hinchee et al, 1984; van den Ende et al, 1984; Tran Thanh Van et al, 1985).

The osmolalities of the media containing either sucrose or maltose or cellobiose were measured over a range of 28-175mM. The osmolarities of the media containing equimolar concentrations of the different sugars did not differ significantly over the range examined (Section 2.16.2).

5.3.1 The effect of maltose concentration, and the addition of mannitol, on anther culture response

The effect of maltose concentration on anther culture was investigated in an initial experiment. Six concentrations were used over a range of 0-175mM. Duplicate maltose treatments were prepared, but with the addition of sufficient mannitol to make their osmolality equal to that of the 175mM maltose treatment. Therefore, a total of 12 treatments including two maltose-free controls were prepared.

Anthers were excised from pretreated spikes and randomised among the treatments (Appendix 2.9). They were orientated flat on the medium. The treatments were replicated five times and the results analysed statistically (Section 2.14). Data are not presented.

Maltose concentration affected anther response and green plant production. The addition of mannitol was inhibitory for all parameters recorded. The most noticeable effect of mannitol in this

experiment was its influence on the quality of androgenic structures. For the corresponding maltose-only treatment, the addition of mannitol produced structures that, although organised, were vitrified in appearance. Consequently, the addition of mannitol depressed plant yields.

5.3.2 The effect of sucrose concentration +/- mannitol on anther culture response

In a similar experiment to 5.3.1, maltose was replaced by sucrose. No response was obtained.

5.3.3 The effect of sugar concentration on anther culture response

The effects of sugar concentrations on anther culture were examined in three experiments. The sugar concentration was over the range of 0-10%, (the equivalent molar concentrations are presented in Table 5.3.). No mannitol was added to any of the treatments.

Table 5.3.
Equivalent mMolar concentrations of sugar treatments in Experiment 5.3.

Treatment	Concentration			
	% (w/v)	mM		
		Maltose	Cellobiose	Sucrose
1	1	28	29	29
2	2	56	58	58
3	3	83	88	88
4	4	111	117	117
5	5	139	146	146
6	6	167	175	175
7	7	194	205	205
8	8	222	234	234
9	9	250	263	263
10	10	278	292	292
11	0	0	0	0
12	0	0	0	0

For each of the experiments anthers from selected pretreated spikes were randomised between the treatments according to a randomised block experiment design (Appendix 2.9).

No androgenesis was observed in the sucrose concentration experiment. In contrast, both maltose (Figure 5.3.1-3) and cellobiose (Figure 5.3.4-6) treatments supported high levels of androgenesis and green plant production. The results of the maltose and cellobiose concentration experiments were similar, but not identical. Anther responses on maltose or cellobiose media were consistently high at concentrations of three percent (w/v) and above, but below this

Figure 5.3. The effect of sugar concentration on barley anther culture.
Figures 5.3.1-3. maltose concentration.

Figure 5.3.1. Anther Response

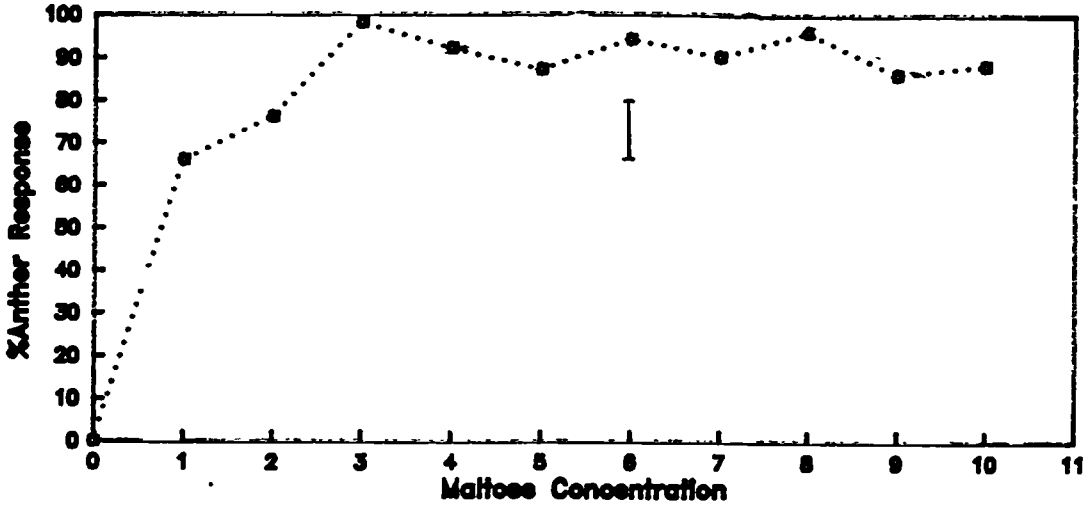


Figure 5.3.2. green plants

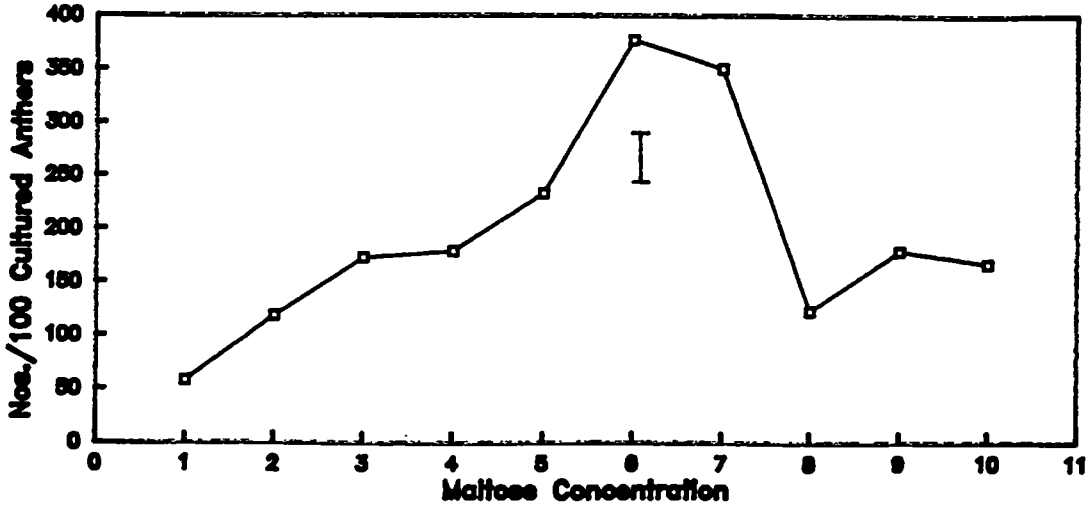


Figure 5.3.3. Green Plants

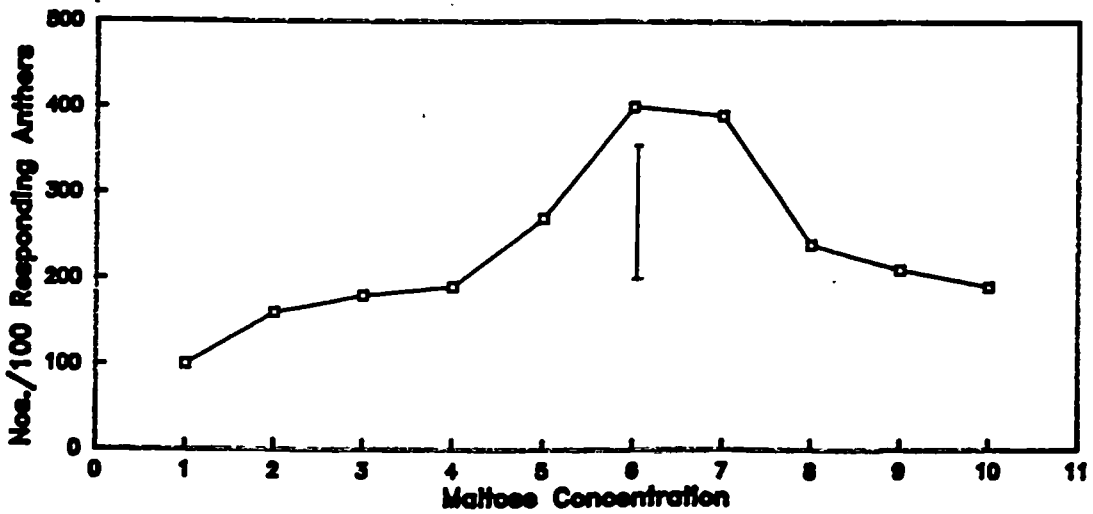


Figure 5.3. continued.
Figures 5.3.4-6.cellobiose concentration

Figure 5.3.4. Anther Response

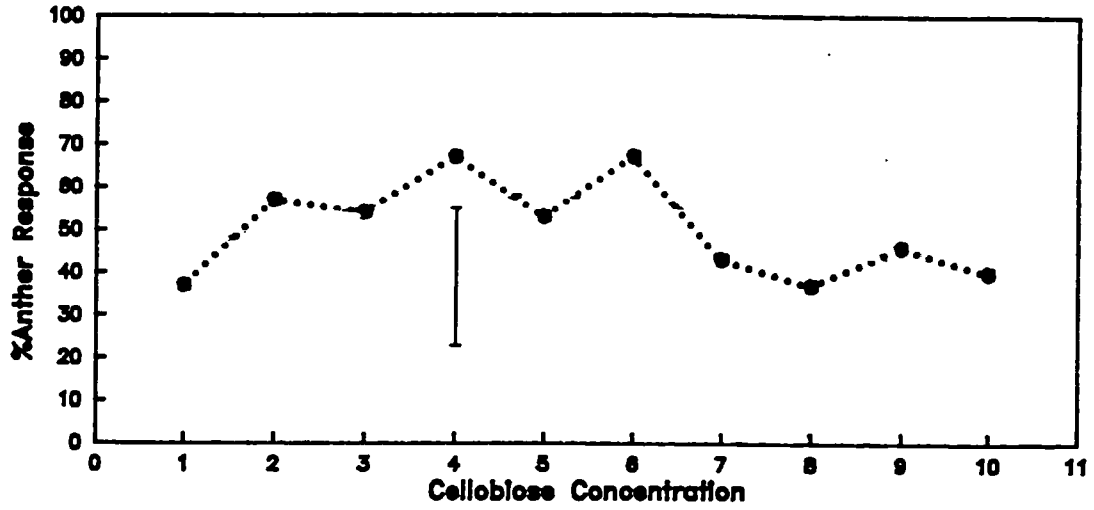


Figure 5.3.5. green plants

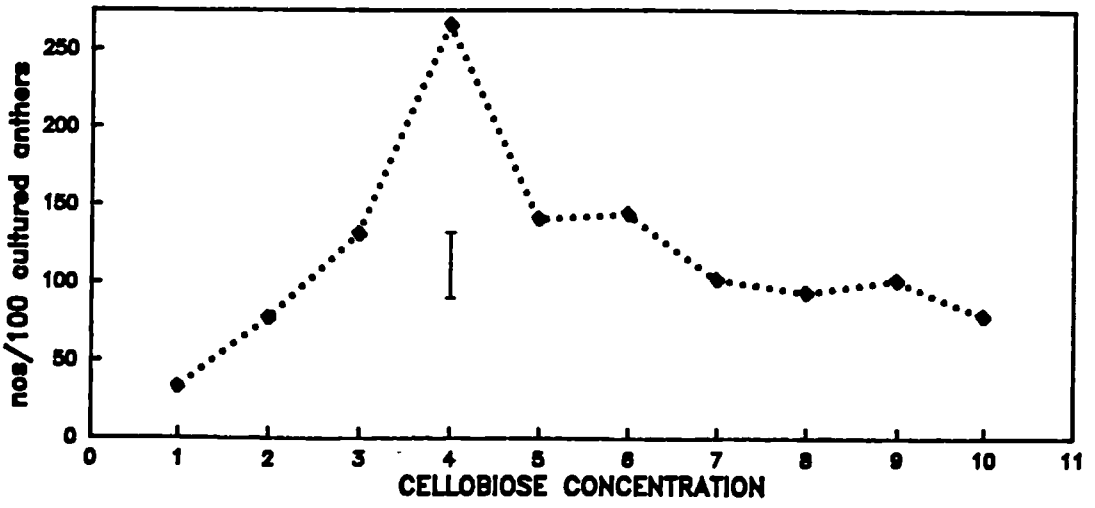
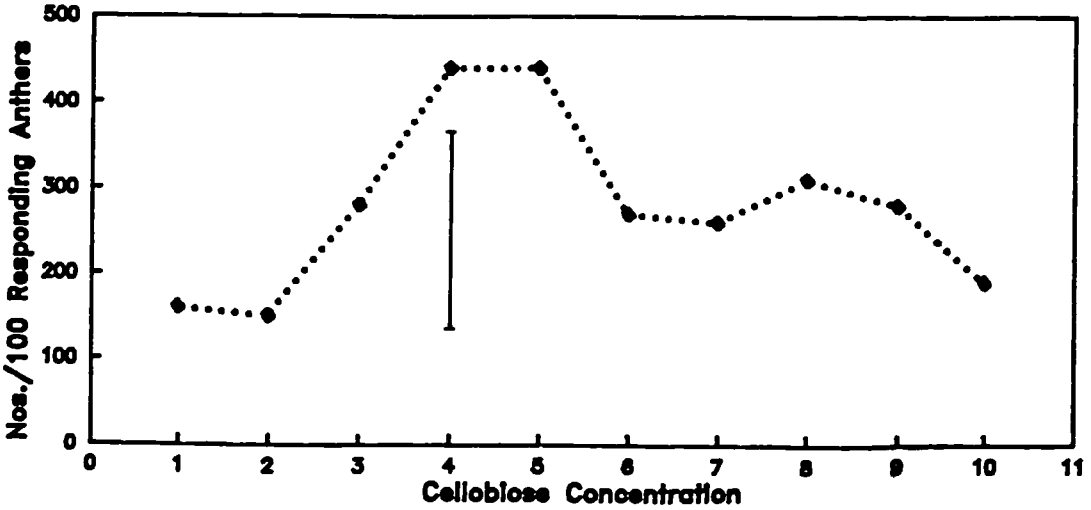


Figure 5.3.6. Green Plants



anther response tended to be lower (Figures 5.3.4 and 5.3.7).

There were clear peaks for optimal green plant production on both maltose and cellobiose treatments. For maltose, the peak was between five and seven percent (140-200mM) (Figures 5.3.5 and 5.3.6), whereas for cellobiose the peak was at about four percent (Figure 5.3.8 and 5.3.9).

The morphology of the androgenic structures which developed on maltose and/or cellobiose media was affected by the sugar concentration. As the sugar concentrations increased, so the androgenic structures became more compact and their colour changed from an almost snowy white to a creamy yellow appearance. The observations were most prominent at cellobiose concentrations >7% and <2%.

5.4 The effect of sugar mixtures on anther culture response

5.4.1 Experiment 1: Maltose/sucrose mixtures

Anthers were excised from pretreated spikes and randomised between the eight treatments (Table 5.4.1). The randomisation was according to a randomised block experiment design (Appendix 2.10). Each treatment received 24 anthers. Four spikes were used for each replicate and the experiment was replicated five times. Anthers were cultured flat throughout the experiment.

Table 5.4.1
Experiment 1: sugar concentration in sucrose/maltose mixtures

Treatment	Sucrose % (mM)	Maltose % (mM)
1	0	0
2	6 (175)	0
3	5.9 (172)	0.1 (3)
4	3 (88)	3.2 (89)
5	1 (29)	5.3 (147)
6	0.1 (3)	6.2 (172)
7	0.01 (0.3)	6.29 (175)
8	0	6.3 (175)

The results were analysed statistically (Section 2.14) and are summarised in Figures 5.4.2-4.

The carbohydrate-free treatment did not respond; neither did the treatment which contained 175mM sucrose alone. The addition of 29mM sucrose to the maltose-containing medium reduced anther response. Additions as low as 0.1% (3mM) sucrose inhibited plant production significantly. Conversely, the addition of 0.1%(28mM) maltose to a sucrose medium supported the response of some anthers. This did not, however, significantly improve green plant production.

5.4.2 Experiment 2: maltose/sucrose and cellobiose/sucrose mixtures

In this experiment sucrose was added to media containing either maltose or cellobiose as the primary carbohydrate source (Table 5.4.2).

Figure 5.4. The effect of sugar mixtures on anther culture.
Figures 5.4.1-4. Sucrose/maltose mixtures, experiment 1

Figure 5.4.1. **Sugar Conc.for Treatments**

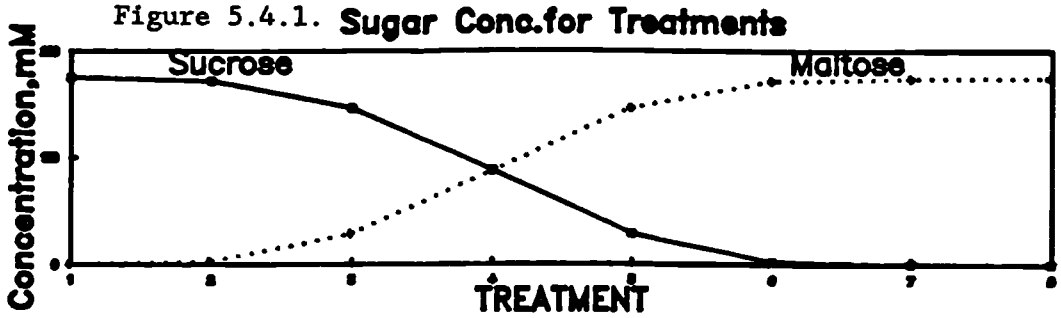


Figure 5.4.2. **Anther Response**

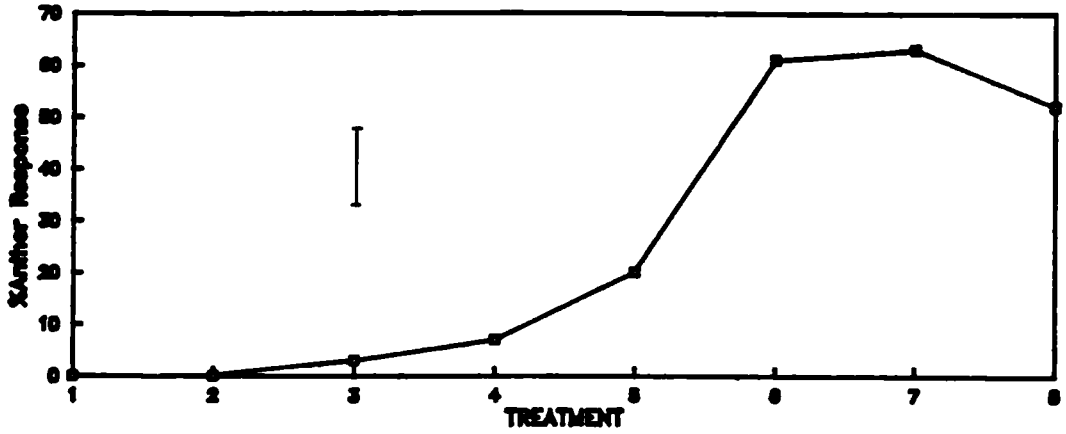


Figure 5.4.3. **Green Plants**

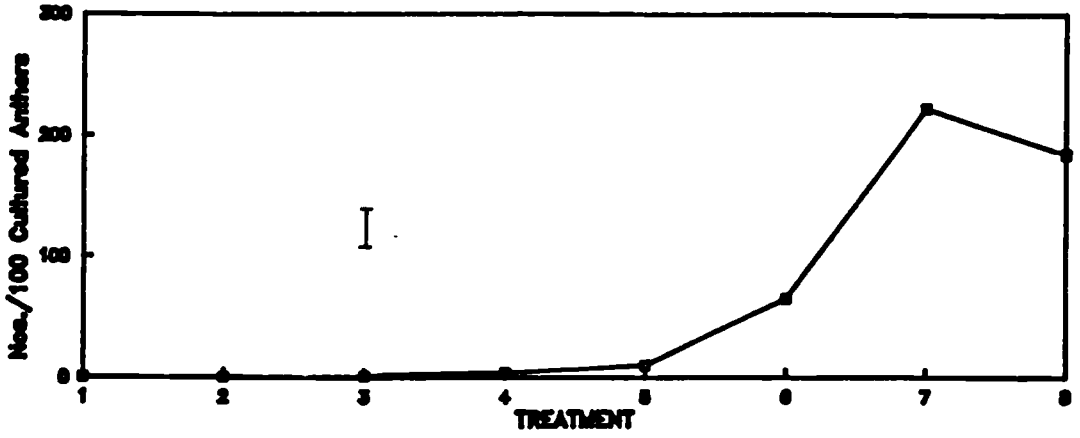


Figure 5.4.4. **Green Plants**

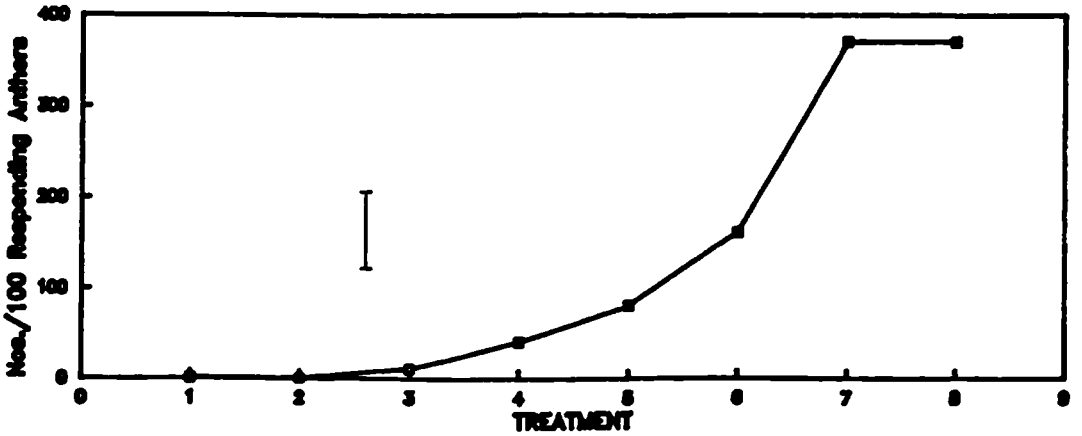


Table 5.4.2
Sugar concentrations in sucrose/maltose mixtures and sucrose/cellobiose mixtures in Experiment 2

Treatment	Sugar Concentration					
	Sucrose		Maltose		Cellobiose	
	%	(mM)	%	(mM)	%	(mM)
1	0	-	0	-	-	
2	0.05	(1.5)	6.25	(173)	-	
3	0.1	(2.8)	6.2	(172)	-	
4	0.2	(6)	6.09	(169)	-	
5	0.5	(15)	5.77	(160)	-	
6	1.0	(29)	5.25	(146)	-	
7	0	(0)	-		6.0	(175)
8	0.05	(1.5)	-		5.95	(174)
9	0.1	(3)	-		5.9	(172)
10	0.2	(6)	-		5.8	(169)
11	0.5	(15)	-		5.5	(160)
12	1.0	(29)	-		5.0	(146)

Maltose containing treatments were numbers 1-6 and those containing cellobiose 7-12. Anthers from selected pretreated spikes were randomised between treatments according to a randomised block design (Appendix 2.9).

The effects of sucrose in the presence of maltose were confirmed in this second experiment. Addition of sucrose at concentrations as low as 0.2% (6mM) or above, significantly inhibited anther response and green plant yields. Similar, although not identical, effects of sucrose were observed in the cellobiose treatments. In cellobiose

Figure 5.4. continued
Figures 5.4.5-8. Sucrose/maltose mixtures, experiment 2

Figure 5.4.5. **Sugar conc. for Treatments**

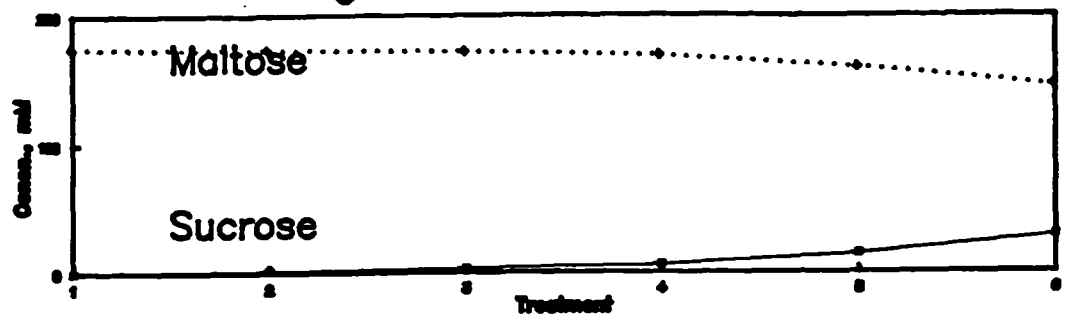


Figure 5.4.6. **Anther Response**

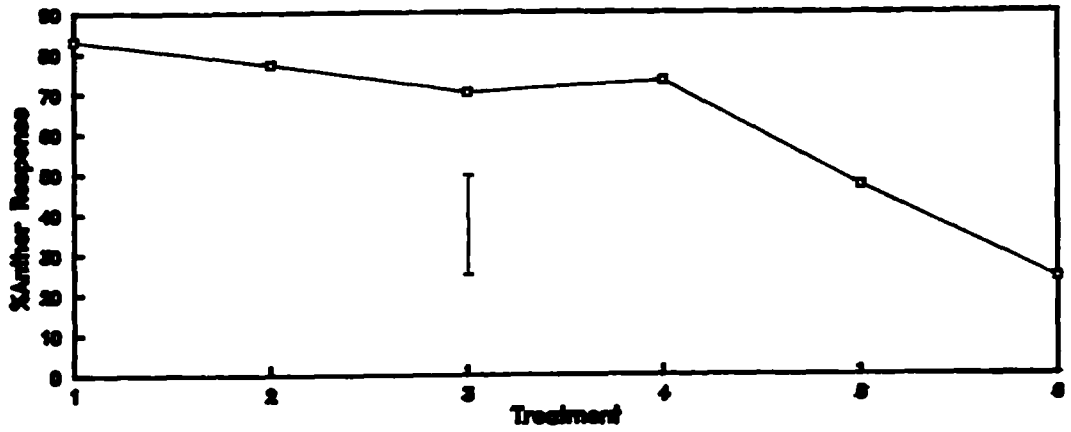


Figure 5.4.7. **Green Plants**

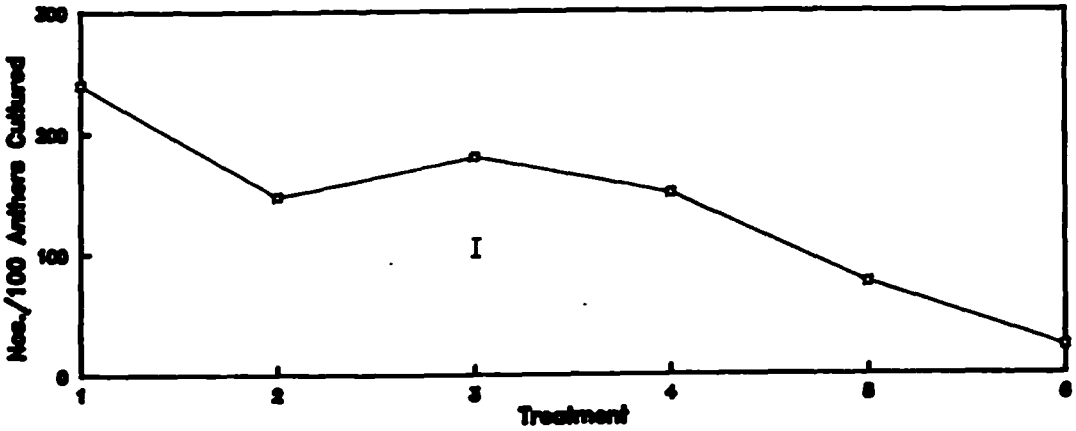


Figure 5.4.8. **Green Plants**

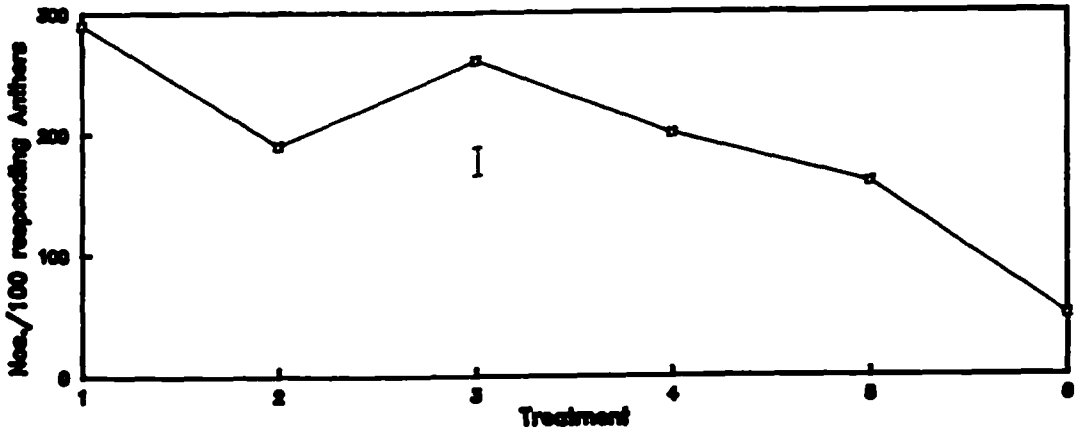


Figure 5.4. continued.
Figures 5.4.9-12. Sucrose/cellobiose mixture experiment.

Figure 5.4.9. Sugar conc. for Treatments

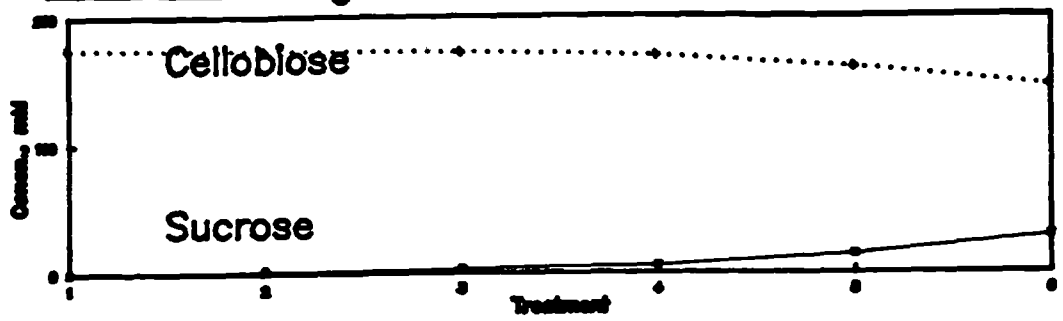


Figure 5.4.10. Anther Response

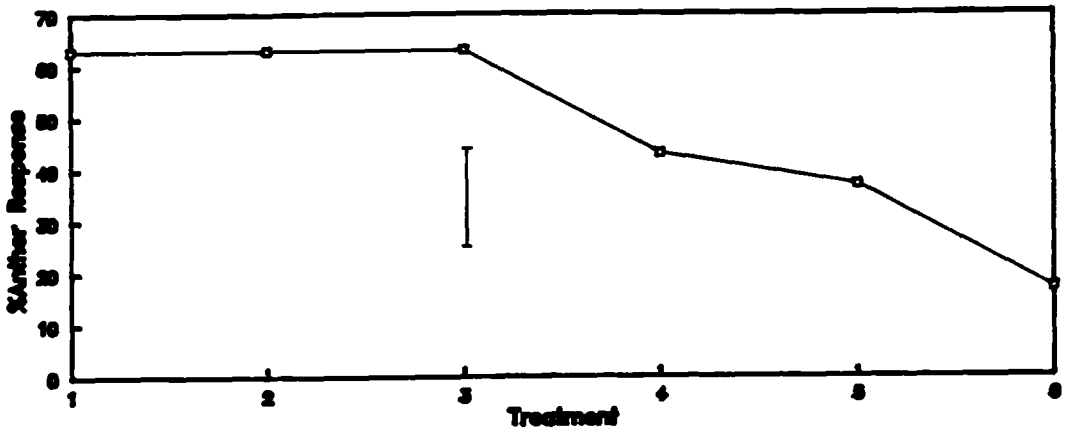


Figure 5.4.11. Green Plants

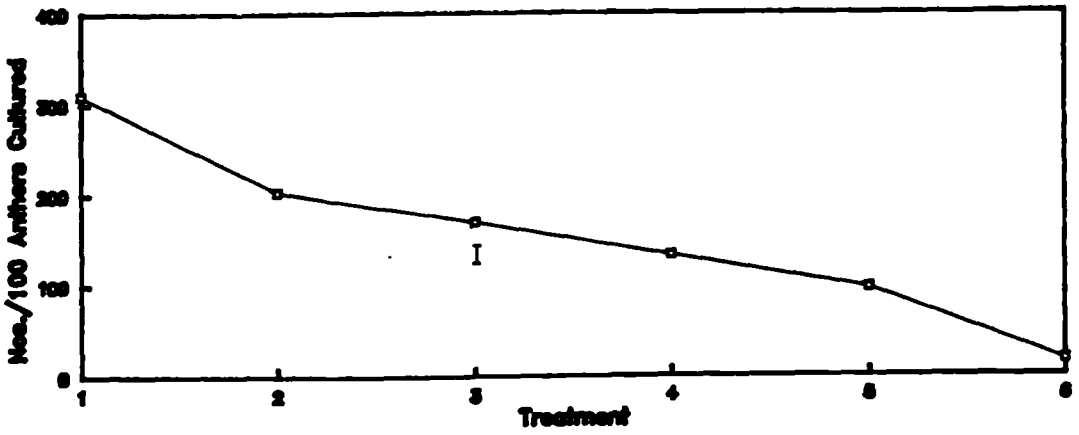
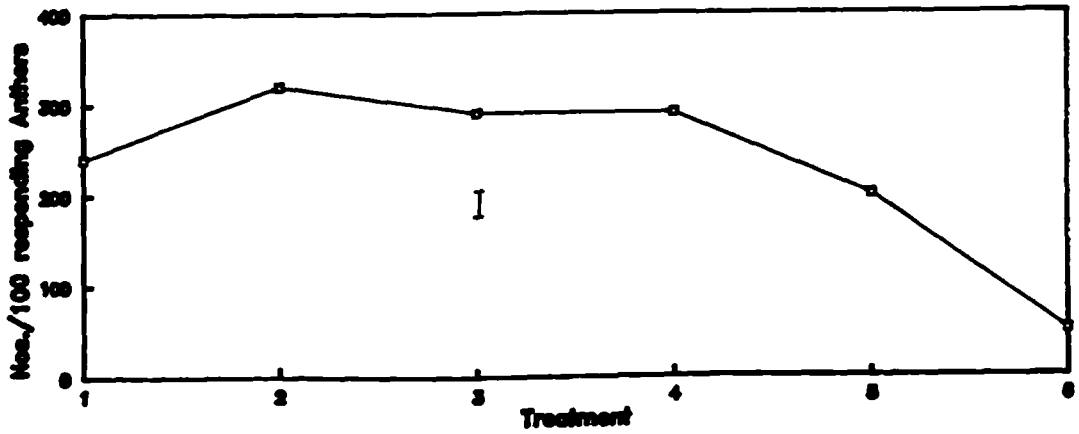


Figure 5.4.12. Green Plants



treatments anther response appeared to be unaffected by sucrose additions of less than 0.1% (2.8mM). However, inhibition of plant production increased with sucrose concentration. The results are summarised in Figures 5.4.9-12 .

5.5 Radiolabelled sugar experiments

5.5.1 Washout of radiolabelled sugar from anthers cultured in liquid media

Before the rate of sugar uptake into anthers could be determined, it was necessary to remove the radiolabelled sugars from within the free space of the anthers. This experiment was carried out to determine the number of washes required to remove the labelled sugar from the free space. This method was adapted from Lyne (1971).

Forty five anthers were incubated for one hour in 1.0ml of culture medium containing the appropriate $U^{14}C$ labelled sugar. After incubation, the labelled medium was removed and the anthers washed with five successive 1ml aliquots of "cold" medium. Each wash was for 1 minute. The time taken to remove the medium and add the next wash was 40 seconds. Radioactivity in the medium was determined by liquid scintillation spectrometry.

The radioactivities of the original media and each of the washings are given as disintegrations/minute for ^{14}C in Table 5.5.1.

Figure 5.5.1.
A semi log plot of the radiolabelled sugar washout

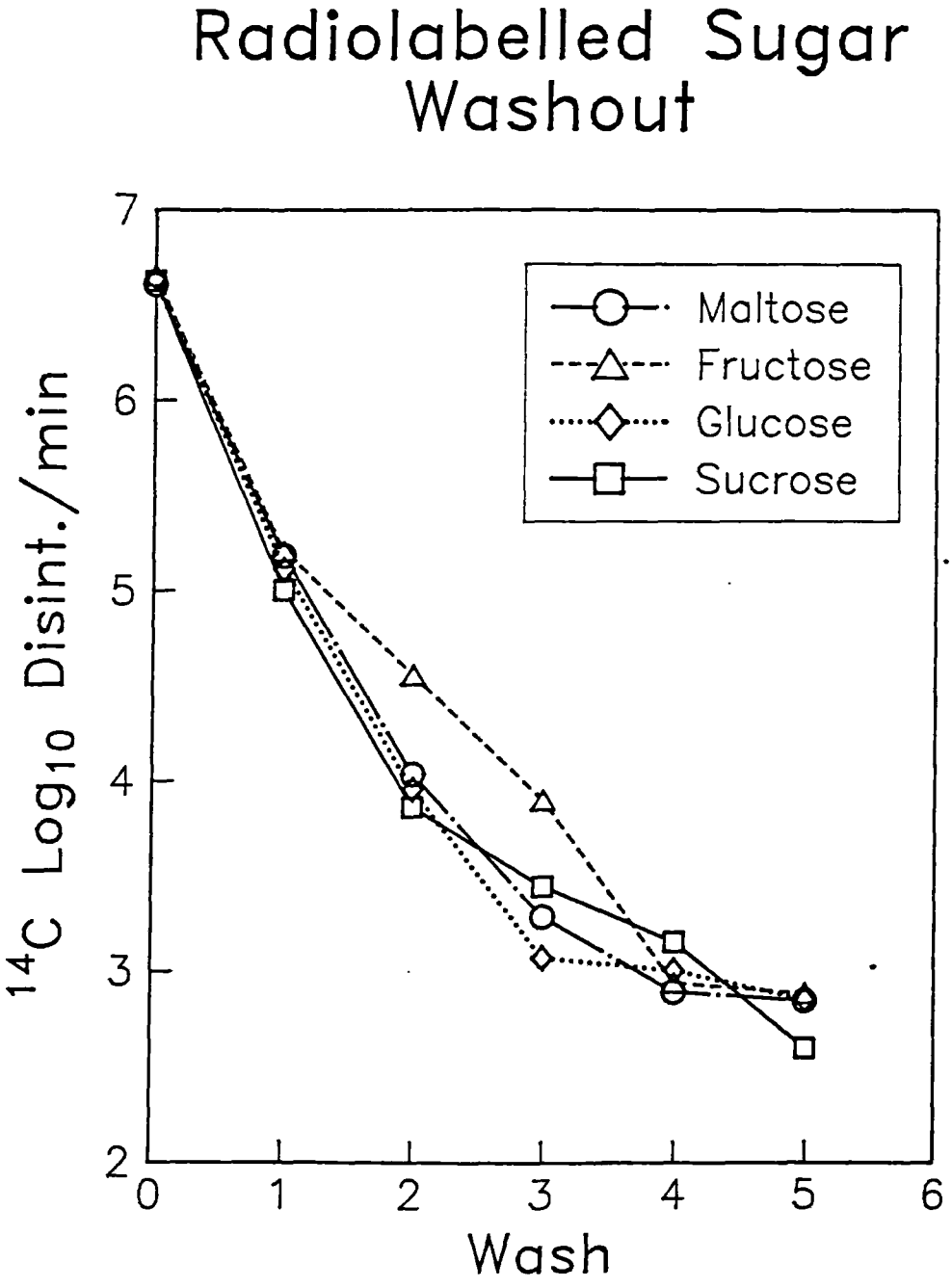


Table 5.5.1

^{14}C Disintegrations/minute in washout experiment

Treatment	Sucrose	Glucose	Fructose	Maltose
Medium	4242518[a]	4400426	4500699	4069506
Wash 1	100995	131449	161202	153002
Wash 2	7330	9148	35813	10904
Wash 3	2823	1196	7949	1952
Wash 4	1437	1023	870	785
Wash 5	399	720	767	708

a) Mean of five replicates

The data are presented as semi-log plot (Figure 5.5.1.), following the method described by Lyne (1971).

The steep phase on the curve corresponds to ^{14}C loss from the free space. Therefore, most of the ^{14}C is removed from the free space during the first three washings.

5.5.2 Experiment to determine the uptake of four different sugars into anthers during the early stage of culture

The uptake of sucrose and maltose and the monosaccharide products of their hydrolysis, glucose and fructose, was determined using $U^{14}C$-labelled sugars (Method Section 2.18.1).

Anthers were excised from selected pretreated spikes and allocated to each treatment according to a randomised block experiment design (Appendix 2). Each treatment received 24 anthers per time-point sample. Culture dishes were allocated to time points according to the treatment randomisation (Appendix 2.12). The treatments were replicated three times.

Sample anthers were washed three times in label-free medium as described earlier (Section 5.5.1) before being plunged into boiling 80% ethanol:water to extract the soluble sugars. Labelled sugar uptake was determined by liquid scintillation spectrometry. The cummulative uptake of sugar into the anthers is presented in Figure 5.5.2.

The rate of sugar taken into the anthers over a 54-hour period is presented as:-

$$n-(n-1)/\text{time difference between the two assessments (hours).}$$

where n = the level of sugar within the test anthers at a time-point.

n-1 = the level of sugar within the test anthers at the previous time-point.

All assessments were measured as Disintegrations/minute measured for ^{14}C and presented as nmoles of sugar (Table 5.5.2).

Table 5.5.2
The rate of sugar uptake/hour into anthers during the early stages of anther culture

Incubation Time(hours)	Sugar			
	Sucrose	Glucose	Fructose	Maltose
1/3	88	86	85	59
1	79	108	68	47
2	83	76	107	59
6	125	229	187	79
18	90	96	82	73
30	75	69	103	93
54	4	33	4	29

The data are presented graphically in a semi-log plot showing the rate of total sugar uptake in nmoles/hour (Figure 5.5.2).

Figure 5.5.2.
A comparison of the cumulative uptake of different sugars into barley anthers during culture, using $U^{14}C$-labelled sugars.

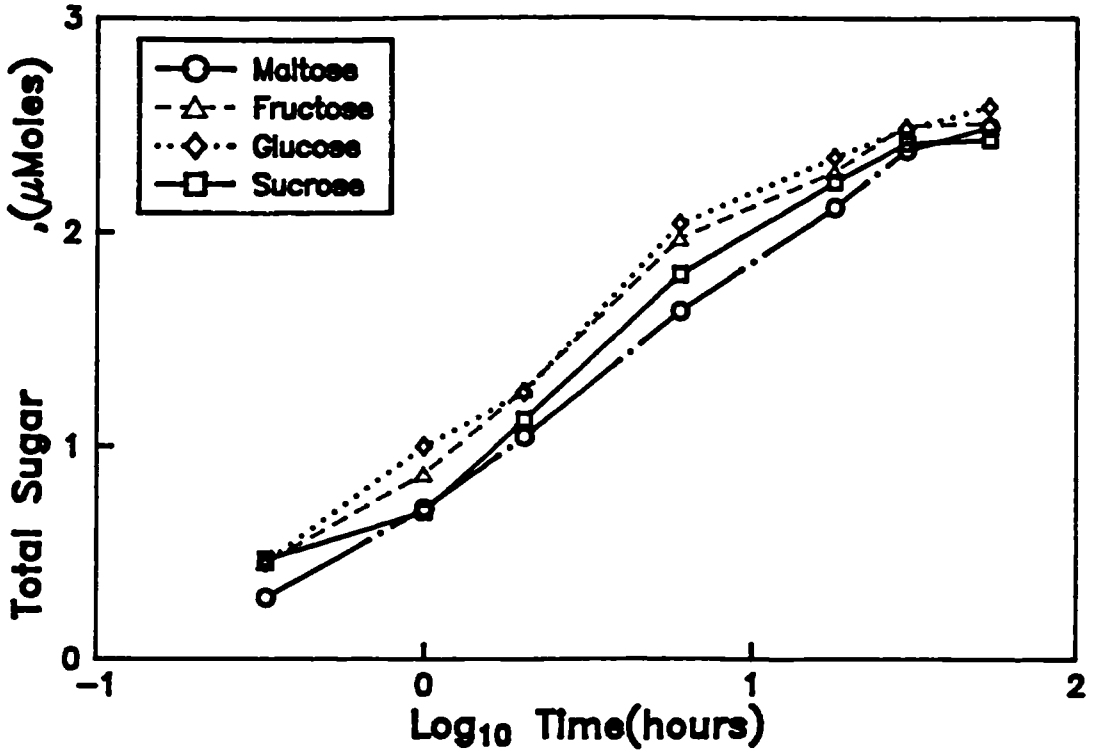
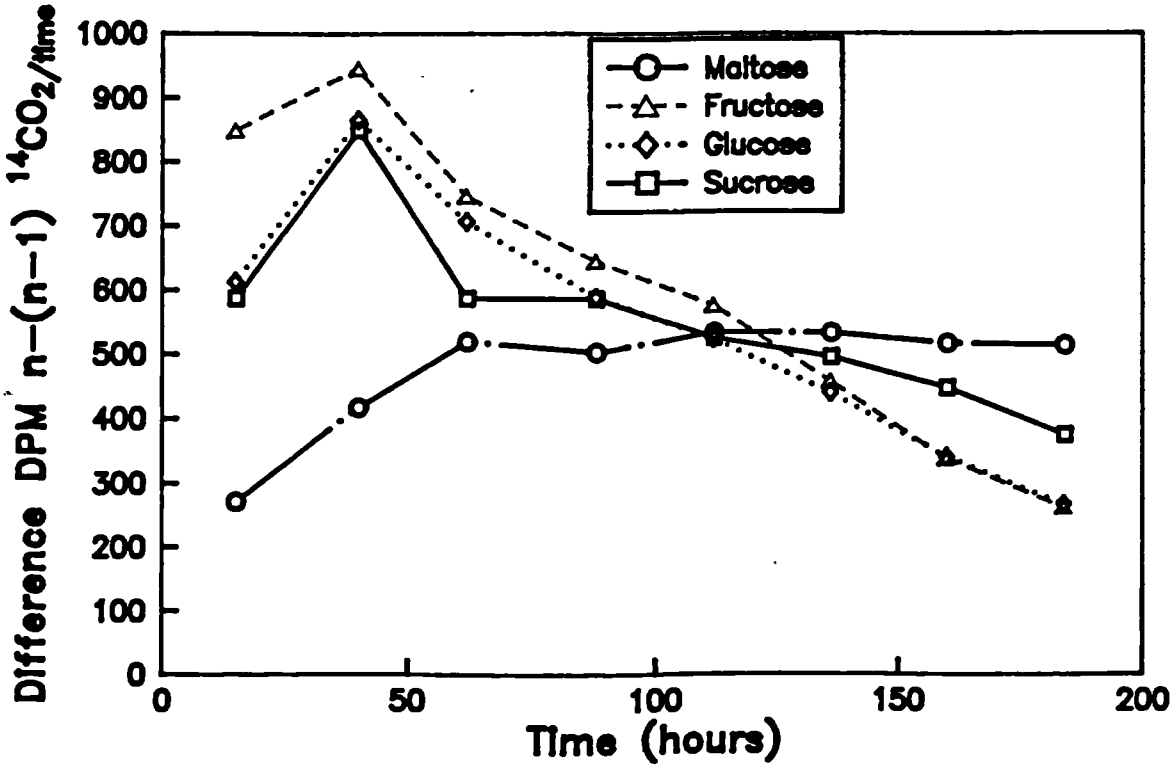


Figure 5.5.4
A comparison of the rate of $^{14}CO_2$ evolution from barley anthers cultured on media containing different $U^{14}C$-labelled sugars



Glucose and fructose were taken up at similar rates throughout the time-course studied. Sucrose was taken up at a slower rate, but faster than maltose. The data presented are the means of three replicates. All four sugars appear to be taken up into the anthers at similar rates (Figure 5.5.2.1).

5.5.3 Determination of sugar composition in the medium at time zero and seven, 28 and 40 days after culture initiation

Samples of the media were taken from the cultures aseptically and prepared as described earlier (Section 2.18). The sugar composition of the inital media and the media after seven, 28 and 40 days was determined using TLC (Section 2.20). The results for the seven and 40 day incubation samples are presented in Figures 5.5.3.1-5.

It was confirmed that sucrose and maltose were the only labelled sugars present in the culture medium when the experiment was initiated. However, after seven days culture both glucose and fructose were detected in the sucrose sample (Sample S_1, Figure 5.5.3.1 and Figure 5.5.3.2). Glucose was also detected in the maltose sample (Sample M_1, Figure 5.5.3.1 and Figure 5.5.3.4), but at a much lower level than in the sucrose sample. A similar picture was observed in the samples after 28 days culture (results not presented). In the sucrose medium between 60 and 70% of the label present was in the glucose and fructose spots. Only ca 10% of the label in the maltose medium had been hydrolysed to glucose. After 40 days' culture the distribution of label between sucrose, glucose and fructose was similar to that observed on the 28th day, but no label was detected in the maltose medium. Plants had only developed in the maltose containing medium.

5.5.4 The determination of $^{14}CO_2$ evolution from anther cultures fed with U.^{14}C-labelled sugars

Anthers were excised from selected pretreated spikes and randomised amongst the four treatments, sucrose, glucose, fructose, and maltose (Appendix 2). Samples were cultured in sterile, gas-tight, vessels as described in the Materials and Methods (Section 2.2).

CO_2 was absorbed into 0.2ml aliquots of 10% KOH. The aliquots of KOH were replenished regularly and $^{14}CO_2$ levels absorbed were assessed using liquid scintillation spectrometry.

The relative levels of $^{14}CO_2$ evolution/hour for each sugar were calculated as described earlier (Section 5.2.2), and are presented as ^{14}C DPM absorbed by the KOH/hour (Figure 5.5.4).

$^{14}CO_2$ evolution from both monosaccharide samples and the sucrose sample were similar. Initially $^{14}CO_2$ evolution was between 2-3 times greater than that of the maltose sample, peaking at ca 40 hours then gradually declining. In contrast, $^{14}CO_2$ evolution from the maltose sample started off gradually and increased until it plateaued after ca 62 hours when it remained constant until the end of the experiment (Figure 5.5.4). The maltose treatment was the only one that supported embryo and plant development in this experiment.

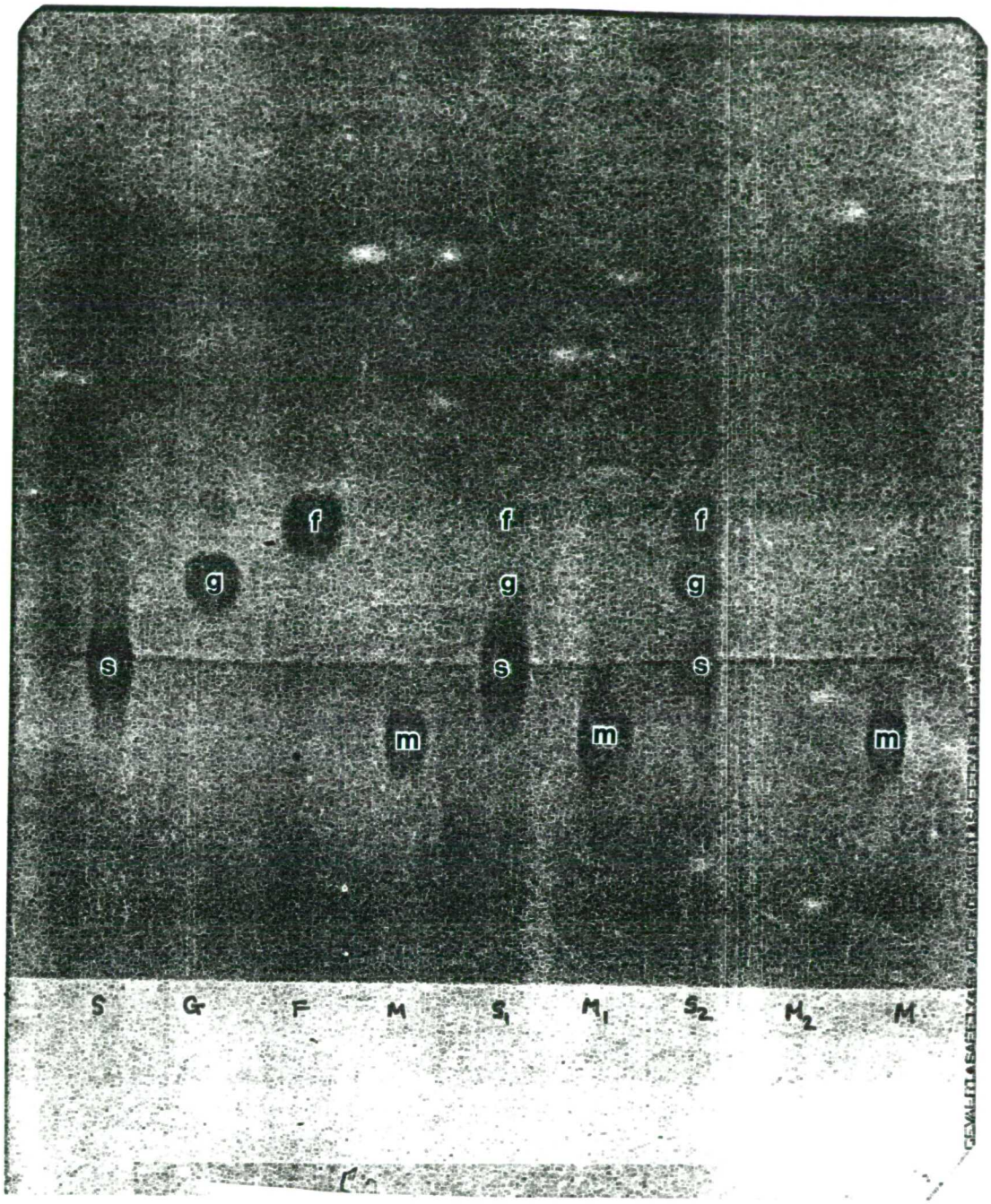


Figure 5.5.3.1

Autoradiograph of TLC plate with U ^{14}C labelled sugar

Table 5.5.3
Track label identification

Sugar	Incubation period (days) after which sample taken		
	0	7	40
Sucrose **s** (S)		(S_1)	(S_2)
Glucose **g** (G)		-	-
Fructose **f** (F)		-	-
Maltose **m** (M)		(M_1)	(M_2)

Figures 5.5.3.2-5 Radiolabel scans. Quantitative measurements of radiolabelled sugars.

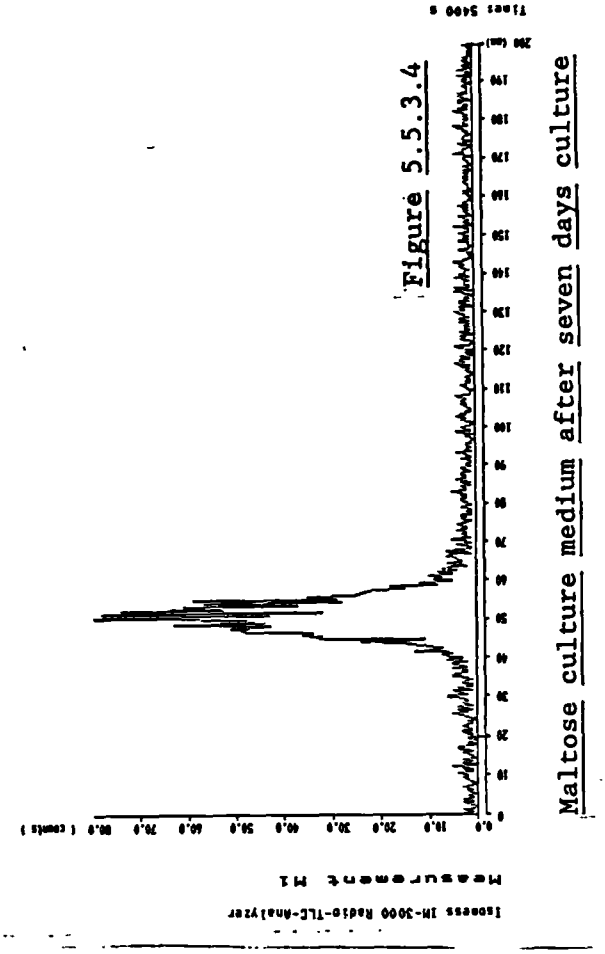


Figure 5.5.3.4

Maltose culture medium after seven days culture

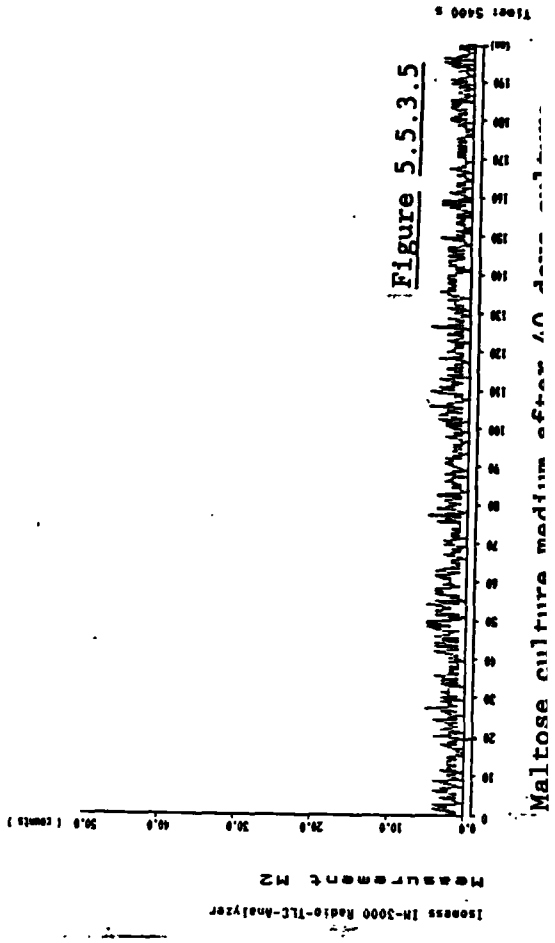


Figure 5.5.3.5

Maltose culture medium after 40 days culture

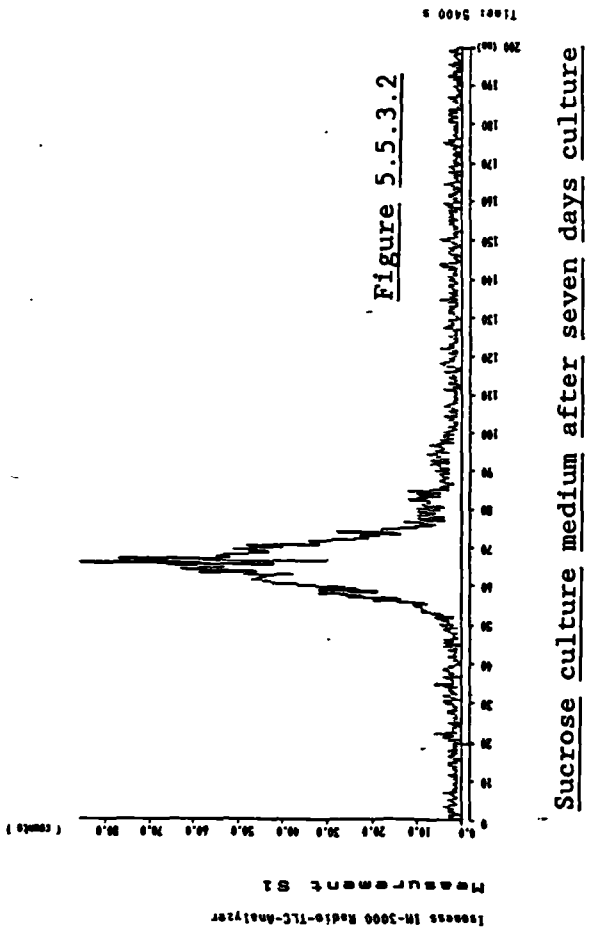


Figure 5.5.3.2

Sucrose culture medium after seven days culture

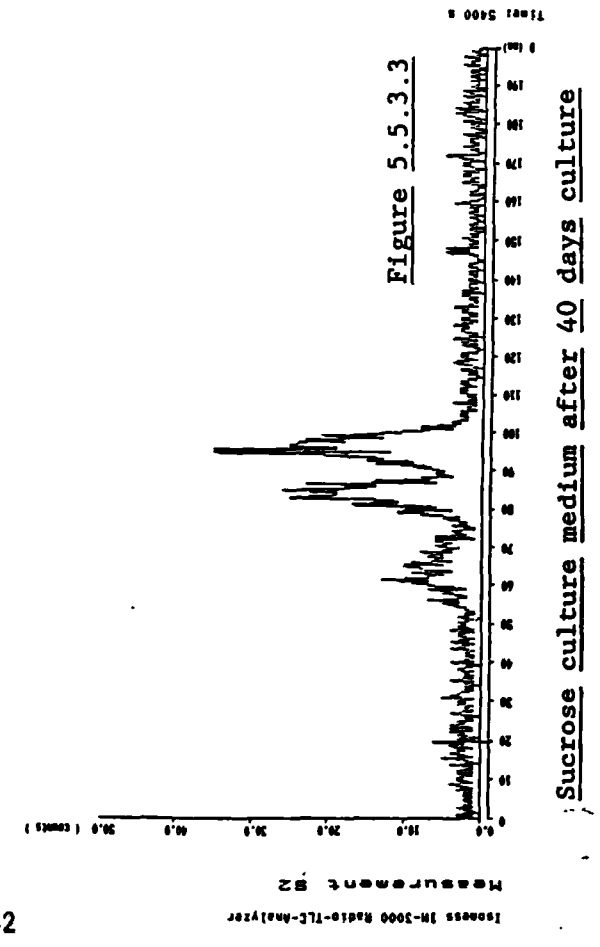


Figure 5.5.3.3

Sucrose culture medium after 40 days culture

5.6 Discussion

The induction, development and morphology of androgenic structures in barley anthers during culture varied with the type and concentration of primary carbohydrate supplied. The predominant effect appeared to be carbohydrate type, more particularly sugar structure, which determined initial androgenic response and the developmental morphology of the androgenic structures. The diglucose sugars cellobiose, maltose and trehalose supported significantly higher levels of anther response and green plant production than the remaining sugar treatments, glucose, fructose, sucrose, raffinose, and lactose (Section 5.2, Table 5.2.2).

Carbohydrate concentration also affected androgenic induction, the rate of development, and the morphology of androgenic structures. For cellobiose, the optimal concentration for maximum green plant yields was between 3-5%, whereas for maltose the optimal concentration was 5-7%.

In common with the results for barley, maltose was found to support the highest levels of embryogenesis in cultures of Petunia (Raquin, 1983) and Medicago sativa (Strickland et al, 1987). The yields of embryos for Petunia anthers cultured on sucrose, glucose, or fructose media were, respectively, 80, 66 and 44 per cent of those obtained on maltose containing medium (Raquin, 1983). Somatic embryo yields from Medicago callus cultures on sucrose media were ca 50 per cent of those obtained on maltose medium (Strickland et al, 1987). Yields on glucose or fructose media were between 20-50 per cent of those obtained on maltose medium. The detrimental effects of all but the diglucose sugars on barley anthers cultured flat on the medium

(Section 5.2) appeared to have no parallel in either of the other culture systems.

As in barley, sugar concentration was found to influence alfalfa somatic embryo development and yields. Lower levels of maltose supported optimal embryogenesis with "Optimal embryo morphology, yield and conversion on media with maltose at concentrations of 4-6%" (Strickland et al, 1987). Both sucrose and maltose, when used separately produced high yields of somatic embryos. In combination, maltose was reported to have a "dominant" effect on embryo yield and morphology over sucrose alone. The stimulatory effect of maltose at low concentration and the significantly higher levels of embryogenesis obtained on media containing equimolal maltose compared to sucrose led Strickland et al, (1987) to conclude that the effect of maltose was primarily nutritional and not osmotically mediated.

Other differences between the alfalfa and barley systems included their responsiveness to sugar mixtures. Somatic embryogenesis in alfalfa cultures grown on sucrose/maltose mixtures was almost tripled. The alfalfa results were presented in such a way that embryogenesis was expressed relative to the 3% sucrose (100%) control. Each treatment within an experiment was inoculated with a fresh weight of callus equal to 1.0g/30ml of solid medium or 30mg/ml of liquid medium (Strickland et al, 1987). In contrast, when similar sugar mixtures were used for barley anther cultures in the present study the previous observation (Section 4.10) that early androgenesis was inhibited by sucrose, was confirmed. Sucrose inhibited androgenesis in anther lobes in direct contact with the medium at

concentrations as low as 3mM in the presence of 174mM maltose or 174mM cellobiose (Section 5.4).

When the possible metabolites of sucrose and maltose (glucose-1-phosphate and glucose-6-phosphate) were used as primary carbohydrate sources in alfalfa callus cultures (Strickland et al, 1987), they did not influence embryogenesis . This was probably due to the inability of the calli to take up the phosphorylated sugars since most phosphate esters do not pass readily across cell membranes (Yudkin et al 1973).

The effects of sugar concentration in alfalfa and barley systems cannot be compared directly since only diglucose sugars supported androgenesis in barley anthers cultured flat on media. No androgenesis was observed on media containing 1-10% sucrose. Diglucose concentration did influence the induction, morphology and development of androgenic structures, and green plant yields in barley (Section 5.3). Optimal plant yields were obtained on media containing 5-7% maltose and 3-5% cellobiose.

Clearly there are differences in the carbohydrate requirements of these three (Petunia, Medicago and Hordeum) tissue culture systems. These differences may be due to one, or a combination of several, genetic, physiological, and/or biochemical differences, not least the fact that the plants belong to widely different genera.

The inhibitory nature of sucrose in barley anther cultures was investigated further using uniformly ^{14}C-labelled sugars (Section 5.5). Maltose, sucrose, glucose and fructose were all found to be taken up into the cultured anthers at relatively similar rates

(Section 5.5.2).

Further investigation is necessary to determine the exact nature of the label taken up into the anthers since both sucrose and maltose media constituents were found to be hydrolysed to their component monosaccharides during the course of culture (Section 5.5.3). The relative rates of hydrolysis, however, varied considerably. Sucrose was hydrolysed much more quickly than maltose. At the end of the experiment, the sucrose had supported no development but 80% of the sugar in the medium had been hydrolysed to glucose and fructose. In contrast, the maltose treatment which had supported high levels of plant production contained no detectable labelled sugar.

The metabolic fates of the different carbohydrate sources should be established to determine whether the differential effects between maltose and sucrose are the result of differences in the rate of metabolism of the two sugars by the same biochemical pathways or the result of metabolism by completely different metabolic pathways.

$^{14}CO_2$ evolution from anthers cultured on maltose media was initially ca one third of the rate of the other sugar treatments tested. $^{14}CO_2$ evolutions in the maltose treatment increased gradually until, after ca 60 hours, it reached a constant level (Figure 5.5.4.). $^{14}CO_2$ evolution from the other treatments peaked after 50 hours then dropped-off dramatically; this may have been related to pollen death, although this has to be confirmed by cytological studies. Androgenesis and plant production only occurred in the maltose treatment (Section 5.5.4). This supports the hypothesis that maltose may act as a slowly metabolised source of carbohydrate.

It may be that glucose is, in fact, the carbohydrate utilised. High concentrations of glucose (175mM) were found to be toxic (Section 5.2). Sucrose is rapidly hydrolysed to glucose and fructose, and therefore, may provide glucose to the developing microspores at concentrations which are toxic to them. Maltose appears to be hydrolysed at a slower rate (Section 5.5.3) and may act as a constant, but low concentration, glucose supply: perhaps sufficient to support androgenesis and sufficiently low so as not to be toxic.

This hypothesis could be supported by an experiment to investigate the effect of glucose concentration (0-175mM) on anther culture response. However, further studies of the effects of different sugars and sugar concentrations may be necessary to increase our understanding of how these substances influence the induction of androgenesis in barley and the subsequent development and morphology of the products.

In view of the developments in isolated microspore culture described in this thesis (Section 7), it may be possible to obtain a clearer picture of these effects in the absence of any anther tissues which may confound the interpretation of experimental data.

The importance of maltose in developing cereal seeds is unclear (Kandler *et al*, 1980). Maltose is synthesised *de novo* via photosynthesis and is also produced, to a lesser extent, by starch degradation (Kandler *et al*, 1980). It is thought that maltose acts as a carbohydrate source during early embryogenesis and as a primer for oligomer synthesis, (DeFekete, *et al*, 1974; Kandler *et al*, 1980).

5.7 Conclusion

The differential effects of sucrose and maltose appear to be related to their structure. The diglucose sugars, maltose, cellobiose, and trehalose were able to support relatively high rates of androgenesis in both lobes of anthers cultured on solid medium independent of anther orientation. With sucrose, androgenesis only occured in the upper lobes of on-edge anthers.

Levels of androgenesis in anthers cultured on maltose and those cultured on cellobiose appeared to differ. Androgenesis was generally most prolific on cellobiose-containing media but green plant yields were not found to differ significantly between the two treatments. Further work to compare the effects of these sugars on androgenesis may help to explain the differences which were often observed between anther cultures cultured on media containing a variety of sugars.

Chapter 6

An improved anther culture method

6.1 Introduction

Substantially improved yields of green plants were obtained when sucrose was replaced by one of a range of diglucose sugars (Section 5). Maltose supported high green plant yields and it was relatively inexpensive. In subsequent experiments, maltose was used as the primary carbohydrate source. This made it prudent to reassess the influences of the other media components on androgenesis reported earlier (Section 4).

The experiments on basal media and plant hormones, reported in Section 4, were repeated. The effects of media composition on androgenesis (Section 4) were confirmed. Plant yields, generally, were improved for all treatments when maltose was the primary carbohydrate source. Nevertheless, the most productive treatments identified previously, were consistent,in terms of androgenesis and plant yields. The amino acid studies were extended and are reported here in more detail. In addition, the effects of anther density during culture and the physical properties of the medium were also evaluated.

6.2. The effect of different amino acids on anther cultures with maltose as the major carbohydrate source

Treatment and anther randomisation were as described earlier (Appendix 2.9). Each plate received six anthers and each treatment was replicated ten times. Four amino acid treatments and one amino acid-free control were compared to assess their affects on embryogenesis in the "maltose" system.

In this experiment, the amino acids glutamine, asparagine, arginine and proline, were all included in the media at 5mM. The proline treatment was intended as an

internal comparison with the conditions used in an earlier experiment (Section 4.8).

The data from these experiments were analysed statistically (Section 2.14). The results were summarised and are presented graphically (Figures 6.2.1-3).

Anther responses were affected by amino acid media composition (Figure 6.2.1). All of the amino acid treatments supported significantly higher responses than those obtained in the amino acid-free controls. They did not, however, differ significantly from each other.

The production of green plants was influenced by the amino acid source. Proline and asparagine produced significantly more plants than did either the arginine or the amino acid-free control treatments. The glutamine treatment sustained almost twice the plant yields of any of the other treatments (Figure 6.2.2). This was also true when considering green plant yields per 100 responding anthers (Figure 6.2.3).

6.3 Determination of the importance of the glutamine carbon skeleton by comparison of the effects of glutamine and glutamic acid on anther cultures.

It has been assumed that enhancement of plant production from anther culture by glutamine is due to its primary role as an organic ammonium source (Stuart et al, 1984,a,b). The effect may also be due to metabolism of the carbon skeleton.

The effects of 5mM glutamine and glutamic acid were compared with an amino acid-free control. Anthers were dissected from selected

Figure 6.2. The effect of different amino acids on anther cultures with maltose as the primary carbohydrate source

Figure 6.2.1. **ANTHER RESPONSE**

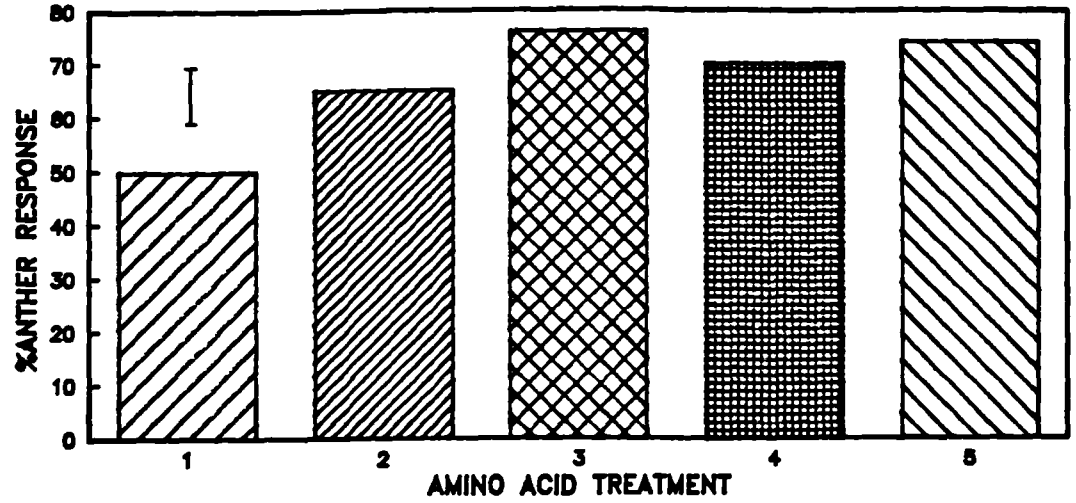


Figure 6.2.2. **GREEN PLANTS**

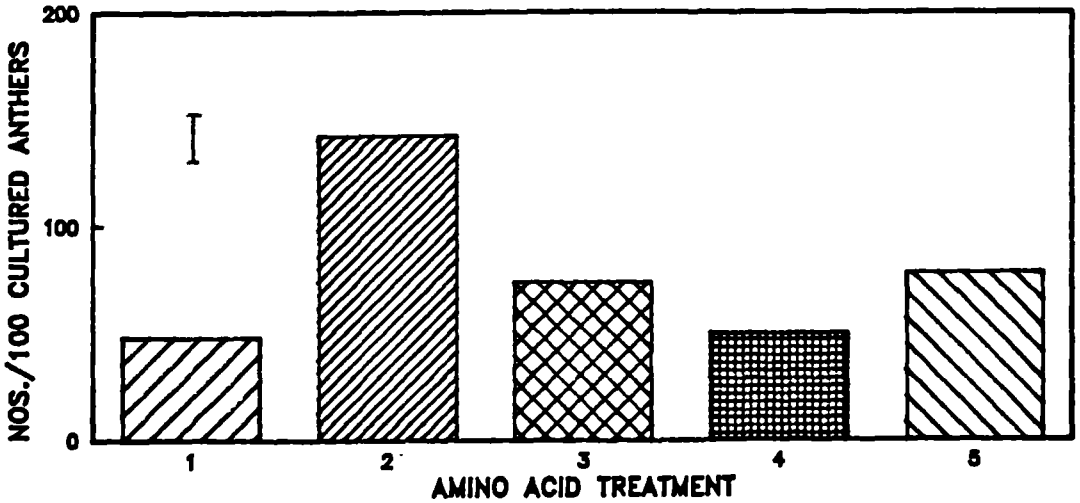
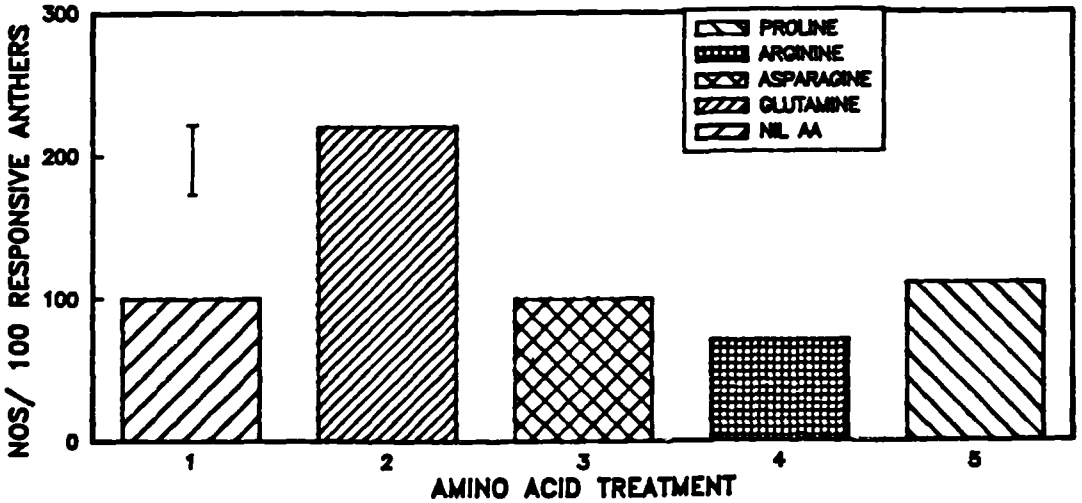


Figure 6.2.3. **GREEN PLANTS**



pretreated spikes and randomised between the treatments (Appendix 2.9). Six anthers were assigned to each treatment and the experiment was replicated ten times. The results were analysed statistically (Section 2.14) and are summarised (Figures 6.3.1-6.3.3).

The promotive affect of glutamine demonstrated in earlier experiments (Section 4.6. and 6.2) was confirmed (Figures 6.3.1-3). Anther response and green plant yields per 100 cultured anthers, were significantly higher in the glutamine treatment than in the other treatments. The addition of glutamic acid to the culture medium inhibited anther response, embryo production and plant production (Figures 6.3.1 and 6.3.2). Green plant yields per 100 responding anthers tended to reflect a similar picture to the yields per 100 cultured anthers. However, differences between plant yields per 100 responding anthers were significant only between the glutamine and glutamic acid treatments (Figure 6.3.3).

6.4. The effect of glutamine concentration on anther culture productivity

Anther selection and culture were as previously described (Section 2.12). Anthers were randomised among the six treatments using a randomised block design (Appendix 2.9). Each of the treatments contained 3ml of the appropriate medium in a 3.5cm diameter Petri dish. Each dish received six anthers and the experiment was replicated ten times. Five glutamine concentrations (1, 2, 5, 10 and 25mM) were compared with an amino acid-free control. The media were prepared as described earlier (Section 2.5).

Figure 6.3 A comparison between the effects of glutamine, glutamic acid and an amino acid-free treatments on barley anther culture.

Figure 6.3.1. **ANTHER RESPONSE**

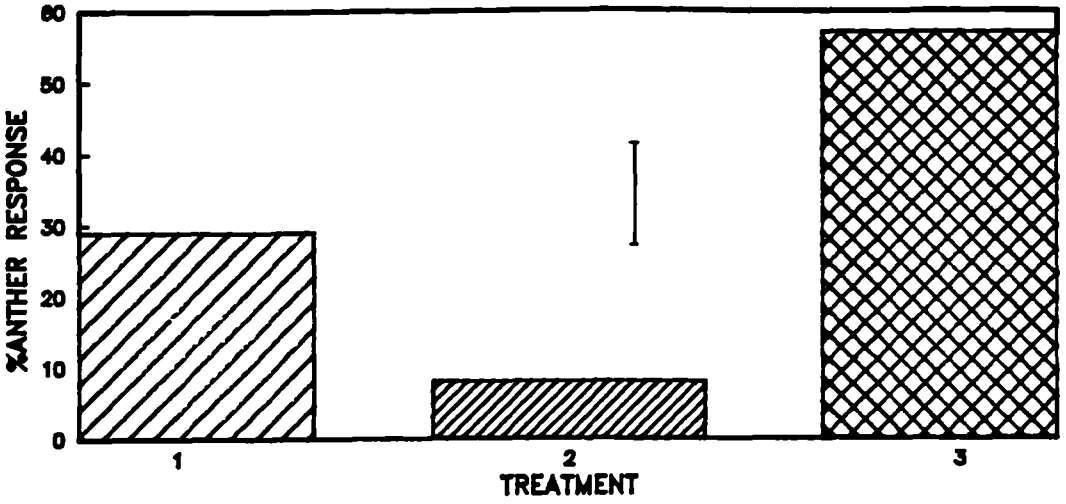


Figure 6.3.2. **GREEN PLANTS**

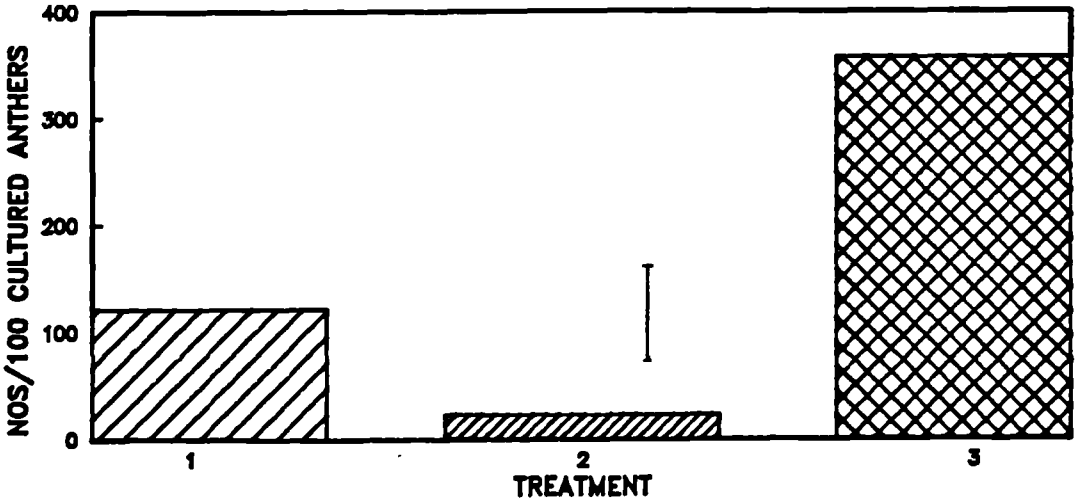
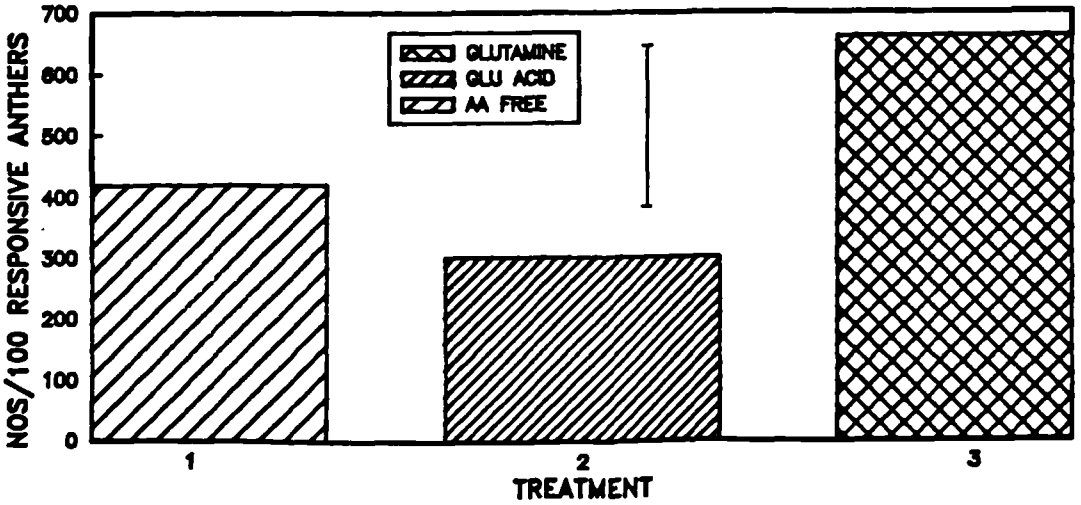


Figure 6.3.3. **GREEN PLANTS**



The results were analysed statistically (Section 2.14). A summary of the results is presented graphically in Figure 6.4.1-3.

Glutamine concentration clearly affects the induction of androgenesis and the morphology and development of the androgenic structures produced (Figures 6.4.1-3). Anther response and green plant production per 100 cultured anthers was highest in the 5mM treatment (Figures 6.4.1 and 2). This was statistically significant when compared with the other concentrations examined. Green plant production per 100 responding anthers gave a similar trend (Figure 6.4.3) although the difference between the 1mM and 5mM treatments was not significant when the data were analysed statistically. In response to the findings of this experiment a 5mM glutamine supplement was added to the media used in subsequent experiments. This modified medium was referred to as FHG medium (Appendix 1.3).

Figure 6.4. The effect of glutamine concentration on barley anther culture.

Figure 6.4.1. Anther Response

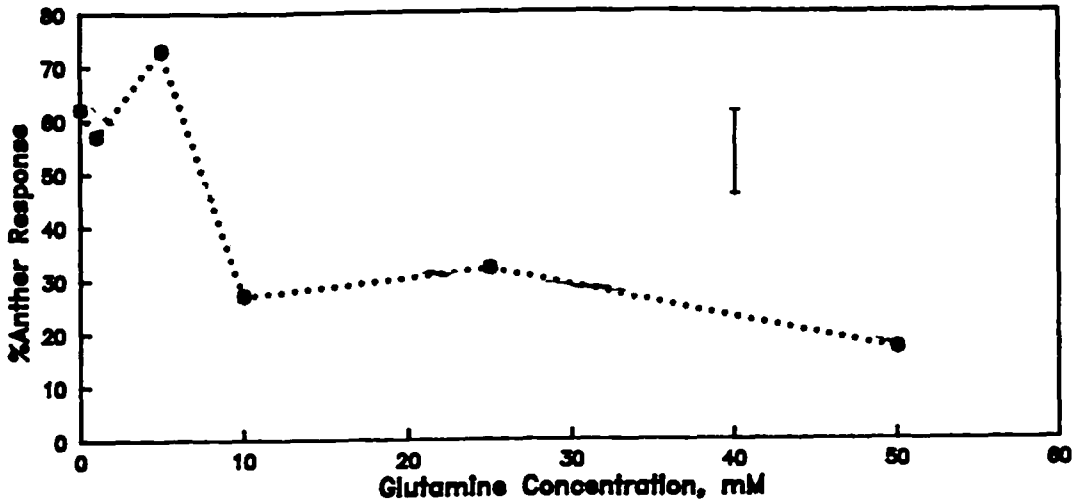


Figure 6.4.2. Green Plants

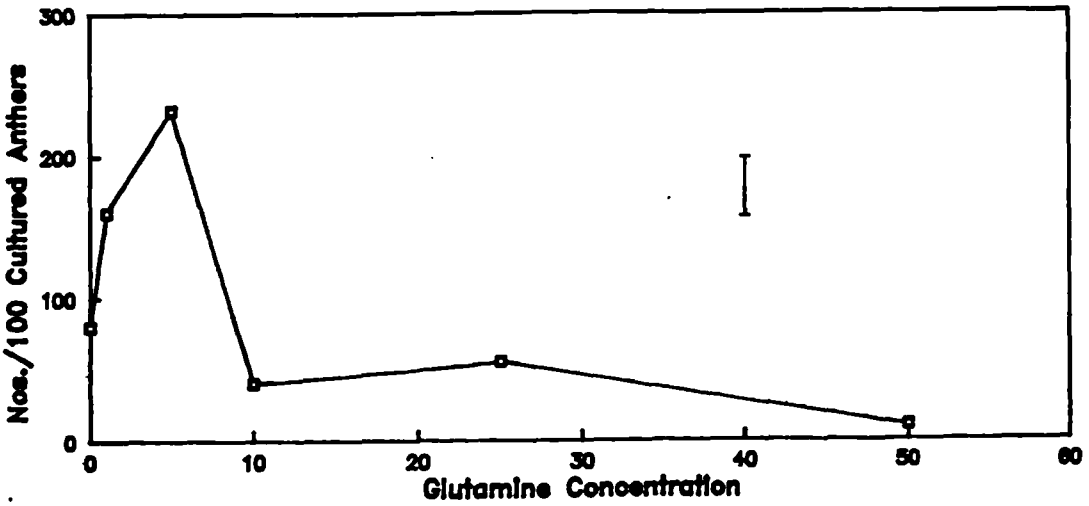
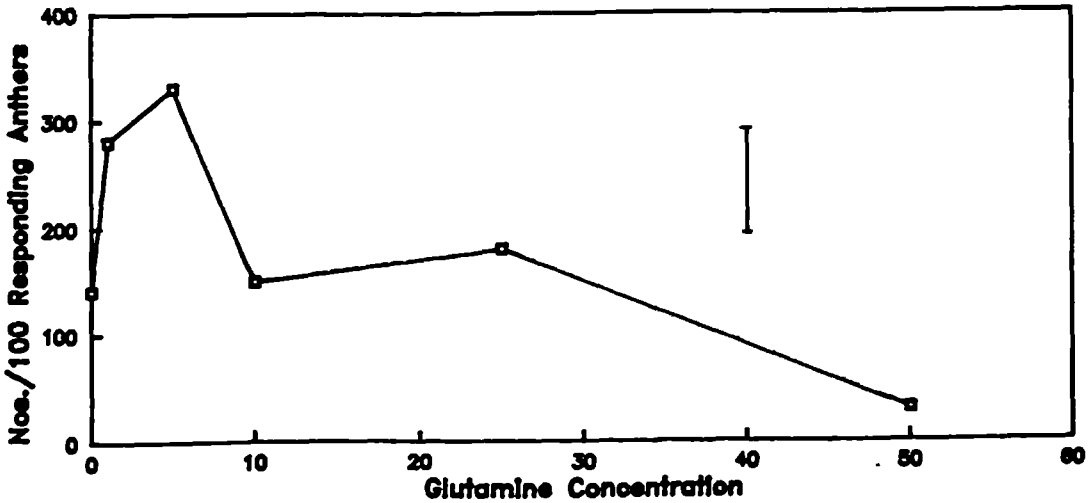


Figure 6.4.3. Green Plants



6.5. The effect of anther density on anther culture success

Anthers were selected and cultured as described earlier (Section 2.10). Anther randomisation was as described earlier (Appendix 2.14). The affect of anther density was assessed over a range of 0.025-4 anthers per ml. of medium (ie 1 anther in 40ml of medium up to 12 anthers in 3ml of medium). The medium used for these experiments was prepared and poured at the same time, although the experiments were set up on three consecutive days. Twenty eight days after culture initiation any structures present were spread evenly across the surface of the culture medium. The dishes were resealed and returned to the incubator. Plantlets were harvested once a week for the next five weeks. Data from each of the experiments were analysed statistically (Section 2.14). The pooled data are also presented in Figures 6.5.1-3.

Optimal green plant production (16.5-17.5 per anther, Figure 6.5.2) was achieved in this culture system at densities of one anther in 15 ml of culture medium and three anthers in 40 ml of medium, ie equivalent to 0.066 and 0.075 anthers per ml of culture medium. Green plant yields declined at densities below two anthers per 40 ml of medium (0.05/ml), but more work needs to be carried out to confirm this. Almost one hundred green plants were obtained from a few individual anthers.

Figure 6.5. The effect of anther density on anther culture.

(Results from three experiments.).

□ - Expt.1. 0.33-4 anthers/ml of medium.
■ - Expt.2. 0.066-0.8 " " " .
✱ - Expt.3. 0.025-0.15 " " " .

Figure 6.5.1. **Anther Response**

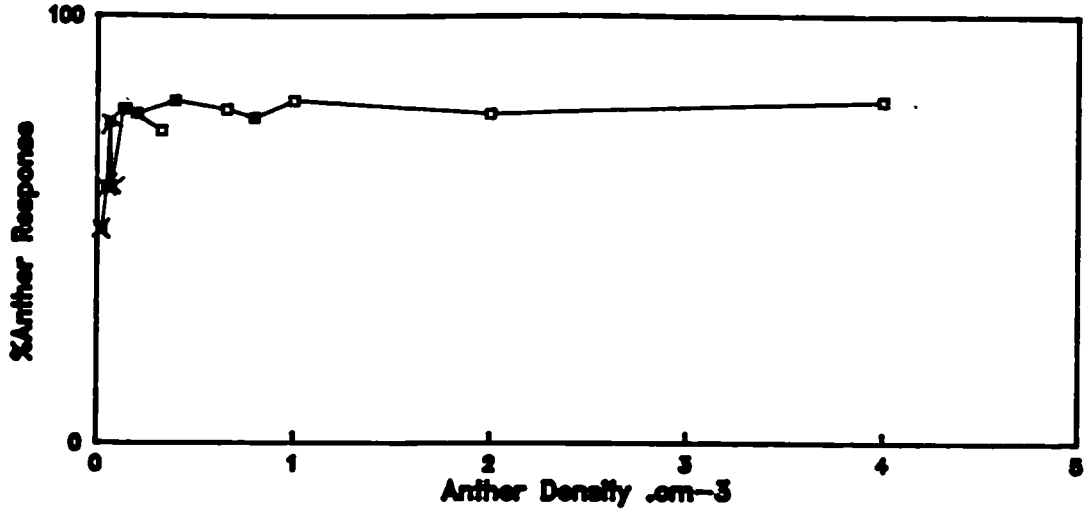


Figure 6.5.2. **Green Plants**

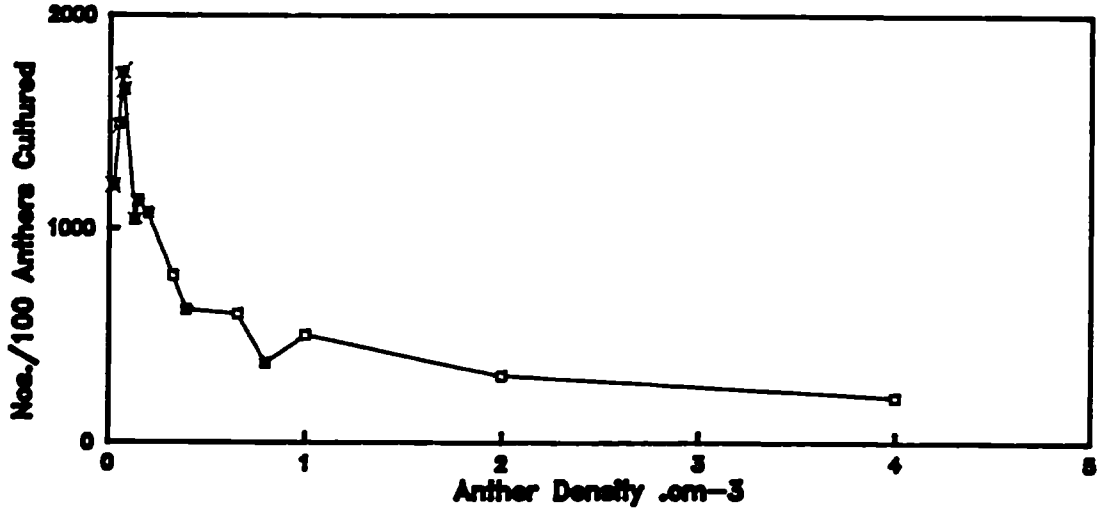
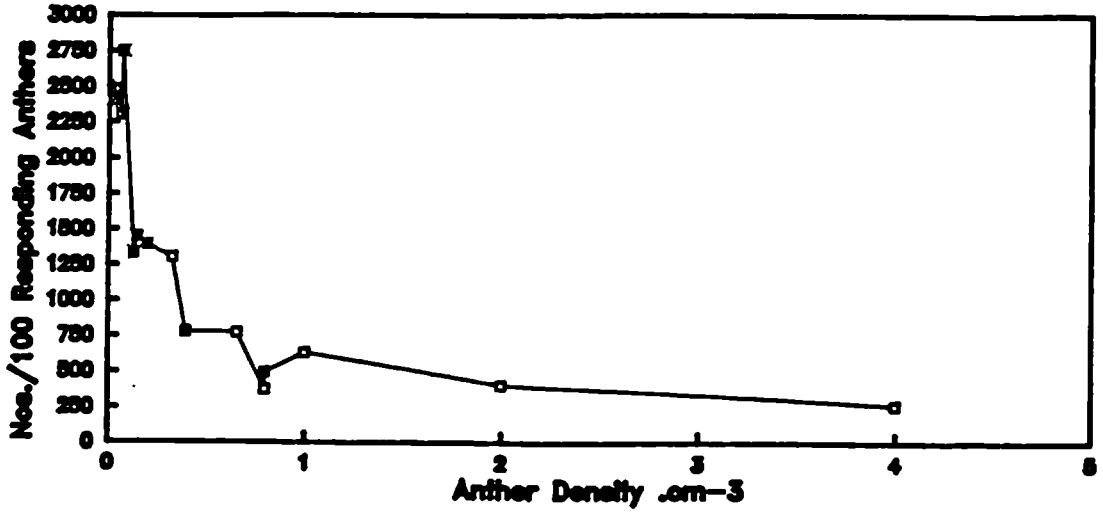


Figure 6.5.3. **Green Plants**



6.6 The effect of the physical properties of the media on anther culture

Liquid media containing Ficoll $_{400}$ had been reported to be superior to solid media for barley anther culture (Kao, 1981). By using maltose as the primary carbohydrate source it was no longer necessary to orientate anthers on-edge on solid medium to support androgenesis. The influence of liquid media on barley anther cultures was examined. The characteristics of the treatments used in this experiment are given in Table 6.6.

Table 6.6.
The physical property characteristics of media treatments investigated in Experiment 6.6.

Treatment	Abbreviation	Characteristics
1	FHAuto	FHG medium (Appendix 1). All media components, with the exception of glutamine, were autoclave sterilised together. Glutamine was added after cooling to ca 35C.
2	FHS/F.F	FHG medium (Appendix 2).All media components were prepared at double strength and filter sterilised (Section 2.5). This was mixed with an equal volume of 1.6% Sea Plaque Agarose solution previously autoclave sterilised and cooled to ca 35C before mixing. In this treatment anthers were cultured "flat" on the medium.
3	FHS/F.UR	As for 2 but anthers cultured on-edge.
4	FHF10	As for 2 and 3 but 0.8 % Sea Plaque Agarose replaced by 10% Ficoll $_{400}$.
5	FHF10/SPA	As for 4 but contains a mixture of 10% Ficoll and 0.8% Sea Plaque Agarose.
6	FHF20	As for 4 but contains 20% Ficoll.

The anthers were selected, randomised and cultured as described earlier (Section 2.10 and Appendix 2.9). Twenty four anthers were allocated to each treatment. Four spikes were used per replicate and the experiment was replicated five times.

It was observed that variation within the same treatments in different replicates was particularly high. This may have been due to the formation of condensation on the lids of some of the dishes. This, from experience, can cause considerable problems with culture response since the condensate drips onto the surface of the medium causing a localised reduction in Ficoll concentration which results in a reduced medium density and surface tension which, in turn, results in an increased tendency for anthers to sink into the medium.

Despite these difficulties, the data from these experiments were analysed statistically (Appendix 3). The results are summarised in Figures 6.6.1-3. Because of the variability of the results obtained in this experiment little can be deduced from these results. However, the quality of the embryos that developed on two replicates of the FHF20 treatment were exceptionally good (see Figures 6.6.3-5). A mean green plant yield of almost 10 per anther cultured was achieved.

Figure 6.6. The effect of the physical properties of the medium on anther culture.

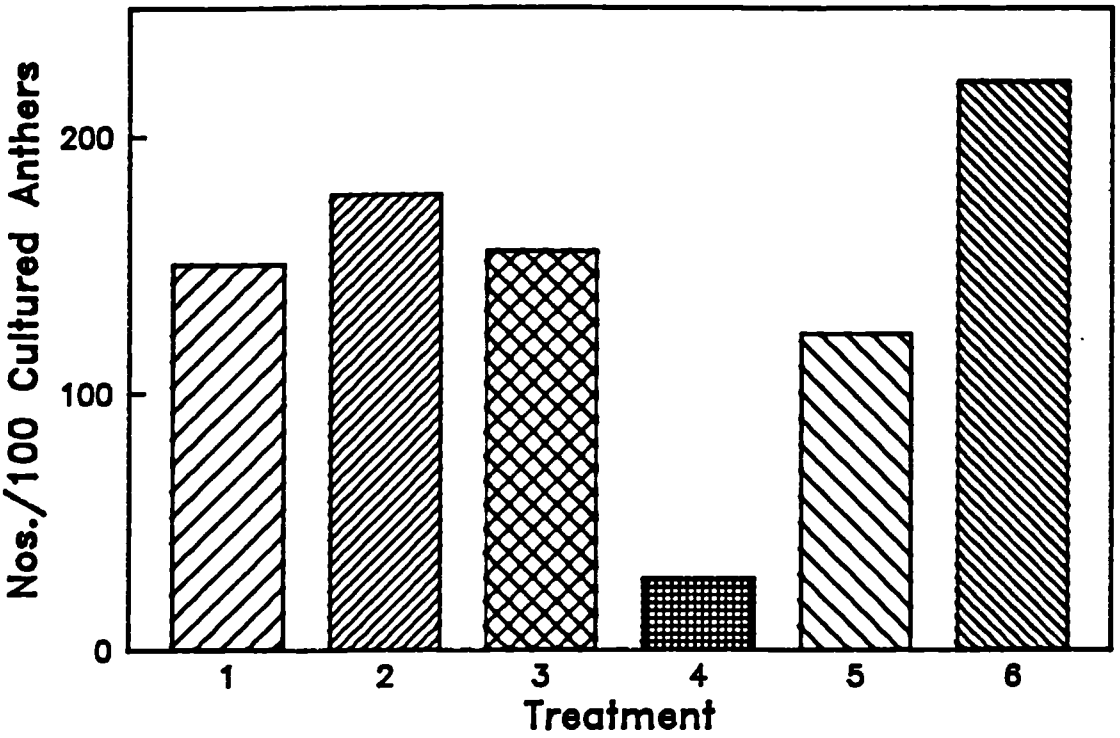


Figure 6.6.1. Green plant production.

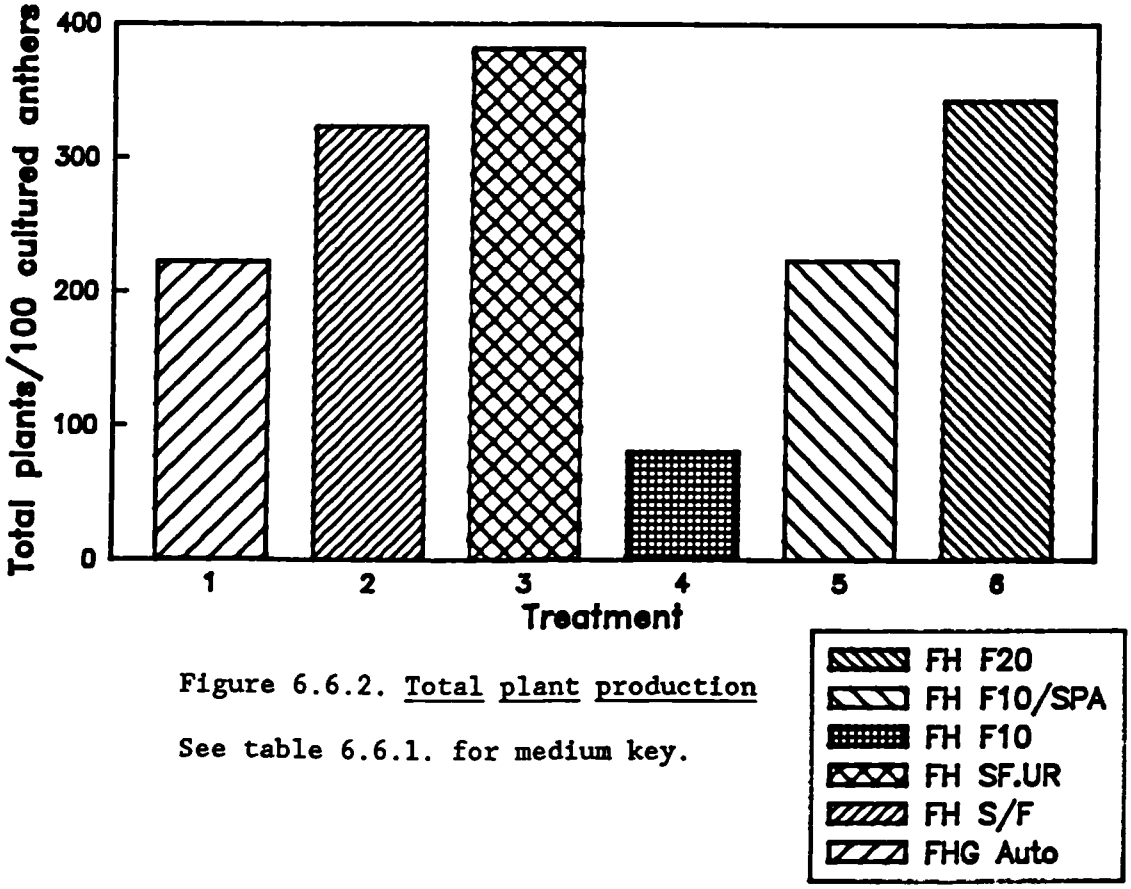


Figure 6.6.2. Total plant production

See table 6.6.1. for medium key.

Figure 6.6. Continued



Figure 6.6.3. Large numbers of barley microspore-derived embryos which have developed on the surface of maltose medium containing 20% $Ficoll_{400}$.

Figure 6.6.4,a,b. Microspore-derived barley embryos on the surface of the medium in figure 6.6.3. (Embryos pictured areca 0.5-2mm long).

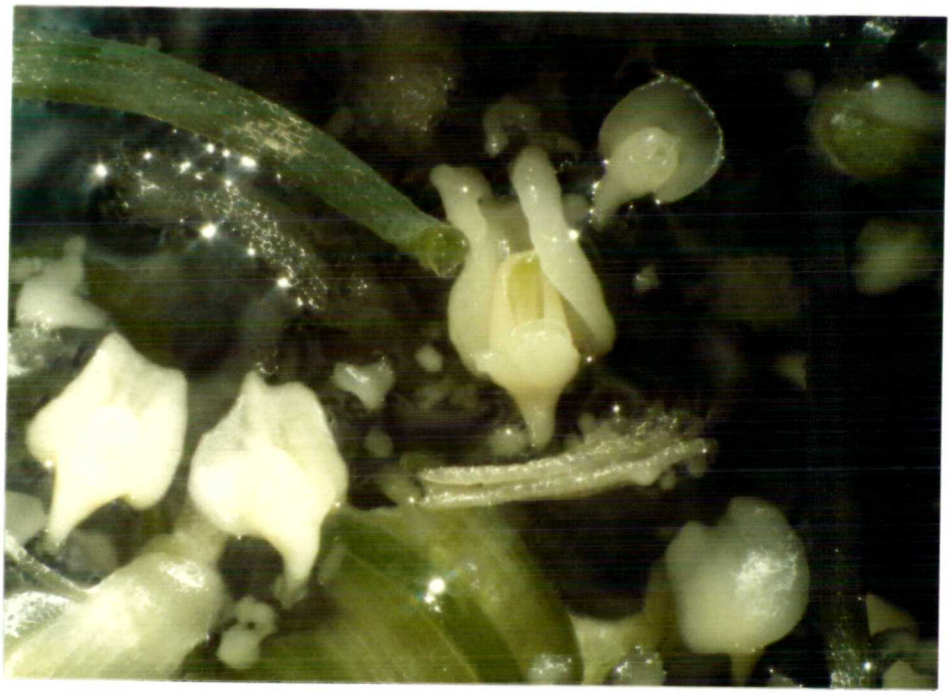




Figure 6.6.5. Microspore-derived plant from figure 6.6.3.



6.7 Discussion

Amino acids, previously reported to have either positive or negative or no effect on embryogenesis in a number of tissue culture systems, at concentrations of ca 5mM, were selected for this study (Xu et al, 1981; Harada et al, 1983; Sangwan, 1983; Stuart et al, 1984a). Response in this barley anther culture system, where maltose was the primary carbohydrate source, was dependent upon amino acid type used (Section 6.3 and 4).

Glutamine consistently enhanced embryogenic response and development in a variety of tissue culture systems (Harada et al, 1983 Sangwan, 1983; Stuart et al, 1984a,b). The influences of other amino acids investigated varied with the tissue culture system (ie some amino acids have positive effects on one sysem while having no effect or being inhibitory in another (Harada et al, 1983; Sangwan, 1983; Stuart et al, 1984a,b;).

The optimal concentration of glutamine for the induction of androgenesis and green plant production in barley was found to be 5mM. This was in agreement with the observation of Harada et al (1983) who found 5mM glutamine optimal for embryo induction in anther culture of Datura innoxia. Glutamine enhanced somatic embryogenesis in callus cultures of alfalfa from 1-30mM, but was inhibitory at 100mM. Glutamine also enhanced callus production in protoplast cultures of Solanum brevidens, also at an optimal concentration of 5mM (Nelson, 1983).

Glutamine appears to enhance androgenesis and embryo development in a variety of species (for reviews see:- Sunderland, 1982 Hu, et al,

1986). Glutamine is synthesised by the enzyme glutamine synthase from glutamic acid and ammonia substrates. It is a protein amino acid and a key intermediate in several metabolic pathways including:- the synthesis of histidine, purine, tryptophan and glucosamine-6-phosphate. It is also utilised as an amino-group donor for transamination reactions (Dougall, 1980; Graham, 1980), including the resynthesis of glutamic acid. This is catalysed by the enzyme Glutamine OxyGlutarate Amino Transferase (GOGAT) which converts one molecule of glutamine and one molecule of oxyglutarate into two molecules of glutamic acid. However, when glutamic acid was added to barley anther cultures as the sole organic nitrogen source, anther response and green plant production were severely inhibited. This was consistent with earlier reports in which glutamic acid inhibited somatic embryogenesis in alfalfa (Stuart et al, 1984a) and seed embryo development of Capsella ursa-pastoris in vitro Rijven, 1955). However, glutamic acid was reported to enhance androgenesis and embryo development in anther cultures of N. tabacum (Harada et al, 1983) and Datura metel (Sangwan, 1983).

Clearly, nitrogen source and concentration are important factors influencing induction of androgenesis and subsequent embryo development. Although glutamine enhanced somatic embryogenesis in callus cultures of alfalfa independent of NH_4^+, a number of other amino acids required the presence of NH_4^+, the optimal concentration of which varied with amino acid (Stuart et al, 1984a). A similar requirement may explain the differential effects of glutamine and the remaining amino treatments in this barley anther culture system.

Anther density also influenced anther culture response and green

plant production. In common with earlier reports of barley anther culture (Xu et al, 1982; Sunderland, 1983,b), anther density was found to influence the levels of androgenesis and plant production in the current study (Figures 6.5.1-3). However, the anther density at which optimal anther response and green plant production was observed differed greatly between the two systems.

Sunderland, (1983,b) reported anther densities of 60-120/ml to be optimal for callus and plantlet production in droplet cultures. In contrast, anthers cultured at densities of between 0.066-0.075/ml of FHG medium solidified with 0.8% Sea Plaque Agarose yielded the greatest number of green plants per cultured anther (Figures 6.5.2). At very low densities, below ca 0.05/ml (Figure 6.5.1), anther response declined, but anther density appeared to have little effect on anther response at densities above this (Figure 6.5.1).

Green plant yields declined at densities above and below 0.06-0.077/ml (Figure 6.5.2). The decline in plant production observed in low density cultures may be a direct result of the reduced level of anther response observed at these densities (Figures, 6.5.1). However, the decline in green plant yield/cultured anther observed in higher densitiy cultures may well be the result of competition between developing embryos. Only the most morphologically advanced embryos developed into plants. As they were harvested from the cultures so the next-most-advanced embryos developed into plantlets. After several harvests large numbers of developing embryos in the higher anther density treatments either stopped developing and became moribund or callused and died. More work is necessary to explain these observations, but studies using isolated microspore cultures

(see next Section 7) may enhance the knowledge of those employing anther cultures.

The substitution of sucrose with maltose permitted the development of androgenic embryos in both lobes of anthers in culture even if they were in contact with the medium. Thus it was no longer necessary to use solid medium to orientate anthers "on-edge". However, an earlier report emphasises the importance of anthers remaining on the surface of the air/liquid interface of the media (Kao, 1981), presumably to maintain aeration.

Several different methods have been adopted to achieve this, including nylon rafts (Lyne et al, unpublished). One of the most effective has been the addition of Ficoll $_{400}$ to the medium (Kao, 1981). This increases the density of the medium which, in turn, also increases the surface tension of the medium, thus preventing the anther and developing embryos from sinking into the medium. The results of this study (Section 6.6) support the findings of Kao, (1981) and demonstrate some potential advantages and disadvantages of liquid compared to solid medium.

When the problems due to condensation were overcome, large numbers of microspore-derived embryos developed on the surface of the medium containing 20% Ficoll $_{400}$. Of these embryos a proportion were almost indistinguishable from freshly excised immature zygotic embryos (Figures 6.6.3-5). Removal of plants from liquid media had to be carried out with care so as not to disturb the surface of the medium. If the androgenic structures became wetted then they too would sink through the meniscus and then deteriorate.

The ability of relatively large numbers of microspores to develop into embryos which are morphologically indistinguishable from zygotic embryos of the same size (for comparison see Section 3.3.2) has been demonstrated on liquid medium containing 20% $Ficoll_{400}$ (Figures 6.6.3-5). Positive and negative aspects of both liquid and solid culture were revealed. Solid culture is recommended for large-scale commercial production of doubled haploids, where-as liquid culture could prove superior for research.

6.8. Conclusions

The addition of glutamine to the culture medium, at a concentration of 5mM, was found to increase anther responses and green plant yields/anther cultured were more than doubled.

Anther density was optimised for green plant production on solid medium at between 0.066-0.075 anthers/ml of medium. However, optimal anther density remains to be determined for liquid cultures.

Exceptionally good quality embryos developed on liquid media containing 20% $Ficoll_{400}$. Green plant yields exceeding 10/anther cultured were obtained in several replicates; these may be improved still further by determining optimal anther densities for these cultures.

Overall, a number of improvements have been made to the barley anther culture system. These include the replacement of sucrose by either cellobiose or maltose, the addition of 5mM glutamine and the determination of optimal anther density for green plant production on solid media. In addition, the physical properties of the culture medium were found to have a marked influence on the initiation and

morphogenesis of barley microspore-derived embryos.

Barley microspores develop directly into embryos and will germinate to give plants, the majority being both green and phenotypically normal. Mean yields of >1750 green plants/100 cultured anthers were observed (Figure 6.5.5) (in several anthers >1000 microspore-derived embryos developed. Some anthers produced ca 100 green plants) compared with mean yields of 8 green plants/100 anthers cultured at the time that this study was initiated (Section 3.2, Table 3.2.1). The revised protocol, highlighting the above improvements, is now presented (Table 6.7.).

Table 6.7.
Outline of the revised protocol used for the barley anther culture

Time (days)		Step
0		Seeds of Winter barley cv Igri were sown.
7		Seeds germinated and were transferred to 4C to vernalise for 56 days.
"a"		
63		Vernalised plants were transferred to controlled environment conditions to grow on to spike harvest after ca 70 days.
133		Spikes harvested and selected for 28 days pretreatment.
161		Pollen viability was determined and anther cultures initiated.
189		Cultures were transferred into constant light.
190	Early* harvest	
"b"		Plantlets were harvested and transferred to vernalisation.
232	Late** harvest	
246		Vernalised plantlets transferred to soil.
288		

"*" The first plants harvested from each culture.
"**" The last plants to be harvested from cultures before the dishes were discarded. Note that plants were harvested from cultures at weekly intervals over six weeks.

Steps in Section "a" (Table 6.7.) remained essentially unchanged from the methods described in detail earlier (Section 2.8-2.10). The improvements in the steps outlined in Section "b" (Table 6.7.) are described in more detail below.

Anther culture

Anthers containing >60% viable pollen were excised from pretreated spikes and cultured on FHG medium (Appendix 1.3). FHG medium consisted of the salts of Foroughi-Wehr et al (1979) containing maltose and solidified with 0.8% Sea Plaque Agarose and was supplemented with 5mM glutamine, 0.4mg/l thiamine HCl, 100mg/l myo-inositol and 1.0mg/l BAP. The anthers were cultured at a density of between 0.07-0.1 anthers/ml of medium.

Plantlet harvest, vernalisation and transfer of vernalised plantlets to soil

Plantlets and germinating embryos were harvested at weekly intervals over the next six weeks and transferred to 60ml plastic specimen jars containing 10ml FHG medium. Plantlets were returned to the 25C incubator until their shoots had grown to ca 2cm long. These plantlets were then transferred to vernalisation conditions for eight weeks in the coldroom under the following regime:

Temperature: 4C constant
Photoperiod: 8hrs. light/16hrs. dark
Light intensity: ca 70$\mu E.m^{-2}.s^{-1}$.

During this period the plantlets grew strongly with deep green shoots and well developed roots. When transferred to soil their survival rate was superior to that of plantlets transferred to soil before vernalisation.

Chapter 7

Microspore/pollen to plants: a method for the culture of isolated barley microspores/pollen

7.1. Introduction

Work carried out so far had concentrated on the development of barley microspores within the anther. The efficiency of anther culture was increased to ca 18 green plants/anther cultured (Section 6). This still only represented ca 0.6% of the microspores present in the anthers (Sunderland, 1978).

The problems encountered in trying to improve the efficiency of plant regeneration further may be alleviated by the culture of isolated microspores, ie. microspores removed from the anthers (Swanson et al, 1987). The efficiency of green plant production in Brassica napus was found to be higher in isolated microspore cultures than was found in anther culture (Lichter, 1982; Chaung et al, 1985).

There have been a number of reports of isolated microspore culture of barley (Sunderland et al, 1982a; Kohler et al, 1985; Wei et al, 1986), but these tended to give low efficiency and plants regenerated via callus.

In this Chapter a simple method for the large-scale production of green plants via embryogenesis from isolated microspores of cv Igri is described. Also the potential for isolated microspore culture is discussed.

7.2. Plant growth

Plants of the winter barley variety Igri were grown as described previously (Section 2.8 and 2.9).

7.3. Spike selection and pretreatment

Spike selection and pretreatment was as described before (Section 2.10).

7.4. Isolated microspore culture

Anthers were dissected from selected spikes as described previously (Section 2.10). Two hundred and forty anthers were placed into 1ml of liquid FHG medium (Appendix 1.3). The microspores were liberated from the anthers by gently squashing the anthers with a sterile teflon rod (Nitsch, 1972). In the first isolation (Section 7.4.1), the crude preparation was split into two, approximately equal aliquots one of which was cultured directly onto 30 ml of FHG medium solidified with 0.8% Sea Plaque Agarose. Subsequently all isolated microspore preparations were purified as follows:-

The crude suspension was passed through a 50um mesh stainless steel sieve to remove the large pieces of anther debris. The filtrate was transferred to a sterile 10ml centrifuge tube and centrifuged at ca 60g for 5 minutes. After centrifugation most of the remaining anther debris was floating on the meniscus. This was removed with the supernatant. The pellet was resuspended in a known volume of fresh liquid FHG medium and plated out onto FHG medium solidified with 0.8% Sea Plaque Agarose. Thereafter the culture conditions were the same as used for barley anther culture (Section 2.11).

7.4.1 Experiment to determine the feasibility of culturing isolated microspores on maltose containing medium.

Microspores were isolated as described before (Section 7.4). The crude preparation was mixed thoroughly then split into two approximately equal aliquots of ca 0.5ml, A and B. Aliquot A was cultured directly onto FHG medium solidified with 0.8% Sea Plaque Agarose, in a 9cm diameter petri dish. Aliquot B was purified as described earlier (Section 7.4) and then cultured as for aliquot A.

Distribution on the surface of the medium of the pollen and anther debris in sample A, and pollen in sample B, was uneven. The cultures were examined after three weeks. Multicellular structures were visible in regions of higher pollen density in both samples. Conversely no development was visible in regions of lower pollen density. In both samples microspore-derived structures resembling normal zygotic embryos formed and continued to develop into plants. At the end of this experiment, 77 green plants had been harvested from sample A and 43 from sample B.

7.4.2. Some factors that influence the success of isolated microspore culture.

Microspore suspensions were prepared as described earlier (Section 7.4). The pellet was resuspended in liquid medium to give a final volume of ca 0.6ml. Twelve 0.05ml droplets were taken, making sure that each droplet was removed from a homogeneous suspension (ie. so that they were approximately equal). The droplets were pipetted onto the surface of 3.5ml FHG medium in a 3cm diameter petri dish from ca 2mm above it. This was to ensure uniform density of microspores on the surface of the medium. Two dishes received one single droplet. In a third dish, two droplets were added as separate drops. Two further dishes received two droplets, this time one on top of the other. The sixth dish received four droplets one on top of each other.

The single droplet samples and the sample with two separated droplets did not respond. However, in the samples containing two or four droplets, pipetted one on top of the other, organised structures developed. These continued to develop into embryos and subsequently plants (Figures 7.4.1- 7.4.6). Little, if any, callus developed

Figure 7.4.

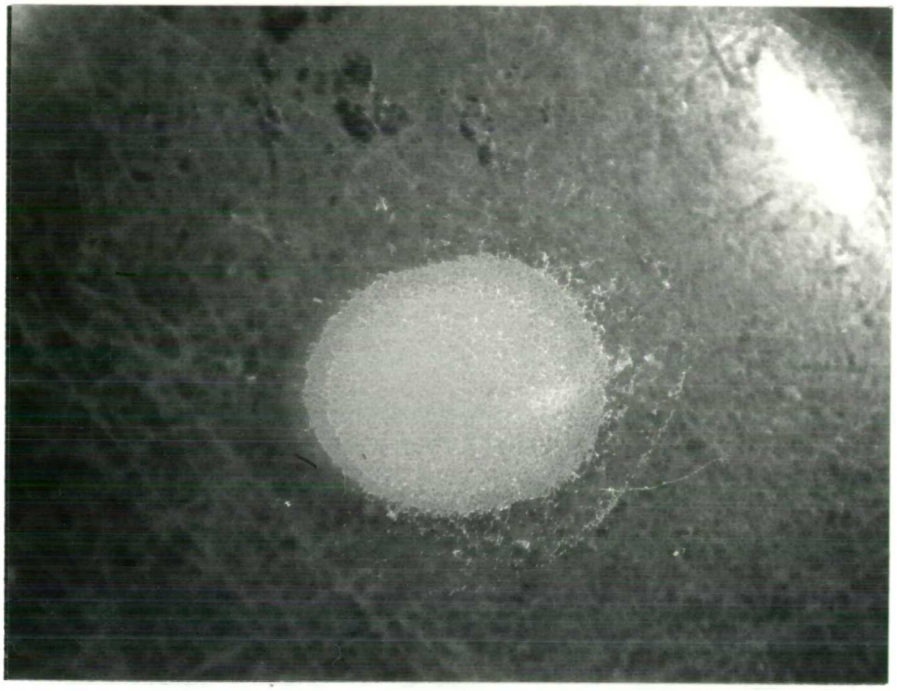


Figure 7.4.1. Isolated microspore culture. 2x0.05ml aliquots of microspore suspension from experiment 7.4.2. pipetted one on top of the other on the surface of solid medium. Photographed at culture initiation in a 3.5cm diameter petri dish. x5 magnification

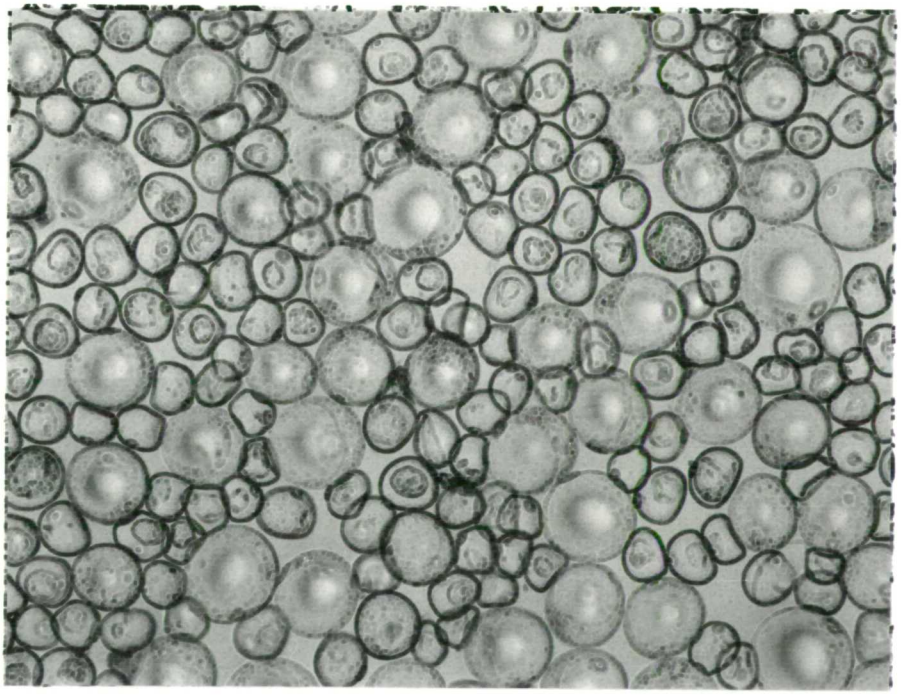


Figure 7.4.2. A similar culture as Figure 7.4.1., but after 3 days dark culture at 25C. x250 magnification

Figure 7.4. continued

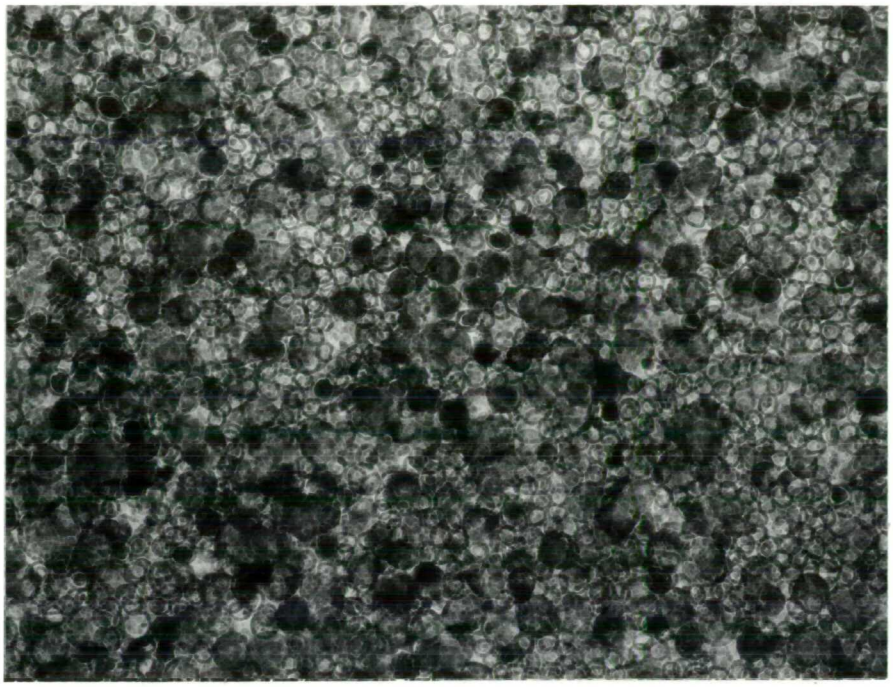


Figure 7.4.3. Isolated microspore culture on solid medium after 14 days culture. Dense dark structures are multicellular microspores. Some have ruptured the sporopollenin case. ×50 magnification.

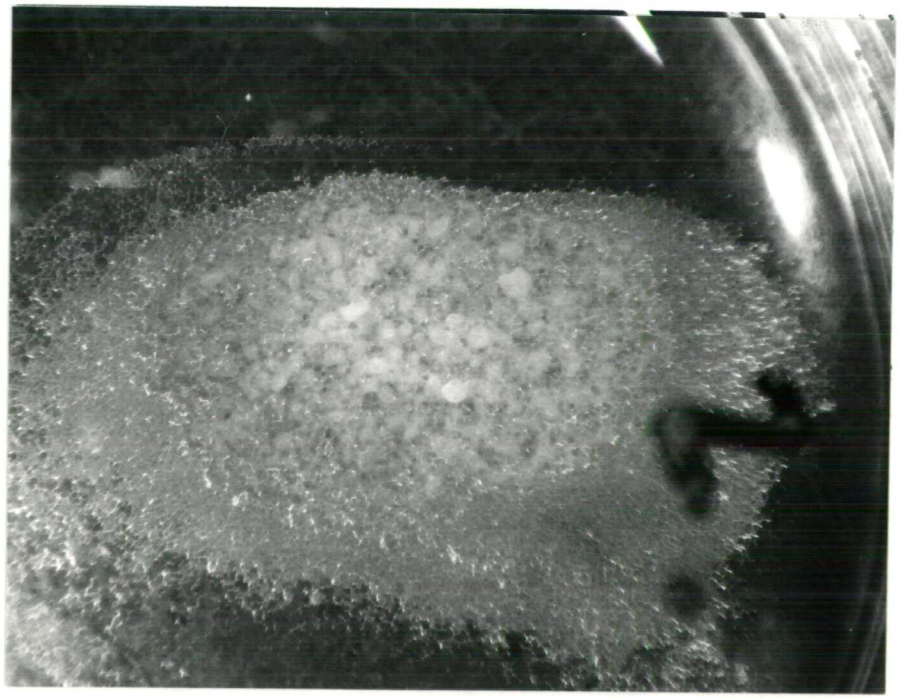


Figure 7.4.4. Similar sample to Figure 7.4.1., but 21 days after culture initiation. Microspore-derived structures recognisable as barley embryos. ×5 magnification

Figure 7.4. continued



Figure 7.4.5. Sample similar to figure 7.4.1., but 28 days after culture initiation. Large embryo-like structures visible and plants are recognisable. x5 magnification



Figure 7.4.6. An isolated microspore culture containing 4x0.05ml aliquots of microspore suspension pipetted one on top of each other onto the surface of solid medium (Section 7.4.2). Twenty eight days post culture initiation. Note: structures are not as advanced as those in Figure 7.4.5. x 5 magnification

during the early stages of this culture system.

Embryos were transferred from one of the two double aliquot samples on to fresh FHG medium after four weeks culture. Those on the second double-aliquot and the four-aliquot sample were spread over their original plate. Plant yields from these plates are presented in Table 7.4.

Table 7.4
Plant production from isolated microspore cultures

Sample	Green Plants	Albino Plants
Double aliquot embryos transferred to fresh medium after 4 weeks	635 (38)	109 (6)
Double aliquot embryos spread on original medium	166 (10)	33 (2)
Quadruple aliquot embryos spread on original medium	669 (40)	73 (4)

(Figures in parentheses % of total plant yield).

Considerably more plants developed from the double aliquot sample where the embryos had been transferred onto fresh FHG medium after four weeks culture.

The proportion of pollen grains, present in the original anthers, that developed into green plants may be estimated as follows:

The numbers of pollen grains per barley anther have been assessed for several varieties. They range from 2500 cv Akka (Dale, 1985) to 3000 cv Sabarlis (Sunderland et al, 1982a). These assessments are in line with rough estimates made for Igri using scanning electron micrographs (not presented). In this study all

calculations assume 3000 pollen grains per anther.

The highest green plant yield obtained and shown in Table 7.4. was 635 from 0.1ml of isolated microspore suspension. Since this was from a total volume of 0.6ml which had been isolated from 240 anthers it is possible to obtain an estimate of the proportion of the pollen originally present in the anthers that developed into plants by the following calculation:

635/(240x0.1/0.6)= 15.9 green plants/anther .

15.9x100/3000= 0.53% of the pollen present in the anthers developed into green plants.

7.5 Microspore isolation efficiency

A major problem encountered with this technique was the efficiency of microspore isolation. A large proportion of the microspores remained associated with the anther debris when the preparations were purified. The efficiency of microspore isolation was calculated in five subsequent experiments with the aid of an improved Neubauer haemocytometer (Table 7.5). (Volume of each square, 1/16 mm^2x0.2mm deep).

Thus,

Count per 16 squares x 5000 x volume, mls.= Density/ml of medium

Table 7.5
Microspore Isolation Efficiency

Experiment	Number Anthers	Number pollen grains/ 16 squares		Pollen Density/ml of medium	Sample Volume (ml)	Isolation Efficiency (%)*
		Count 1	2			
1	240	24	22	113,750	0.6	16
2	240	22	21	107,500	1.0	15
3	240	29	33	156,250	1.0	22
4	240	25	29	135,000	1.0	19
5	240	29	29	145,000	1.0	20

* % of total pollen in donor anthers, assuming 3,000 pollen grains per anther.

The microspores isolated in the fifth experiment were purified and diluted to 1.0ml final volume. The suspension was split into two x0.5ml samples. One was used to determine the pollen density and the other was cultured as the most successful double droplet samples described above (Section 7.4.2.). Yields of 27 green plants per anther were obtained. Assuming the average number of pollen grains to be 3000 per anther the percentage of the total population of pollen that developed into green plants was:- 0.9%. However, if this was estimated as a percentage of the isolated microspore population (Table 7.5) this would increase to 4.5%.

In an effort to improve the efficiency of isolated microspore culture, microspores were isolated from anthers by two sequential isolation steps. As before, microspores were isolated from anthers by mechanically rupturing the anthers (Nitsch, 1974). Approximately

137,000 microspores/ml (19% of the available pollen) were obtained. The process was repeated using fresh medium with the anther debris. This time ca 108,000 microspores/ml (15% of the available pollen) were obtained. Using this method 34% of the pollen was isolated from anthers. The isolation procedure was as gentle as possible to limit damage to the microspores.

Microspores from both extractions were treated as separate samples. They were purified and cultured at approximately equal densities. After incubating the samples for one week, large numbers of multicellular structures were observed in cultures from both extracts. Unfortunately these structures did not develop further. It appeared that microspores, in vitro, were sensitive to light during the first two to three weeks of culture. Further work is necessary to confirm and quantify the apparent sensitivity of isolated microspores to light during the early stages of culture.

7.6. The culture of microspores isolated from unpretreated anthers

In this experiment, microspores were isolated from unpretreated anthers. This proved to be technically difficult since the microspores appeared to be "sticky" and could not easily be separated from the anther debris. For this reason they were cultured with the anther debris. This experiment was unsuccessful since no development of any sort was observed.

7.7. Isolated microspore culture on Ficoll-containing medium.

Microspores were isolated from pretreated anthers and purified as described earlier (Section 7.2). Unfortunately, attempts to layer them on the surface of the meniscus of the culture medium were

unsuccessful since the majority of the pollen tended to sink and, as observed with anther cultures, microspores which sank did not develop further.

7.8 Discussion

The successful development of large numbers of microspore-derived green plants has been achieved via direct embryogenesis from isolated microspores cultured on FHG medium solidified with 0.8% Sea Plaque Agarose. Although the regeneration of green plants from isolated microspore cultures has been reported before (Kohler et al, 1985; Wei et al, 1986), regeneration was via callus and plant yields were low (Table 7.8).

Table 7.8
Comparative efficiency of green plant yields from isolated microspore cultures of Hordeum vulgare, and Brassica napus

Source	Variety	Green plants /100 anthers	Green plants % of isolated microspores	Green plants % of total microspores
Hordeum vulgare				
Kohler et al 1985	Dissa	0.086	5.7×10^{-5}	2.9×10^{-5}
Wei et al 1986	Sabarlis	NA**	3.7×10^{-2}	NA
Hunter This report	Igri	2700	4.5	0.9
Brassica napus				
Swanson et al, 1987 *		5200-13000	up to 4.5	NA

* Embryos not green plants.
** Not available.

Green plant yields reported for barley (Section 7.4) now compare with embryo yields reported from isolated microspore cultures of the highly responsive dicotyledenous species Brassica napus (Swanson et al, 1987).

Although the efficiency of isolated barley microspore culture has been improved, the full potential of the method has probably not yet been realised. A large proportion of the available pollen fails to develop into plants.

In earlier reports the low frequencies of anther and isolated microspore culture response, in all but a few species, have led to suggestions that only a small proportion of the pollen population cultured are capable of embryogenesis (Dale, 1975; Horner et al, 1978; Rashid, 1983). Some authors have proposed that these embryogenic individuals originate exclusively from a particular type of abnormal pollen (eg Rashid et al, 1981a; 1981b, Heberle-Bors et al, 1981). A second possibility is that pollen redifferentiates from gametophytic pollen in response to culture (Sunderland et al 1971) by way of cytoplasm and organelle regression (Dunwell et al, 1974,a,b).

There have been several reports of distinct populations of pollen being present in a single mature anther (Dale, 1975; Horner et al, 1978; Rashid, 1983; Sunderland et al, 1971). This is often referred to as pollen dimorphism. The two populations have been variously referred to as: P-pollen, premitotic pollen (Heberle-Bors et al, 1981), and N-pollen, normal gametophytic pollen (Heberle-Bors et al, 1981); or NS-pollen, non-staining cytoplasm (Dale, 1975) and N-pollen (Dale, 1975); or E grains, embryogenic pollen (Horner et al, 1978)

and N-grains, (Horner et al, 1978).

It has been suggested that, in barley at least, the abnormal pollen is a result of dissociation from the anther wall during development (Idzikowska et al, 1981;1982). The pollen is either starved of nutrition or shielded from developmental controls. Depending upon when the isolation from the pollen wall occurs, the pollen becomes abnormal and dies or becomes embryogenic. Then, by providing a suitable medium it will develop into a plant independent of its origin.

The precise origin of embryogenic pollen remains controversial. Both Dale (1975) and Horner et al, (1978) found a correlation between the number of abnormal pollen grains and the number of embryogenic structures that developed in culture. Evidence that embryogenic pollen originates exclusively from the population of abnormal pollen is largely circumstantial. The observations were made on small sample sizes and with few replicates. This, combined with high variation in the results make these interesting observations which require more investigation. However, a correlation between the size of three-celled embryos and the S-grains was observed (Horner et al, 1978).

By selectively isolating the smaller, abnormal pollen from tobacco anthers and culturing them in vitro, Heberle-Bors et al, (1980) demonstrated that at least a small proportion of abnormal pollen could become multicellular structures.

There is evidence that suggests pollen, other than abnormal pollen, can develop into embryos or tissues. He and Ouyang (1983) showed the presence of dimorphic pollen in old wheat anthers. The maximum

frequency of multiple-celled pollen grains in younger anthers was far greater than the population of abnormal pollen he found in older wheat anthers. The number of embryogenic pollen grains in barley exceeded the number of abnormal microspores (Wilson et al, 1978). More recently, Thurling et al, (1984) reported the total absence of morphologically abnormal pollen in Brassica napus anther cultures, where over 67% of the pollen became multicellular.

The levels of androgenesis observed in barley anther and microspore cultures undertaken in this thesis (Section 5,6 and 7) exceeded the levels of "abnormal" pollen reported for barley (Dale, 1975; Horner et al, 1978). This suggests that morphological characteristics need not be the sole criterion for totipotent pollen.

Solutions to purely practical problems would facilitate further improvements in the levels of dihaploid barley plants produced using this technique. This would support the hypothesis that microspores, other than abnormal ones, could undergo embryogenesis and develop into plants.

Certain factors which currently limit the efficiency of this isolated microspore culture include;

- the efficiency of microspore isolation from the anthers (Section 7.5);

- an apparent pollen density "threshold", below which androgenesis does not occur (Section 7.4) and,

- once androgenesis has been induced; apparent competition between microspore-derived embryos (Section 7.4).

By improving microspore isolation efficiency green plant yields could

be increased upto five-fold.

The apparent competition effect may be similar to the anther density effects observed in anther cultures (Section 6.5). Microspore-derived embryos which remained on the original culture medium either callused or did not develop into plants. This may suggest that either the medium was depleted of nutrients or some byproduct from the cultured microspores was inhibiting further development.

The results reported here describe the successful regeneration of large numbers of green plants from isolated microspores cultured on FHG medium solidified with 0.8% Sea Plaque Agarose. Already green plant yields of 27/anther compare favourably with the most efficient anther culture methods (Table 7.8).

Chapter 8

Morphology of developing androgenic structures

8.1 Introduction

In this chapter, photomicrographs and scanning electron micrographs of androgenic structures from anther cultures and isolated microspore cultures that developed during the course of this study are shown.

8.2. Comparison of androgenic structure morphology with that of barley zygotic embryos at recognised developmental stages.

The stages in barley zygotic embryo development are classified chronologically (Figure 8.2.1, Mericle et al, 1957 as cited by Raghavan, 1976). In this section, the morphologies of androgenic structures from anther cultures (8.2.2-8) and isolated microspore cultures (Section 7.2.) are compared with those of zygotic embryos as illustrated in Figure 8.2.1.

Barley anther cultures were prepared for Scanning electron micrography as described earlier (Section 2.16). Isolated microspore-derived structures were placed onto the surface of fresh FHG medium and light micrographs were obtained using a Leitz M3 dissecting microscope with a phototube attachment.

Anther cultures were examined after 21 days in culture on solid FHG medium (Section 2.11). Androgenic structures of a range of sizes had developed within both lobes of the anthers (Figure 8.2.2). Isolated microspore cultures were examined after 28 days of culture (Figure 8.2.5). A closer examination of androgenic structures from both culture systems revealed a striking resemblance between the morphology of the structures and the reference stages of zygotic embryo development (Mericle et al, 1957 cited by Raghavan, 1976).

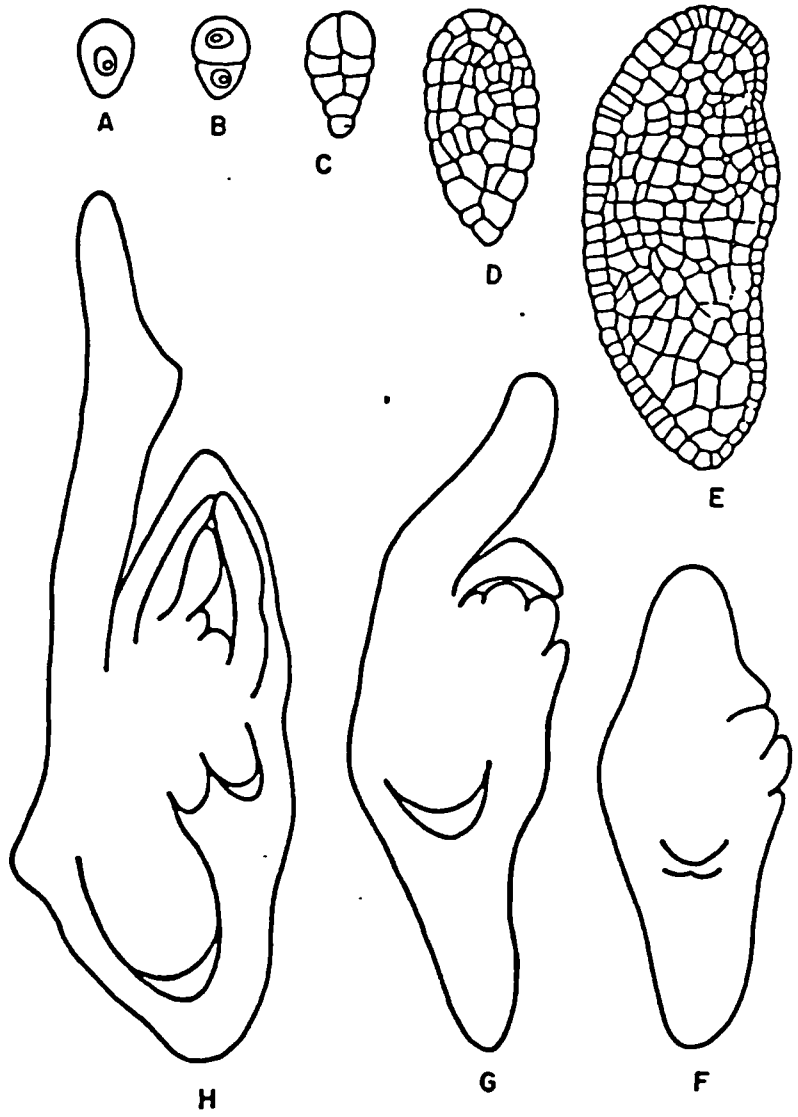


Figure 8.2.1; Developmental stages of barley zygotic embryos designated by their chronological age: A, Zygote; B,C, Early proembryos; D, Mid-proembryos; E, Transition from late proembryo to early differentiating embryo; F, Early differentiation; G, Mid-differentiation; H, Fully differentiated mature embryo. (After Mericle et al, 1957, cited by Raghavan, 1976)

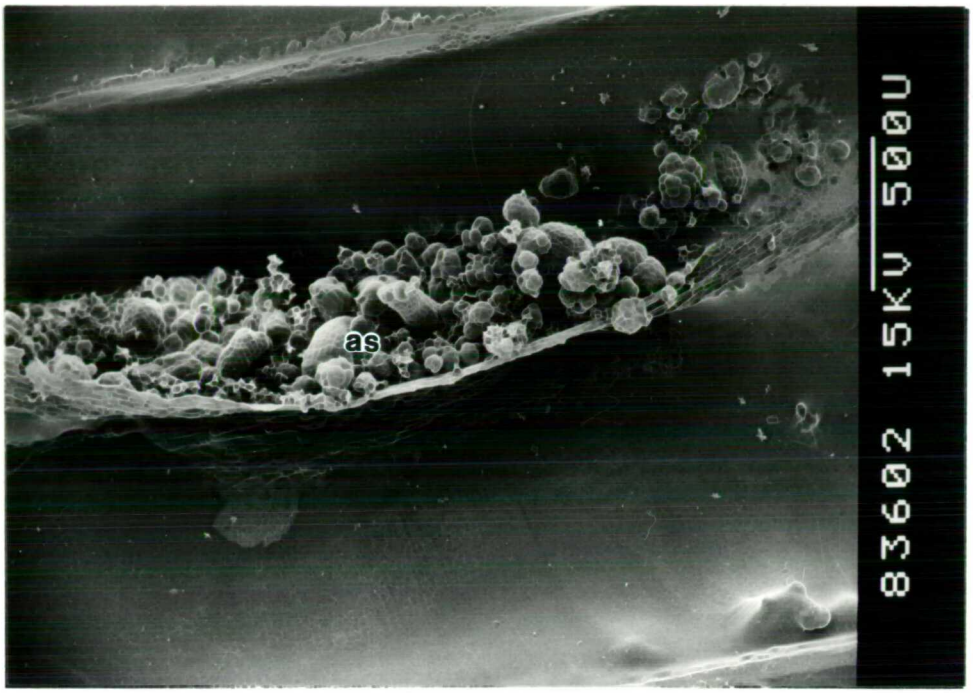


Figure 8.2.2; Androgenic structures of different sizes developing within the ruptured lobe of an anther cultured on solidified FHG medium after 21 days incubation.

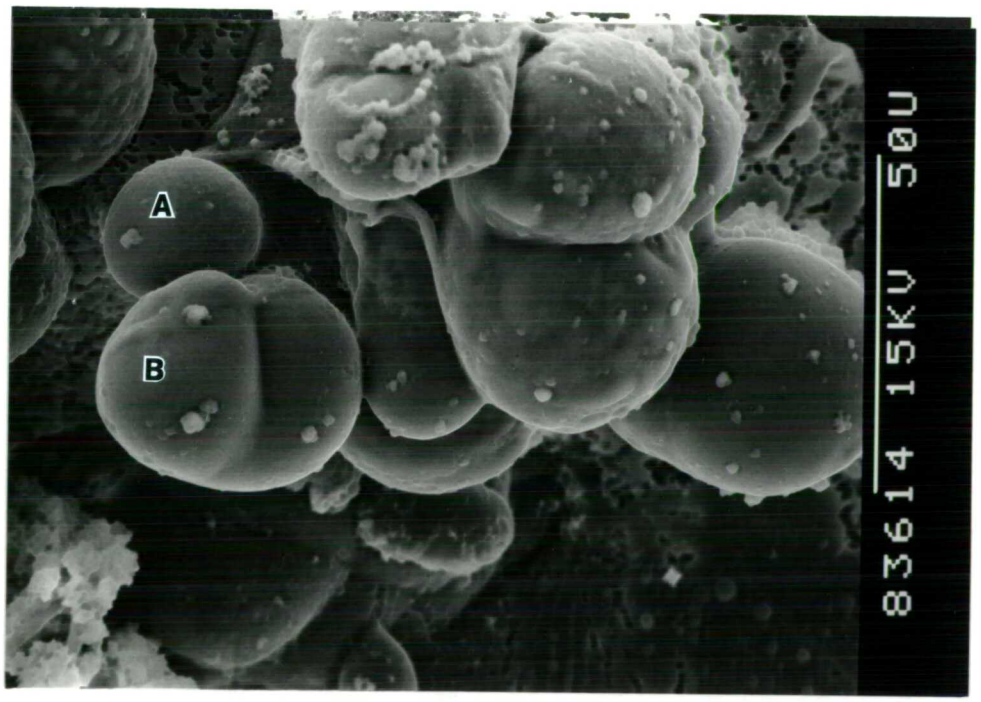


Figure 8.2.3; Androgenic structures corresponding to stages A (zygote) and B (Early proembryo).

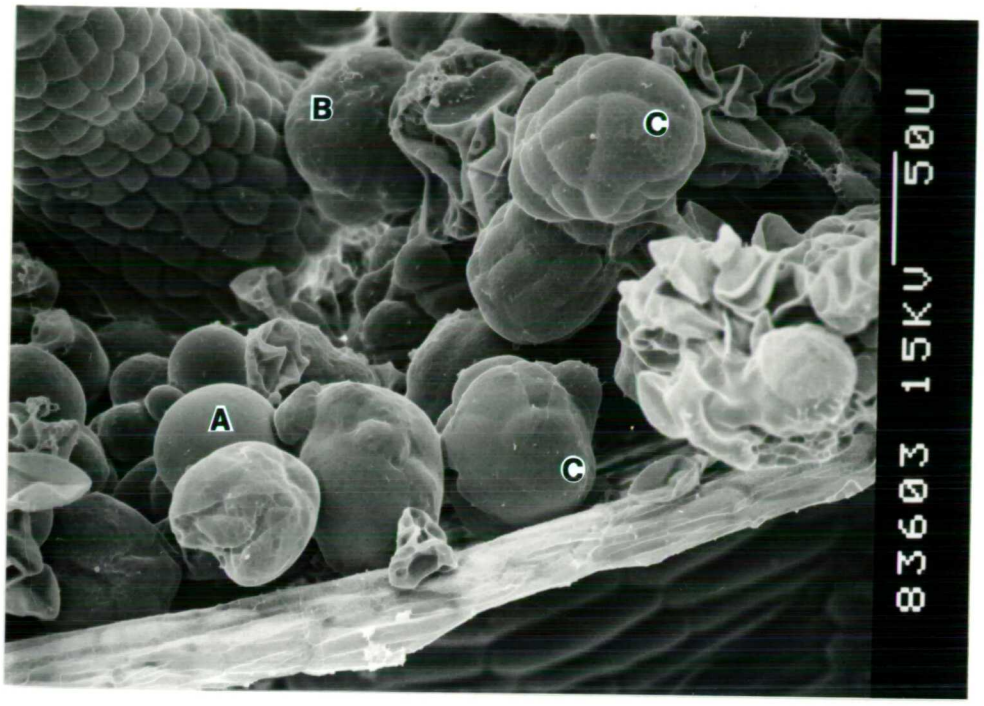


Figure 8.2.4; Androgenic structures equivalent to stages A (Zygote), B,C (Early proembryos), and E (Transition from late proembryo to early differentiating embryo).

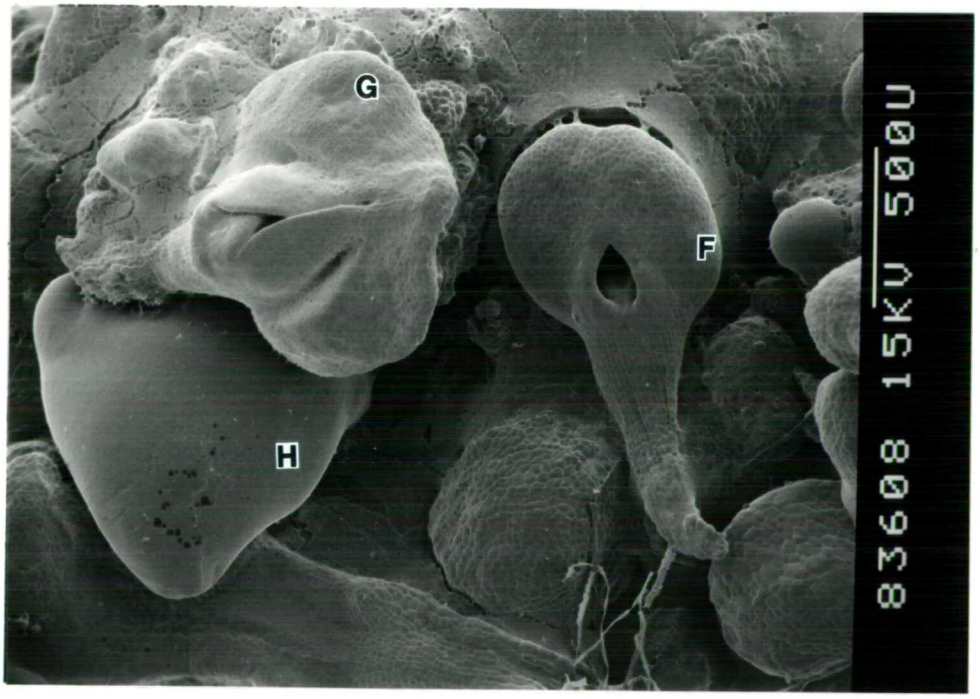


Figure 8.2.5; Androgenic structures corresponding to stages F (Early differentiation) and G (Mid differentiation).

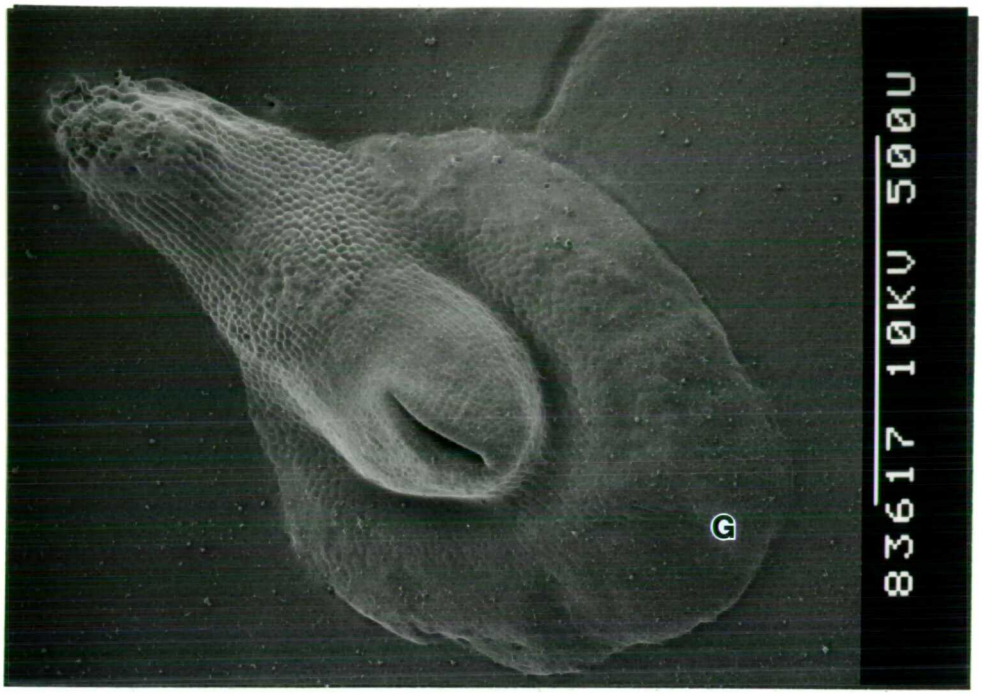


Figure 8.2.6; An androgenic structure equivalent to stage G (Mid differentiation).



Figures 8.2.7; Androgenic structures from isolated microspore cultures after 28 days culture. Corresponding to stages ca C-H (Early proembryo - differentiated embryo).

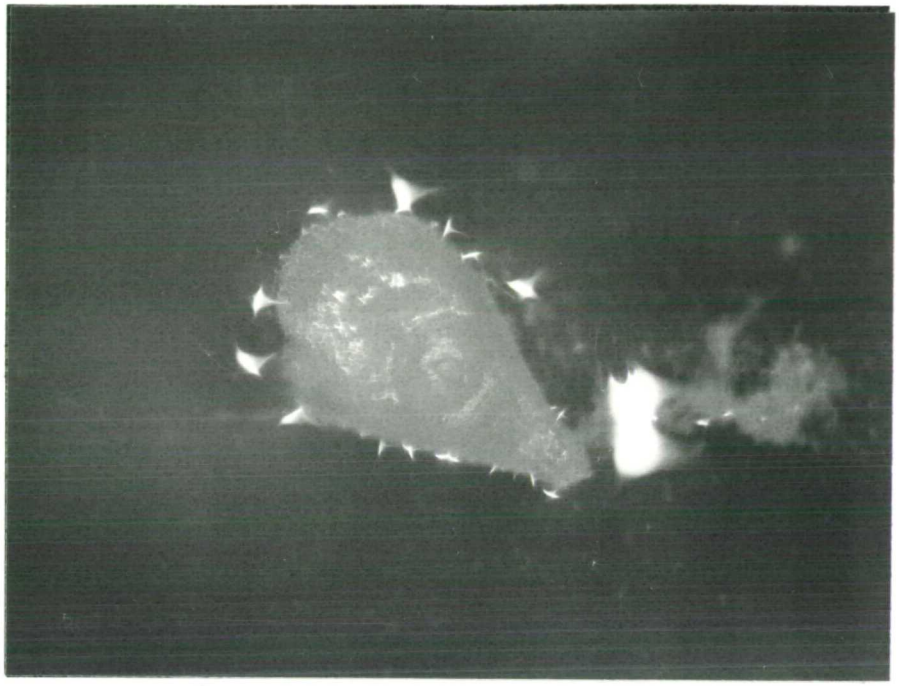


Figure 8.2.8,a; Freshly excised zygotic embryo ca 1.5mm long viewed from the coleoptile.

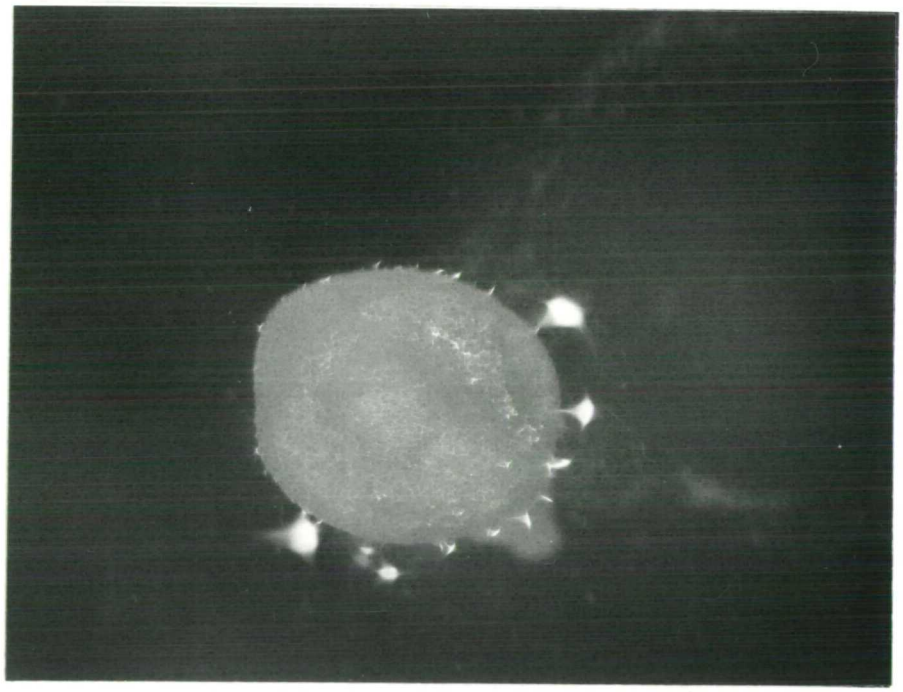


Figure 8.2.8; Freshly excised zygotic embryo ca 1.5mm long viewed from the scutellum.

8.3. Discussion

The pollen of a variety of species (eg. *N. tabacum*; *D. innoxia* and *B. napus*) are known to undergo direct embryogenesis through developmental stages reminiscent of zygotic embryo development to form embryoids and plants (Raghavan, 1986). This has not been observed previously in barley. In earlier reports microspore-derived barley plants had only developed via callus which subsequently regenerated into plantlets by somatic embryogenesis or organogenesis (Raghavan, 1986).

Chapter 9

General Discussion

The world-wide demands for increased crop production continue to outstrip the agricultural industries' ability to fulfil them. Economical, political and environmental pressures are gradually restricting crop management practices so that development of new crop varieties with improved agronomic performance provides the most acceptable and effective method of achieving the escalating targets.

Barley is one of the major crops in the world. It is used primarily for animal feed, but it is also used as food grain and for brewing (Dunwell, 1986,b). In the United Kindom, barley yields have increased from two to five t/ha over the last 40 years (Dunwell, 1986,b). This has been attributed to changes in crop management practices and the use of higher yielding varieties (ie genetic improvement through conventional breeding techniques). The contribution of the genetic component has been assessed to be between 50-73% of the improved yield (Dunwell, 1986,b).

New breeding technologies for the more efficient production of new varieties are emerging, including the production of doubled haploids. There are three techniques for the production of dihaploid barley plants (Section 1). The results obtained in this thesis emphasise the advantages of anther/microspore culture over the other two techniques, ie the "bulbosum method" (Kasha, 1974), and ovary culture (San Noeum, 1976).

At the start of this study the best yields from anther culture were ca five green plants/100 anthers cultured (Huang, 1982; Lyne et al, 1986). When the method of Lyne et al (1986) was repeated a yield of eight green plants/100 anthers cultured was obtained (Section 3.2).

It was recognised that androgenesis only occurred in the upper lobe of barley anthers cultured "on-edge" (Section 3.3) and that by orientating all of the anthers "on-edge" at the beginning of culture it was possible to improve green plant production to 20/100 anthers cultured (Section 3.3). Further studies revealed that neither zygotic nor microspore-derived embryos were able to survive direct contact with the culture medium during the early stages of their development (Section 3.4). In addition it was found that only well-organised embryo-like structures developed into plants.

As a result of these studies it was hypothesised that one or more components of the culture medium were toxic to very young embryos. An investigation of the influence of media components on the development of both microspore-derived and zygotic embryos was carried out to; a) define culture conditions which would encourage direct embryogenesis rather than callus development and; b) to identify any "toxic" component(s) of the culture medium (Section 4).

By replacing sucrose with maltose it was possible to sustain the development of microspore-derived embryos in both lobes of the anther independent of their orientation on solid medium. Thus it was discovered that the levels of sucrose used routinely in culture media were toxic to very young microspore-derived and zygotic embryos of barley.

Sucrose has been used widely as the primary carbohydrate source for the majority of tissue culture systems (Flick et al, 1983; Raghavan, 1986). The successful regeneration of plants from single cells has been limited to a few model species (Flick et

al, 1983), even then many of these respond poorly in conventional tissue culture systems. There are many more that do not respond at all (Evans et al, 1983). From the experience obtained in this study perhaps more attention should be paid to determining the most suitable carbohydrate source to support the type of development desired for each species.

The influence of carbohydrate supply on the morphology and development of plant cells in vitro has been reported before (Tran Thanh Van 1977). The addition of maltose to culture media for both petunia (Petunia hybrida) anther cultures and alfalfa (Medicago sativa) somatic embryo cultures of has been shown to enhance embryogenesis and plant production in both species (Raquin, 1983; Strickland et al, 1987). However, sucrose was not recognised to be inhibitory in cultures of these species. This suggests that the differential influence of sucrose and/or maltose on tissue cultures of these species is, in part at least, due to mechanism(s) other than those observed in barley (Raquin, 1983; Strickland et al, 1987). Sucrose has been reported to inhibit the early stages of development in isolated microspore cultures of tobacco (Wei et al, 1982; Mantell, unpublished results), but any relationship with the barley system remains unclear.

An explanation for the differential effects of maltose and sucrose in barley microspore cultures remains unclear. However, the results from experiments reported in this study support the suggestion that maltose, and its structurally related sugars, may provide a consistent supply of glucose at a low concentration throughout the culture period (Section 5.6).

The use of maltose as the primary carbohydrate source in cultures of barley not only overcame the requirement for anthers to be orientated "on-edge" on solid medium but also facilitated the use of liquid media which supported large numbers of high quality embryos. In addition, isolated microspores (ie essentially isolated from the anther wall) could be cultured successfully. In one experiment, almost 6500 green plants were obtained from 240 anthers (27 green plants/anther) using a very inefficient microspore isolation procedure (Section 7.4). This compared favourably with yields obtained from anther cultures (Section 6.5).

Previously, the in vitro development of dihaploid plants from barley microspores has involved an obligatory callus phase from which plantlets regenerated following organogenesis (San Noeum et al, 1982; Raghavan, 1986). It was anticipated that microspore-derived plants from essentially "true-breeding" parent material would be phenotypically and genetically identical to the parent lines (Evans et al, 1983; Maddock, 1985; Lorz et al, 1986). However, there have been several reports of variation among the progeny of plants regenerated from barley microspores (eg San Noeum et al, 1982; Powell et al, 1984). Such variability has been reported for many species, especially in tissue culture-derived plants regenerated from callus tissues (Lorz et al, 1986). Generally the production of stable homozygous individuals would be preferred.

The morphology of microspore-derived structures which developed in both anther and isolated microspore cultures described in this study ranged from disorganised callus to embryoids which were indistinguisable from zygotic embryos (Section 7). In anther

cultures, callus developed either directly from microspores or indirectly as a result of the dedifferentiation of microspore-derived embryos. But, in isolated microspore cultures, callus appeared to be the result of microspore-derived embryo dedifferentiation alone. In each case, callus inevitably led to tissue death while plants only developed from microspore-derived embryos (Figure 9.1). Since the plants only developed from single microspores via direct embryogenesis they may be expected to be homozygous and, as such, to be true breeding (Snape et al, 1986). To substantiate this, one hundred microspore-derived plantlets of Igri winter barley were grown to maturity in the glasshouse at Shell Research and compared with ten Igri pedigree seed derived plants grown under the same conditions (Section 6.7.4). All plants developed and survived to maturity. Seventy percent of the microspore-derived plants were fertile and appeared to be normal when compared with the pedigree seed-derived Igri plants. The remaining 30 percent were only distinguishable from the seed-derived plants after tiller emergence. These plants were phenotypically haploid. They were infertile, approximately two thirds the size of their fertile siblings and produced many more tillers over a prolonged period.

The high proportion of spontaneous dihaploids occurring was advantageous, since the inconvenience of using chemicals to induce chromosome doubling in haploid individuals was avoided. Chemically induced chromosome doubling would have been time consuming, inefficient, inconsistent, expensive and has been reported to induce mutations in cereal crops (Franke et al, 1952). Thus chemically induced chromosome doubling should be avoided if possible.

Possible pathways by which barley microspores can develop into plants when cultured according to the method developed in this thesis.

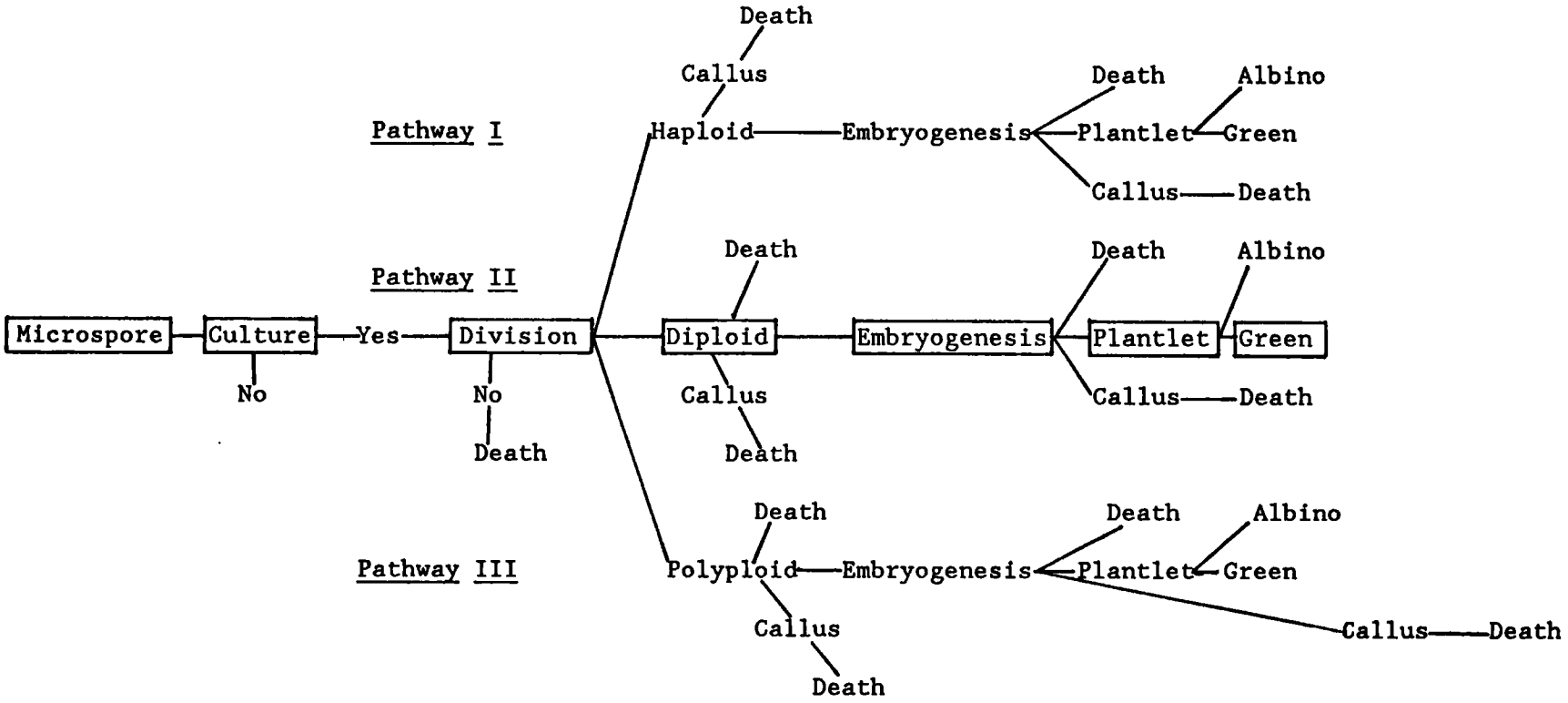


Figure 9.1

Footnote: Graph to illustrate decision points in microspore development and how they influence the type of development. Boxed pathway represents major developmental routes.

Several hundred plantlets of a number of pedigree lines and crosses have been regenerated from microspores using the protocols described in this thesis. They have been grown to maturity and their seed bulked for the assessment of any subsequent phenotypical segregation. To date, the progeny appear to be uniform and essentially true breeding (Dr.P.Gymer, Nickerson Rothwell Plant Breeders, pers. comm.).

The potential impact of this protocol, for the efficient production of large numbers of dihaploid plants in barley breeding programmes, is very significant. In addition, a greater understanding of the events which initiate and control embryogenesis should be possible if the efficient system for barley microspore embryogenesis described in this thesis, is combined with studies at the biochemical and molecular level. It may also facilitate the application of emerging techniques such as genetic engineering (Schell, 1987; Cocking et al, 1987), in vitro mutation (Chaleff, 1983; Sunderland, 1983a; Tyagi et al, 1984) and in vitro selection (Chawla, 1987).

In the past, monocotyledonous species were considered to be recalcitrant in tissue culture. The cellular origin and developmental pathways of plants together with problems associated with reproducibility have limited the applications of tissue culture techniques for monocotyledonous species. However, the work in this thesis, in combination with the increasing number of reports elsewhere (for recent review see; 7th. Int Protoplast Symp. 1987) suggest that monocotyledonous species are not as unresponsive as first thought.

Table 9.1
Examples of monocotyledonous crop species from which plants can be regenerated in vitro

Species	Culture system	Reference
Anther/Microspores		
Barley		This study
Rice		Karim (1985)
Wheat		Armstrong et al (1987)
Protoplasts		
Rice		Toryama et al (1986)
Fescue		Dalton (1987)
Barley	Albino plants only	Luhrs et al (1987)
Wheat	shoots only	Lee et al (1987)
Somatic embryos/ Complex explants		
Millet		Vasil (1982,b)
Wheat		Maddock (1985)
Rye		Nitzsche et al (1977).
Barley		Norstog (1970)
Orchard grass		Gray et al (1984)

The improved tissue culture techniques for cereal crop plants now makes genetic transformation of these species a realistic goal.

Various methods for introduction of foreign genetic material into plant cells have been reported recently (for reviews see Schell, 1987; Cocking et al, 1987). The majority of the reports have been in dicotyledonous plants (eg Solanaceae). However, there have been reports recently of transformation in monocotyledonous species (Table 9.2).

Table 9.2
Examples of transformation in monocotyledonous crop species

Lolium multiflorum	Schell (1987)
Oryza sativa	Baba et al (1986)
Secale cereale	de la Pena et al (1987)

Tissue culture-mediated methods for the transformation of plants may be divided into two groups.

1) Methods for the transformation of intact microspores. For example, microinjection (Spangenberg, 1987; Weber et al, 1987)/ electroporation (Mishra et al, 1987), may begin to compete with Agrobacterium infection (Cocking 1987). Other methods which remain to be tried on intact microspores include laser microinjection (Weber et al, 1987), electroinjection (Tao et al, 1987) and the "shotgun technique" (Bryant, 1987).

2) Methods for the transformation of microspore-derived embryo cells. The above techniques may be applied to whole embryos, single cells isolated from the embryos or protoplasts prepared from microspore-derived embryos.

3) A third method for transformation of cereals has already been employed successfully in the transformation of Secale cereale (de la Pena et al, 1987). This involves the microinjection of a suitable DNA preparation into the immature inflorescences of haploid or dihaploid plants.

The tissue culture system developed in this thesis provides an ideal tool for the transformation of barley. There are initially very large numbers of individual cells. They are initially haploid and a high proportion of them spontaneously double to produce apparently homozygous individuals. Thus transformation of 1% of the pollen

grains from a single isolated microspore preparation (ie 240 anthers) may be expected to produce approximately 60 transformed plants, which could be easily selected *in vitro*.

The improved efficiency characteristics obtained in this study will facilitate the use of barley anther culture and isolated microspore culture for the large scale production of doubled haploid lines of barley and for the *in vitro* study of barley embryogenesis.

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APPENDICES

Appendix 1: Culture media

Appendix 1.1 Basal components

(Component mg/litre)

Basal Medium

	F/G	E	C	H	J
Macroelements					
KNO_3	1900.0	-	-	950.0	470.0
NH_4NO_3	165.0	-	400.0	82.5	-
KH_2PO_4	170.0	910.0	68.0	540.0	-
KCl	-	750.0	-	375.0	700.0
$MgSO_4.7H_2O$	370.0	740.0	246.0	555.0	730.0
$CaCl_2.2H_2O$	440.0	720.0	293.0	580.0	-
$Ca(NO_3)_2.4H_2O$	-	-	-	-	290.0
$NaSO_4$	-	-	-	-	200.0
$NaH_2PO_4 2H_2O$	-	-	-	-	800.0
FeNaEDTA	40.0	-	40.0	20.0	-
Microelements					
$MnSO_4.5H_2O$	22.3	3.0	0.17	12.7	3.0
H_3BO_3	6.2	0.5	6.2	3.4	0.5
$ZnSO_4H_2O$	8.6	0.5	0.28	4.6	0.5
FeCitrate	-	10.0	-	5.0	10.0
$CoCl_2.6H_2O$	0.025	0.025	0.024	0.025	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.25
$NaMoO_4.2H_2O$	0.25	0.025	0.024	0.025	0.25
Organics					
Sucrose	60,000.0	60,000.0	60,000.0	60,000.0	60,000.0
Inositol	100.0	50.0	180.0	75.0	50.0
Thiamine HCl	0.4	0.25	-	0.33	0.25
Ca Pantothenic acid	-	0.25	0.25	0.13	0.25
Pyridoxine HCl	-	0.25	2.5	0.13	0.25
Malatic acid*	-	1000.0	-	500.0	1000.0
NH_4OH	-	+	-	+	+
Alanine**	400/-				

All media used in this thesis were prepared with double distilled water and had a final pH of 5.6. This was achievied by adjusting the pH with 0.1 or 1.0M KOH. Media with the suffix "D" contained 0.4mg/l of each of the plant hormones IAA and BAP. Those with the suffix "C" contained 1.0mg/l IAA and BAP. In later experiments IAA was omitted from the prepation as stated.

Medium E0.01 was the E formulation with an addition of 0.01mg/l ABA. Medium F contaied 4.7mM alanine, whereas in medium G this was omitted.

* Ammonium malate was prepared by dissolving 1.0g of malic acid in 30ml of double distilled water and adjusting the pH to 5.0 with (0.88 specific gravity) ammonium hydroxide.

** The 400mg/l alanine was omitted from FH medium.

Appendix 1.2

Inorganic Media Components
Macroelement Ion Concentration (mM)

Ion	F	E	C	H	J
NO_3^-	21.1	-	5.0	10.43	7.4
NH_4^+	2.1	7.5	5.0	4.8	0.8
K^+	20.3	16.8	4.5	18.4	14.3
PO_4^-	1.3	6.7	0.5	4.0	5.0
$2Cl^-$	6.0	19.9	4.0	13.0	9.4
SO_4^-	1.5	3.0	1.0	2.3	4.5
Mg^+	1.5	3.0	1.0	2.3	3.0
Ca^+	3.0	4.9	2.0	4.0	1.2
Fe^+	0.1	0.03	0.1	0.07	0.03
Na^+	0.3	-	0.2	0.1	6.5

Appendix 1.3

FHG medium

Component	
Macroelements	
KNO_3	1900.0
NH_4NO_3	165.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
$CaCl_2.2H_2O$	440.0
FeNa.EDTA	40.0
Microelements	
$MnSO_4.5H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
$CoCl_2.5H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$NaMoO_4.2H_2O$	0.25
Organics	
Maltose	62,000.0
Inositol	100.0
Thiamine HCl	0.4
Glutamine	730.0
Hormones	
BAP	1.0

All media were prepared with double distilled water and had a final pH of 5.6. This was achievied by adjusting the pH with 0.1 or 1.0M KOH.

Appendix 2

Treatment randomisation

Introduction

Because of the variation between anthers from different spikes and different spikelets within a spike it was necessary to randomise anthers from a spike between the treatments. Anthers from the middle spikelets of each spike were developmentally more advanced than those from spikelets at the extremeties of the spikes. As the efficiency of plant production improved so it became necessary to modify the randomisation of the anthers among the treatments. This was to minimise the effects of anther variability and to maintain managable levels of plant production.

A.2.1 Randomisation for anther reorientation experiment (Section 3.4)

In this experiment there were seven treatment dishes and one blank. Eight spikes were used without replicates. To compensate for the variability in anther material spikes were divided into eight segments, each containing two spikelets (Figure A.2.1). Anthers from each of the segments were allocated to each of the treatments such that each of the treatments and the blank received 48 anthers from each of the spikes and each of the segments.

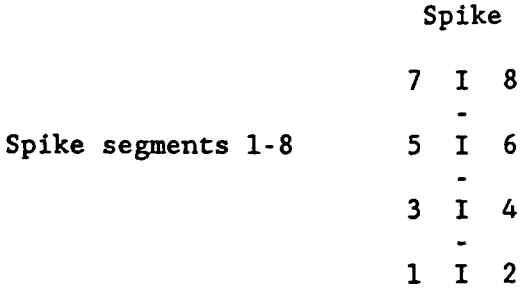


Figure A2.1

Diagram to represent spike segmentation

Anthers were assigned to the treatments according to a 4x4 randomised block design from random number tables (Fisher et al, 1963).

A.2.2. Basal media experiment (Section 4.4)

In this experiment four spikes were used for each of ten replicates. The spikes were divided into four quaters (Figure A.2.2). Anthers were harvested from the three spikelets nearest the middle of each spike in each quater and assigned to a treatment according to a four by four randomised block design (Fisher et al, 1963). Each treatment received a total of 60 anthers with anthers from each of the spike and from each of the spike quaters.

A.2.3. Randomisation for experiment (Section 4.5), the effects of different cytokinin treatments on anther culture response

There were six treatments in this experiment. Six spikes were used for each of five replicates. The experimental design was similar to those described above, but the spikes were split into sixths, rather than eighths or quaters. Each sixth contained five spikelets and, therefore, 15 anthers. Hence each dish received 60 anthers from each of the spikes and segments.

A.2.4. Auxin experiment (Section 4.6)

Anther randomisation as described earlier (Section A.2.3).

A.2.5. IAA concentration experiment (Section 4.7)

Anther randomisation as described earlier (Section A.2.3).

A.2.6 Amino acid treatment experiment (Section 4.8)

Anther randomisation as described earlier (Section A.2.3)

A.2.7. The comparison of sucrose and maltose as primary carbohydrate sources

This experiment was replicated ten times. Two spikes were used per replicate. Twenty four anthers were removed from the central region of the spikes and assigned alternately to the two treatments.

A.2.8. The effect of different sugars on barley anther culture response

Eighteen spikes were used per replicate. Anthers were excised from 18 spikelets per spike starting towards the bottom of the first spike anthers were assigned to treatment A and moving up the spike to each treatment up to R. The same was repeated with the second spike, but this time starting at treatment b and so on until each of the treatments had received anthers from each of the spikes and spikelets 1-18, a total or 54 anthers on 10ml of medium solidified with 0.6% w/v Sea Plaque Agarose per plate. This was repeated for each of the replicates with the treatments randomised according to table A.2.7.

Table A.2.7.

Treatment randomisations for the experiment to evaluate the effects of different sugars on barley anther culture response

Replicate	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	2	5	13	6	1	8	14	4	16	9	11	15	7	17	10	18	3	12
2	10	2	17	11	14	1	6	12	7	15	8	5	16	3	18	13	4	9
3	3	14	1	15	2	13	17	8	11	12	16	9	18	6	4	5	10	7
4	7	4	12	8	11	5	10	15	18	6	3	13	2	14	9	16	17	1
5	9	18	3	13	4	17	12	16	2	14	15	1	8	10	7	11	6	5

A.2.9. The effect of sugar concentration on barley anther culture response.

Because the barley anther culture system had become much more productive it was possible to reduce the number of anthers cultured per treatment. It was, therefore, necessary to devise an alternative

procedure for anther randomisation between the treatments to compensate for variation in anther material. To facilitate this, anthers within each spikelet were identified by number from right to left, I,II,and III. Starting towards the base of the spike and moving up the spike, anthers within each spikelet were dissected in order I,II and then III and allocated to each treatment according to the table A.2.9. Treatments randomisation was after (Fisher et al,1963)

Table A.2.9
Anther randomisation for the experiment to evaluate the effects of sugar concentration barley anther culture response

Treatment											
A	B	C	D	E	F	G	H	I	J	K	L
I_1	II_1	III_1	I_2	II_2	III_2	I_3	II_3	III_3	I_4	II_4	III_4
II_8	III_8,	I_5	II_5	III_5	I_6	II_6	III_6	I_7	II_7	III_7	I_8
III_{11}	I_{12}	II_{12}	III_{12}	I_9	II_9	III_9	I_{10}	II_{10}	III_{10}	I_{11}	II_{11}

This was repeated with a second spike ,
but starting at treatment G and finishing a treatment J.

I_1.....

..... III_{12}

The treatment randomisation for the experiments 5.3.1, 5.3.2, 5.3.3 were after Fisher et al (1963).

A.2.10. The effects of sugar mixtures on barley anther culture response, experiment 5.4.1

An alternative method of anther randomisation was employed for this experiment. Anthers were excised from four spikes per replicate and

allocated to each treatment according to the treratment randomisation (Table A.2.10). The experiment was replicated six times.

Table A.2.10
Treatment randomisation for the first sugar mixture experiment.

Replicate	Treatment							
	A	B	C	D	E	F	G	H
1	7	3	8	1	6	4	2	5
2	1	5	3	7	8	2	4	6
3	2	4	6	8	1	5	3	7
4	4	6	1	5	3	7	8	2
5	5	7	2	6	4	3	1	8
6	6	1	4	2	5	8	7	3

Starting towards the base of the first spike anthers were removed from the first suitable spikelet and allocated to plate A and so on up the spike until each treatment had recieved six anthers. This was repeated with the second, third and fourth spikes, but starting at plates C, E and G respectively.

Sugar mixture experiment 2 (Section 5.4.2).

There were 12 treatments in the second experiment in this section, these were replicated five times. Anthers were excised from a single spike for each replicate so that each treatment received six anthers randomised (randomisation table A.2.9). Treatment randomisation was after Fishert et al (1963).

A.2.11. Anther randomisation for radiolabelled sugar washout experiment (Section 5.5.1).

Anthers were randomised between the four treatments in a similar manner to that described earlier (A.2.8.).

A.2.12. Anther randomisation for experiment 5.5.2

Anthers were randomised between four treatments and eight time points (Table 5.5.2) using a similar randomisation to A.2.8, but this time designed for 24 treatments.

A.2.13. Anther randomisation for $^{14}CO_2$ evolution experiment (Section 5.5.4).

Anthers were excised from four pretreated spikes and randonised between the four treatments in a simialr fashion to that described above (A.2.8.).

Figure A.3. Results from preliminary experiments investigating the effect of basal media on anther culture.

Figure A.3.1. ANTHER RESPONSE

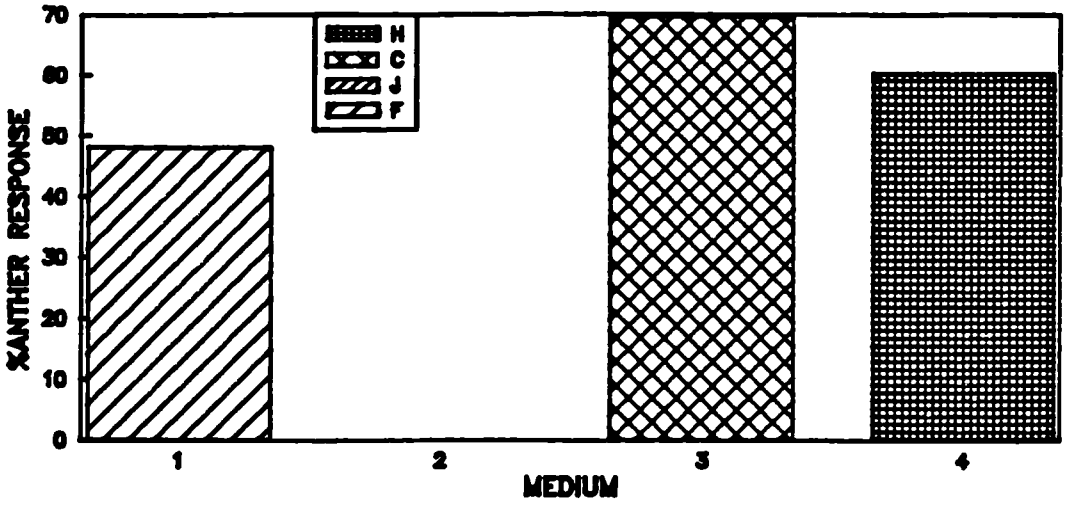


Figure A.3.2. GREEN PLANTS

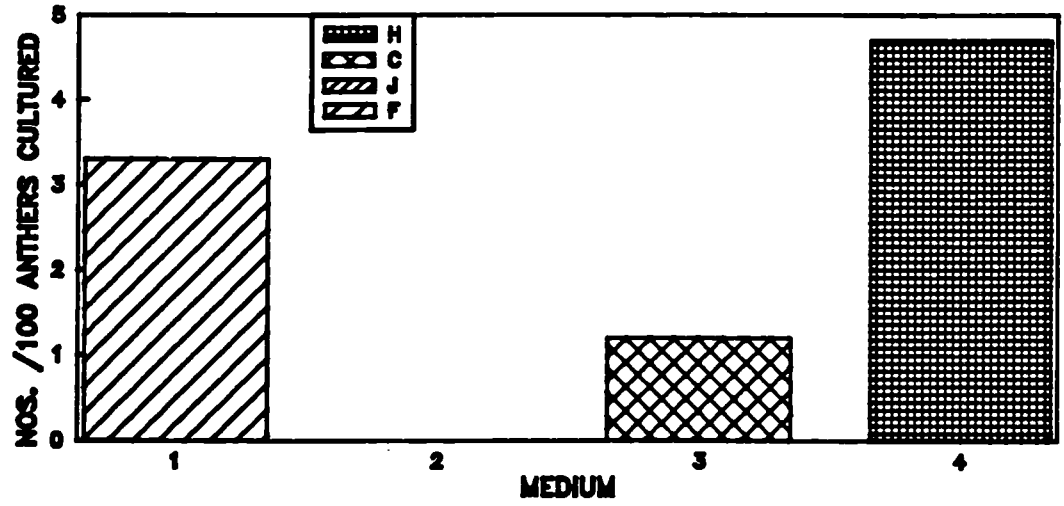
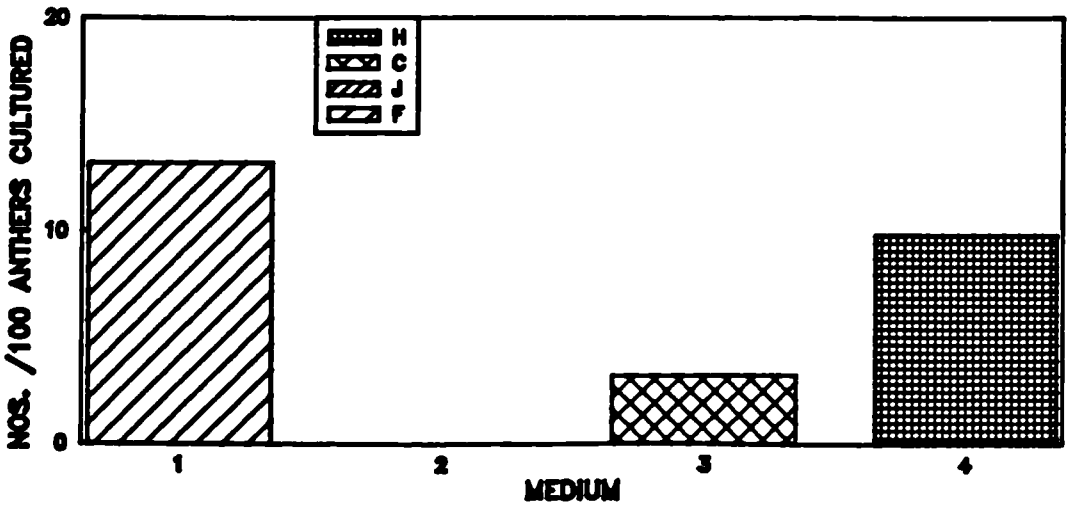


Figure A.3.3. TOTAL PLANTS



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The effect of anther orientation on the production of microspore-derived embryoids and plants of *Hordeum vulgare* cv. Sabarlis

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Abstract

The orientation of barley anthers on culture medium had a marked effect on their response. Embryoids developed only in the upper lobes of anthers cultured on edge, i.e. with a single lobe in contact with the culture medium. Anthers cultured flat, i.e. with both lobes in contact with the medium, did not respond. Careful orientation of anthers at the start of incubation resulted in a mean yield of 20 green plants per 100 anthers cultured.

Introduction

Techniques for the regeneration of plants from microspores of barley via anther culture are well established in the literature (for review see Dunwell, in press). However, current methods are very inefficient when compared with the alternative bulbosum method of producing doubled haploids (Huang et al. 1984). The efficiency of anther culture needs to be improved substantially before the potential of the technique as a breeding tool can be properly evaluated.

A research programme on direct embryogenesis from barley anther culture has been undertaken in this laboratory (Lyne et al, in press). During the course of this work it was observed that orientation of the anthers on the culture medium affected their response. The results of experiments designed to quantify this effect are reported here.

Materials and Methods

Seed of Barley (Hordeum vulgare L.) variety Sabarlis, was kindly donated by Dr. N.Sunderland, John Innes Institute, Norwich, U.K. Plants were grown in controlled environment cabinets (12 °C; 350μ $E.m.^{-2}$ s^{-1} at top of pot; 16h day; 60–80% RH). Spikes containing anthers at the mid-uninucleate stage were selected and pretreated for 28 days at 4 °C as described by Huang and Sunderland (1982). At the end of the pretreatment microspore viability was assessed by acetocarmine staining and spikes were discarded if the viability was less than 40%. Anthers were cultured in 5 cm Petri dishes containing 10 ml of the callus induction medium of Foroughi-Wehr et al. (1976) solidified with 0.5% Sea Plaque agarose. Cultures were incubated in the dark at 25 °C. After 28 days, the anthers bearing callus and/or embryoids were counted (= number of responding anthers). The cultures were then transferred to continuous light. Any callus or embryoid larger than 2mm, that was observed during the next 4 weeks was transferred to regeneration medium (Foroughi-Wehr et al. 1976) solidified with 0.5% Sea Plaque agarose. The numbers of green and albino plants that developed from these were recorded.

Results and Discussion

Embryoid development was only observed in the upper lobes of anthers placed on edge, i.e. with a single lobe in contact with the culture medium (Figure 1). No development was seen in the lower lobe. When anthers were lying flat, i.e. with both lobes in contact with the medium, the microspores were generally shed onto the medium and did not develop further (Figure 2).

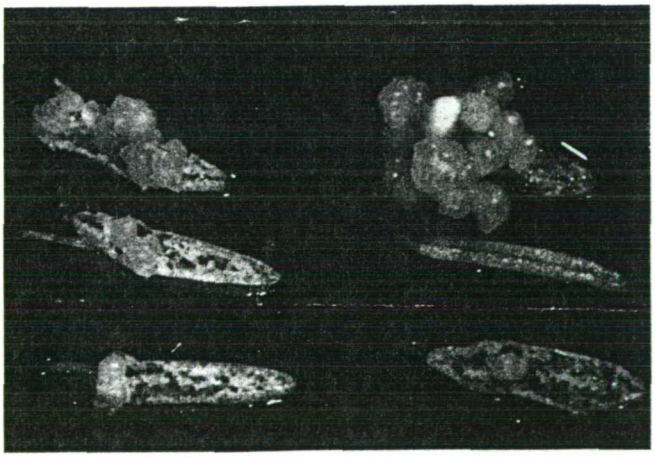


Figure 1. Embryoid development only observed in the upper lobes of anthers placed on edge, i.e. with a single lobe in contact with the culture medium.

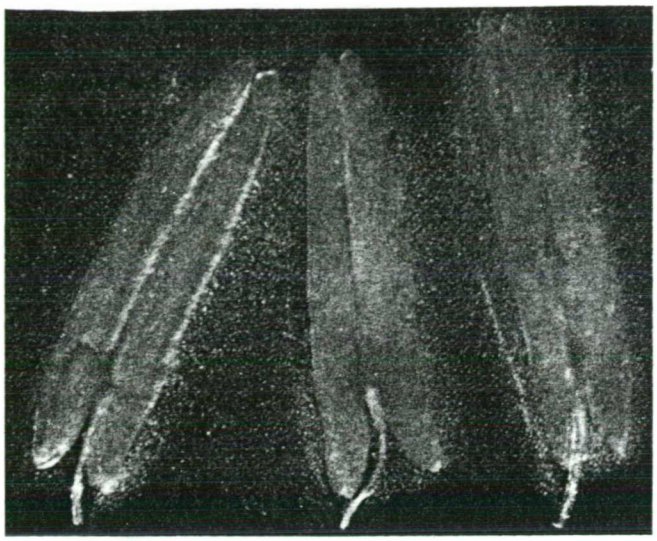


Figure 2. Anthers lying flat, i.e. with both lobes in contact with the medium generally shed microspores onto the medium. These did not develop further.

Table 1. Effect of orientation on anther response and plant regeneration.

	Experiment 1.		Experiment 2.	
Orientation after 28 days culture	On edge	Flat	On edge	Flat
Number of anthers	99	252	553	230
Anthers responding	60	1	480	0
Plants regenerated				
- green	19	0	160	0
- albino	23	0	408	0
Plants regenerated per 100 anthers responding				
- green	32	0	33	-
- albino	38	0	85	-

Experiment 1. Anthers from 5 spikes, random orientation.

Experiment 2. Anthers from 12 spikes, on edge orientation.

Table 1 shows results from two experiments carried out to examine the relationship between anther orientation and plant regeneration. In the first experiment anther orientation was random, while in the second experiment anthers were placed on edge. As some anthers placed on edge fell into the flat position, orientation was checked after 28 days incubation. The proportions of anthers remaining on edge were 28% and 71% respectively. The data show clearly that little or no response was obtained from anthers lying flat on the medium. However, a high proportion of anthers lying on edge responded, producing green and albino plants.

Mean yields of 5 and 20 green plants per 100 anthers cultured were obtained in experiments 1 and 2 respectively. Most of this difference is attributable to the higher proportion of anthers remaining on edge, and therefore capable of response, in the second experiment. Anthers that did respond produced plants at a similar frequency in both experiments. This emphasises the importance of maximising the numbers of anthers responding when seeking to improve the overall yields from anther culture.

Effects of anther orientation on response in culture have been reported previously for Datura (Sopory and Maheshwari, 1976) and Nicotiana (Misoo et al. 1981). Anthers of Datura responded better cultured flat than on edge, in contrast to our results with barley. The experiments with tobacco indicated that anthers responded best when cultured vertically, with one end partially embedded in the medium. Thus it appears that orientation is an important factor in determining the efficiency of plant regeneration from cultured anthers, although the preferred orientation varies with the species.

Acknowledgements

This paper will form part of a thesis to be submitted for a postgraduate degree to the University of London, School of Agriculture, Wye College, Kent.

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EMBRYOID AND PLANT PRODUCTION FROM CULTURED BARLEY ANTHERS

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Introduction

Much effort has been put into the development of anther culture techniques for barley. However, despite considerable progress, current techniques lack the required efficiency, in terms of green plant production and/or genotype insensitivity, to allow their proper evaluation as an aid to plant breeding. Our aim is to seek further improvements in all aspects of the process, from mother plant growth to media and conditions used during culture. The ultimate objective is to provide breeders with a practical tool applicable to a wide range of genotypes.

Two groups have been in the forefront of research in this field in the last decade. Sunderland and co-workers have demonstrated the importance of spike pretreatment prior to anther culture (Huang and Sunderland, 1982) and have done much fundamental work on the early stages of microspore morphogenesis (for review *see* Sunderland, 1982). The group headed by Foroughi-Wehr has concentrated on the identification of responsive genotypes (Foroughi-Wehr, Friedt and Wenzel, 1982) and application of the techniques to practical ends (Foroughi-Wehr, Friedt and Wenzel, 1983; Foroughi-Wehr and Friedt, 1984). Elements from the methods of the above groups have been incorporated into the present study in order to identify optimal growing conditions, spike pretreatments and culture media.

Experimental material

PLANT GROWTH

Seed of barley (*Hordeum vulgare* 'Sabarlis') was obtained from Dr N. Sunderland, John Innes Institute, Norwich, UK and was multiplied in the glasshouse. Plants were grown singly in 5 inch pots in general compost in controlled environment cabinets at 12 °C or 15 °C under a 16 h photoperiod of 350 $\mu E\ m^{-2}\ s^{-1}$ (as measured at top of pot) and 60–80% relative humidity. Plants were fed weekly with a liquid fertilizer solution.

SPIKE PRETREATMENT AND ANTHER CULTURE

Spikes containing anthers at the mid-uninucleate stage were selected and pretreated at 4 °C for 28 days, as described by Huang and Sunderland (1982). Two-compartment Petri dishes were used to separate the spike from the water added to maintain humidity. At the end of the pretreatment microspore viability was assessed by acetocarmine staining and spikes were discarded if their viability was less than 40%. Anthers were cultured on 10 ml solidified medium in 5 cm diameter Petri dishes. To prevent dessication, four dishes, plus one containing water, were placed inside a 14 cm Petri dish which was sealed with 'Nescofilm' (Jencons (Scientific) Ltd). Cultures were incubated at 25 °C in the dark. After 28 days, the proportion of responding anthers was assessed and the cultures were transferred to continuous illumination (70 $\mu E\, m^{-2}\, s^{-1}$).

PLANT REGENERATION

Calli and embryoids (for convenience called structures subsequently) of approximately 2 mm in diameter were transferred to regeneration medium (Foroughi-Wehr *et al.*, 1976) solidified with 0.8% Sea Plaque or 0.5% Type VII agarose and incubated at 20 °C in the light (70 $\mu E\, m^{-2}\, s^{-1}$). Green plants were transferred to hormone-free Murashige and Skoog (1962) medium for rooting, prior to establishment in Jiffy 7 peat blocks.

Comparison of media and gelling agents

Anthers from plants grown at 15 °C were inoculated onto four media solidified with four different gelling agents (used at 0.8%) as detailed below:

Media: K Klc from Kao (1981), without Ficoll.
P-9 Chinese potato medium (Chuang *et al.*, 1978) as used by Xu and Sunderland (1981).
N-9 Chinese defined medium (Chu, 1978) as used by Xu and Sunderland (1981).
FC Callus induction medium of Foroughi-Wehr *et al.* (1976).

Gelling agents: Difco-Bacto agar
Sigma Type VII agarose
Sea Plaque agarose
Sigma Type I agarose.

In a split-plot experiment, the media formed the main plots and the gelling agents the sub-plots, making a total of 16 treatments. Five replicates were set up, using 80 spikes in all. To minimize variation between spikes, and within spikes, the treatments were fully randomized. Groups of four spikes were allocated to each plot. On each spike anthers were divided into four groups of 12, (top left, top right, bottom left, bottom right). Each treatment received 12 anthers from each of four spikes, making a total of 48 anthers per treatment.

The percentages of anthers producing structures after 28 days of incubation are shown in *Table 37.1*. The highest response was on medium P-9 which was

Table 37.1 PERCENTAGE OF BARLEY ANTHERS PRODUCING STRUCTURES AFTER 28 DAYS CULTURE

Gelling agent	*Medium*				*Gelling agent* (means)
	K	*P-9*	*N-9*	*FC*	
Difco-Bacto agar	12	73	54	29	42
Type VII agarose	13	69	59	21	40
Sea Plaque agarose	10	79	56	27	43
Type I agarose	17	79	48	31	44
Medium (means)	13	75	54	27	

Table 37.2 NUMBERS OF STRUCTURES TRANSFERRED TO REGENERATION MEDIUM PER SPIKE (48 ANTHERS)

Gelling agent	*Medium*				*Gelling agent* (means)
	K	*P-9*	*N-9*	*FC*	
Difco-Bacto agar	14	22	32	28	24
Type VII agarose	12	15	29	29	21
Sea Plaque agarose	5	16	23	38	21
Type I agarose	11	17	28	28	21
Medium (means)	11	18	28	31	

Table 37.3 NUMBERS OF GREEN PLANTS PER SPIKE (48 ANTHERS)

Gelling agent	*Medium*				*Gelling agent* (means)
	K	*P-9*	*N-9*	*FC*	
Difco-Bacto agar	0.0	0.0	0.2	1.0	0.3
Type VII agarose	0.2	0.0	0.4	0.6	0.3
Sea Plaque agarose	0.0	0.0	0.6	2.0	0.65
Type I agarose	0.0	0.0	0.0	0.2	0.05
Medium (means)	0.05	0.0	0.3	0.95	

Table 37.4 NUMBERS OF ALBINO PLANTS PER SPIKE (48 ANTHERS)

Gelling agent	*Medium*				*Gelling agent* (means)
	K	*P-9*	*N-9*	*FC*	
Difco-Bacto agar	0.2	1.0	1.6	5.2	2.0
Type VII agarose	0.2	0.8	3.8	3.6	2.1
Sea Plaque agarose	0.0	0.6	3.6	4.4	2.15
Type I agarose	0.6	0.6	2.0	7.4	2.65
Medium (means)	0.25	0.75	2.75	5.15	

significantly better (at the 5% level) than N-9, which in turn was significantly better than medium FC or K. The nature of the gelling agent had no effect.

The ability of the treatments to support further development is indicated by the numbers of structures transferred to regeneration medium (*Table 37.2*). Media N-9 and FC produced more than P-9, and significantly more than K. Again, there was no effect of gelling agent.

Data for green plant production are shown in *Table 37.3*. Media FC and N-9 were clearly superior to P-9 and K. The greatest number of green plants was obtained on medium FC; statistical analysis showed that the difference between FC and N-9 verged on significance at the 5% level. An effect of gelling agent is also apparent. Sea Plaque agarose gave the best result, and was significantly better than Type I agarose. Other differences were not significant.

The data for albino plant production (*Table 37.4*) confirm the superiority of media FC and N-9 for plant regeneration. The difference between FC and N-9 was not significant, nor was there any effect of gelling agent. Overall in this experiment albinos exceeded green plants by a factor of almost seven.

Comparison of plant growth temperatures

Using plants grown at 12 °C and 15 °C, 60 anthers from each spike were cultured initially on medium FC solidified with Sigma type VII agarose (0.5%). Spikes from plants grown at the lower temperature survived the cold pretreatment better, as indicated by the higher proportion that passed the 40% viability test (*Table 37.5*). This may be related to the more robust stature of the plants, which was reflected in their larger anthers. Green plant production was higher from the 12 °C grown apparent effect of temperature was not significant. However, the experimental design was less than ideal and further confirmatory work is needed.

Table 37.5 THE EFFECT OF PLANT GROWTH TEMPERATURE ON ANTHER CULTURE OF BARLEY (60 ANTHERS CULTURED PER SPIKE)

	Growth temperature	
	12 °C	*15 °C*
No. of spikes pretreated at 4 °C	24	48
No. of spikes with pollen viability >40%	19	28
No. of green plants per spike	5.4	1.9
No. of albino plants per spike	8.5	5.2

A wide variety of structural types developed in culture, ranging from unstructured callus to highly organized structures closely resembling zygotic embryos. Scanning electron microscopy confirmed this similarity, with clear differentiation of root and shoot axes (*Figures 37.1* and *37.2*).

In our experience, embryoid formation is closely correlated with plant regeneration. Plants were only obtained from well organized structures and we did not observe regeneration from callus. However, this statement hides the genuine difficulty of classifying the continuum of structural types obtained. Less than half of the embryoid structures produced plants in our experiments.

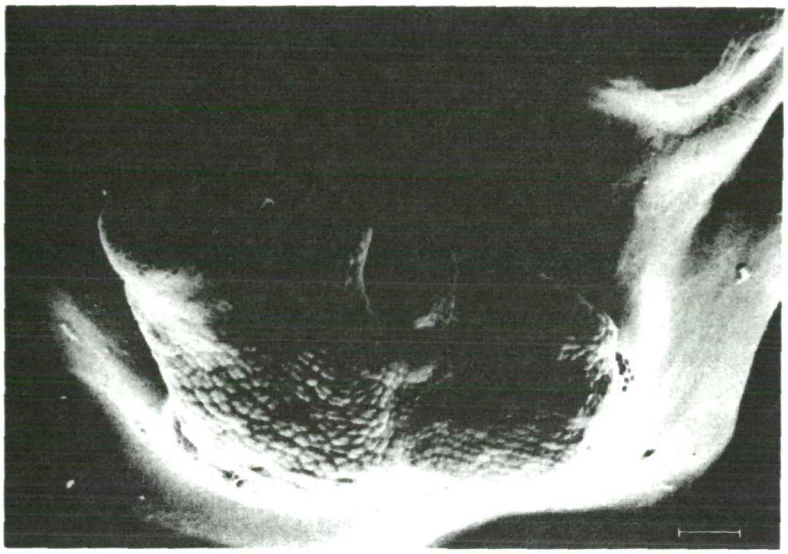


Figure 37.1 Scanning electron micrograph (SEM) of an embryoid after 20 days in culture. The tissue was prepared by setting in Tissue-Tek adhesive (Raymond Lamb Ltd, London), freezing in liquid nitrogen and sputter coating with gold before examining at 30 kV in a Philips 510B SEM. (Bar = 75 μm).



Figure 37.2 SEM of an embryoid 28 days after culture initiation, showing clear differentiation of root and shoot axes. (Bar = 150 μm).

Cytological analysis of 20 regenerant plants revealed 18 to be diploid and two to be haploid. A predominance of diploids is in agreement with previous work on barley (Huang and Sunderland, 1982).

Discussion

The four media varied in their effectiveness for the different stages of culture, medium P-9 giving the best initial response, but media FC and N-9 promoting the formation of much larger numbers of embryoids able to regenerate into plants. The observation that the type of gelling agent does not affect the early stages but does influence the yield of green plants, contrasts with protoplast culture, where the nature of the gelling agent is at its most critical during the initial culture phase (Lörz *et al.*, 1983; Shillito, Paszkowski and Potrykus, 1983). Overall, the best combination of those tested was the medium FC of Foroughi-Wehr *et al.* (1976), solidified with Sea Plaque agarose. The observation that growth of donor plants at low temperatures improves anther response is in agreement with a previous report (Foroughi-Wehr and Mix, 1979). Whether the effect is caused by a change in nutrient status or developmental pattern of the anthers is unknown. Further work will be carried out to confirm and extend this result.

To the authors' knowledge, the embryogenic development obtained here has not been reported previously for reasons which are unclear. The combination of cold pretreatment and FC medium solidified with agarose is probably new, but embryoids were also obtained on N-9 medium, on agar-solidified media, and occasionally in N-9 liquid medium, in an earlier, unpublished study. Significantly, the discovery of an embryogenic route of development holds promise for the

(1984).

In conclusion, we have defined conditions that lead to embryogenesis in anther cultures of the barley variety 'Sabarlis'. An average of over five green plants per spike (60 anthers) or nine green plants per 100 anthers has been obtained from plants grown at 12 °C. Work is in progress to improve the efficiency of these techniques further and to assess the response of other barley cultivars.

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