ASPECTS OF ANTHER AND TISSUE CULTURE IN *HELIANTHHUS ANNUUS* L.

by

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DEDICATION

I would like to dedicate this thesis to my wife

Mumtaz Shaukria

DECLARATION

This is to declare that this thesis has been carried out by myself and has not been accepted for any previous application for a degree. All information and assistance obtained from other sources has been acknowledged in the appropriate places.

Manzoor Ali Akhtar

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ABSTRACT

The experiments undertaken in this study investigated aspects of media and/or other requirements for the establishment of direct embryogenesis from anthers and for shoot organogenesis from callus derived from hypocotyl explants.

Media modifications for anther culture included supplementation with NAA, IBA, IAA, 2, 4-D and BAP. Other treatments examined included complex additive supplementation with coconut water, altered carbon source and the value of pre-culture cold shock on the incidence of embryogenesis and subsequent development on culture. No media additive induced direct embryogenesis. However, each of the treatments affected subsequent development of callus, leading to shoot organogenesis. Cultivar 'Holiday' proved to be a consistent responsive genotype with respect to shoot organogenesis on primary callus. Although considerable numbers of shoots were produced, difficulties were experienced in transferring these to *in vivo* conditions. Those that made the transition were found to be abnormal, consisting of a mixture of haploid and diploid cell lines.

Hypocotyl culture was investigated with a view to establishing a protocol for the initiation of shoots/roots from callus material. Experiments involved media supplementation with NAA, BAP GA_3 and casamino acids. Clear trends for the development of a suitable protocol were difficult to establish. BAP and casamino acids both appeared to have positive effects on shoot organogenesis, but there was no advantage to joint inclusion in the media. The shoots produced in these treatments were, with difficulty transferred to *in vivo* conditions. All were stunted and flowered early.

The value of these two breeding approaches, exploitation of the gametophyte and creation of variability via the callus phase is discussed for the improvement of sunflowers (*H. annuus* L.).

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SECTION 1

GENERAL INTRODUCTION

Pakistan is faced with a serious shortage of edible oil because domestic production is sufficient to meet only 20% of the demand for the 106 million population (Pakistan Economic Survey, 1984-85). Thus, the country is constrained to import edible oil in large quantities involving very large expenditure in foreign exchange. It is a matter of great national concern that the import of edible oil continues to decrease. Given growth in consumption, it is expected that 2.8 million tons will be required by 1994 (Pakistan Economic Survey, 1984-85). If the current international price trend continues, the edible oil import bill of Pakistan would increase to about 2.9 billion US dollars and cast a destructive effect on the overall economy of Pakistan. It is, therefore, imperative for Pakistan to take appropriate measures to improve the domestic production of edible oil and simultaneously reduce import dependency.

The two types of oil seed crops sown in Pakistan could be described as traditional species and newly introduced species. Traditional oil seed crops include rape, mustard, sesame, castor, groundnut and linseed, whereas newly introduced oil seed crops are soybean, safflower, sunflower and jojoba. Amongst these, the newly introduced oil seed crop sunflower (Helianthus annuus L.) appears to have tremendous potential. In Pakistan it is a short duration crop (90-100 days) and can be grown twice a year without interfering with the production requirements of the major crops such as cotton and wheat. Sunflower is drought tolerant and can be successfully cultivated in rainfed areas. As a cash crop it can yield additional income in irrigated areas. Helianthus grows well within a temperature range of

20°c-25°c although environment tests indicate that 27°c to 28°c would appear to be optimum (English et al., 1979). Seed is rich in oil (35-50 percent) and can produce a high quantity of oil per unit area (Popova, 1977). The average constitution of oil from temperate crops is 55-60% linoleic acid and 25-30% oleic acid, but seeds produced under high temperature regimes may contain 65% oleic acid and 20% linoleic acid. The decorticated Helianthus seed is rich in digestible protein and free from toxic elements. The protein content may vary but is usually between 15-20 percent. Oil from the seeds can easily be extracted using available equipment so that no major adjustment would be needed in industrial processing. As an additional bonus sunflower can produce large quantities of pollen when in full bloom and as such the species is good for apicultural development.

As a new crop the major problem constraining its production and development is the non-availability of good quality seed of suitable hybrids and open-pollinated varieties. The relatively recent introduction of hybrid Helianthus varieties has improved the profitability and long term prospects for the crop. Hybrid sunflower has important advantages over open-pollinated varieties. It produces very high seed yield, has increasing seed oil content, flowers over a short period and has a short, sturdy stem with a single head. These characteristics increased the amount of mechanisation possible and improved efficiency. Many hybrids are also resistant to insect pests and diseases. Additional benefits of modern hybrids include the trend towards self-compatibility, (Weiss, 1980), thus reducing the dependency on insects especially on bees, for pollination. This, in turn, facilitates the use of chemical control of insect pests and disease without the risk of low yield from killing pollinating insects while plants are flowering.

The production of Helianthus hybrid seed, although somewhat tedious, is not difficult. The hybrids grown at present in Pakistan are imported. This is very expensive and quality may deteriorate in transition, so it is advisable in the long term, to develop indigenous hybrid varieties suited to the requirements of Pakistan agriculture. The production of hybrid seed of Helianthus requires as a first step the development of inbred lines. Production of inbred lines in Helianthus by conventional procedures may take up to 5-6 seasons. Once produced, inbred lines require progeny testing to indicate potential as parental lines of a hybrid variety. Any mechanism which allows the speeding up of this process would be beneficial. Anther or pollen culture and ovule culture have been recommended by workers in many crops as a potentially rapid system to produce haploid plants which in turn could be encouraged by suitable chemical treatment to become homozygous true breeding lines. It is the aim of this project to investigate the potential for the production of haploids in Helianthus and in particular to investigate the physical and chemical environmental requirements necessary for the development of successful haploids.

SECTION 2

LITERATURE REVIEW

Plant breeding technology has developed considerably over the last 80-90 years. A common feature of these changes in technology has been the move towards increasing the efficiency by which new varieties can be created. *In vitro* culture and the yet newer technologies of gene transfer and RFLP (Restriction Fragment Length Polymorphim) analysis offer significant potential for continuing the progress of plant breeding. The aim of this Review is to examine the potential for and advantages of varying approaches to *in vitro* culture for the production of haploids with particular reference to *Helianthus annuus* L.

2.1 VALUE OF HAPLOID PRODUCTION IN PLANT BREEDING/GENETICS

For many crop species, the trend over the past decades has been production of more or less precisely defined varieties (Simmonds, 1979, Fehr. 1987). These may be inbred lines, based on homozygous true breeding genotypes or F1 hybrid varieties which are highly heterozygous but homogeneous as the product of a deliberate cross between two inbred (homozygous) parental lines. Thus, for many species there is a desire to produce homozygous lines, either for use directly as varieties or indirectly as parents of varieties. In the past the production of inbred lines was largely dependent on the breeding system of the crop species in question. With species which normally self-pollinate, there is no particular difficulty in producing inbred lines, but the time-scale to be successful can be considerable. Cross pollinating species, many of which rely on sophisticated mechanisms to ensure outbreeding, present great difficulties and in addition may also

require many generations. For both these groups exploitation of halpoid plants can be extremely beneficial. The gametophyte generation can represent an array of possible genotypes (provided the parental source is heterozygous). If this generation can be encouraged to develop into haploid organisms or if haploids can be produced by exploiting other forms of *in vitro* culture, particularly callus culture, or via other systems such as genetic mismatching (see below), then this offers huge potential to speed up breeding programmes. Haploid plants can be treated by physical and chemical means (in particular with colchicine) to create doubled haploids which will be homozygous and true breeding. This potential for the production of so called 'instant inbreds' has attracted considerable interest from plant breeders dealing with many species (both inbreeding and cross-pollinating). Haploids have many other attractions both for plant breeders and plant geneticists. For example, haploids are suitable basic material for building monosomic series which may be used for cytogenetics and plant breeding (Sears, 1939), and the decorative effect of small flowers with prolonged blooming, common to haploid plants of *Pelargonium* has attracted commercial interest (Kostoff, 1941). Haploids can also be utilised in mutation breeding (Abel, 1955) and may be useful for the transfer of genes from polyploid to diploid species (Kasha, 1974b).

2.2 METHODS OF PRODUCTION OF FREE LIVING HAPLOIDS

Haploid plants contain a "basic set" of chromosomes and are useful in plant breeding programmes for the direct selection of desirable characteristics. The purpose of anther/pollen culture is to produce haploid plants by the induction of embryogenesis from repeated division of monoploid spores either microspores or immature pollen grains. Various physical and chemical factors and anther pretreatments improve embryo production in some plants.

Low temperature pretreatment of anthers for a period of 2-30 days at a temperature of 3-10°c may stimulate embryogenesis (Sunderland and Roberts, 1977). Other types of pretreatment include soaking the detached inflorescences in water for several days (Wilson, Mix and Foroughi-Wehr, 1978) and cool temperature centrifugation of anthers for approximately 30 minutes (Sangwan-Norreel, 1977). Although haploids have been mostly derived from the haplophase life cycle of the plants following the production of meiocytes giving rise to male and female gametophytes, other approaches have been exploited as outlined below:

2.2.1 Spontaneous Induction

Haploids have been found to arise spontaneously in nature. In most cases the haploids are produced in such a low frequency that they cannot be used in breeding programmes. However, in Brassica species the frequency of production of haploids was found to be sufficiently high enough in small seed to allow utilisation in breeding programmes. For example, in the field of oil seed rape it is possible to obtain 20 haploids from 1,000 plants (Wenzel, 1980). Spontaneous haploids have arisen through mono or polyembryonic parthenogenesis (Magoon and Kanna, 1963), androgenesis (Dunwell and Sunderland, 1973) and semigamy (Turcotte and Feaster, 1963).

Semi-gametic production of haploid is also a form of spontaneous occurrence. It occurs when a microspore nucleus enters an egg cell but nuclear fusion does not occur. Both nuclei divide independently, thereby producing an egg embryo that is sectored for maternal and paternal tissue. Semigamy was first observed in Pima cotton (Gossypium barbadense) in a line that originated as a doubled haploid (Turcotte and Feaster, 1963). The frequency of spontaneous haploid production in Gossypium barbadense is

normally less than 1% (Owings, Sarvella and Meyer, 1964). Over 30% of the progeny of the original semigametic doubled haploids were haploids. The haploid plants containing paternal sector suitable for chromosome doubling are useful for the production of doubled haploid lines (Turcotte and Feaster, 1973 and 1974).

2.2.2 Genetic Mismatching

This is frequently described as the 'Bulbosum method' and is mostly used in barley and wheat. A high frequency of barley haploids can be obtained in the progeny of crosses of cultivated barley, H. vulgare with H. bulbosum (Kasha and Kao, 1970). Haploidy results from the selective elimination of H. bulbosum chromosomes in the developing embryo of interspecific H. vulgare x H. bulbosum and its reciprocal crosses (Bennett et al., 1976; Subrahmanyam and Kasha, 1973). The endosperm develops for 2-5 days and then disintegrates. In the developing monoploid embryo cells, the division and development is slower than the diploid cells. This slower growth leads to the formation of a small embryo which may be dissected out from the fruits and cultured under aseptic conditions. Following embryo culture, the developing plantlets are raised under normal greenhouse conditions and chromosome doubling induced on established plants.

2.2.3 Somatic Chromosome Reduction

In comparison to other methods of production of haploids, somatic chromosome reduction by some physical treatment or chemical mutagen application has the advantage that it is useful for all species. However, a low level of success makes it unattractive in practice. Bla keslee, et al., (1922) were able to produce haploids by influencing mitosis with low temperature.

Applying the same method, Muntzing (1937) was successful with rye. High temperature treatment induced haploidy in maize (Randolph, 1932). Swaminathan and Singh (1958) were able to achieve somatic reduction with x-rays in watermelon. In most cases ionizing rays were applied during meiosis or before fertilisation. Like physical factors, chemical agents can be effectively used to effect somatic reduction. Kasha (1974a) and Zenk (1974) used colchicine (in sorghum), 3-fluorophenylalanine (in Lolium x festuca hybrids), parafluorophenylalanine (in Nicotiana culture) and chloramphenicol (in Hordeum root tips) and were able to reduce the chromosome number in plant cells or tissue.

2.2.4 *In Vitro* Approaches

For the production of artificial haploids, *in vitro* approaches can be utilised through callus derived from gametophytic source material or from somatic explants, or via direct exploitation of gametophyte to produce haploid embryoids.

2.2.4.1 Callus When microspores are cultured on a complex medium, instead of undergoing embryogenesis, they develop into callus which bursts through the anther wall. This mode of development is quite common and is usually caused by complex media and disturbance of polarity. The callus can arise by one of the following three pathways: (i) division of the vegetative cell while the generative cell is non-functional, (ii) division of the microspores in which generative cell is not formed, and (iii) division involving both the vegetative cell and the generative cell (Sunderland, 1980). The callus either differentiates on the same medium or it has to be transferred to another medium.

In many species callus produced from a wide range of somatic explant sources has been stimulated to undergo organogenesis leading to the release of independent plants (Larkin and Scowcroft, 1981). These plants have been found to be haploid/aneuploid/diploid/polyploid depending on the original genetic status of initial explant source.

2.2.4.2 Exploitation of Gametophyte This is based on potential male gametes (microspores) and female gametes (megaspores). The anther or microspore culture has more potential and advantage because there are more potential haploids in the form of male gametophytes than female gametophytes. However, both these methods are based on embryogenesis and development of plants from monoploid embryos, to be followed by chromosome doubling to obtain homozygous diploids. The suitability of each of these techniques to the production of haploid in Helianthus spp. is largely unknown. Most progress has been made in in vitro approaches in this particular group of species and for this reason the rest of this Review will concentrate on these approaches.

2.3 HAPLOID/ANEUPLOID PRODUCTION FROM CALLUS

In recent years there has been considerable interest in the possibilities of exploiting the variants arising following a callus in vitro phase for many plant species. These variants may be selected for use in plant breeding programmes either during the culture phase or later, as mature plants (Bajaj, 1990a).

This approach to plant breeding depends on exploitation of totipotency of somatic plant cells through tissue culture, the possibility of which was discussed by Haberlandt as early as 1902. In fact, the concept of

totipotency was inherent in the cell Theory of Schleiden (1838) and Schwann (1839) and was popular as early as 1858 by Virchow with his famous aphorism "every cell from a cell". Major developments during 1955-65 led to the demonstration of totipotency of many higher plant species. These included the discovery of the role of hormones in cell growth and differentiation (Skoog and Miller, 1957) and development of experimental procedures for the successful culture of tissues (Reinert, 1959), cell suspension (Steward *et al.*, 1958) and eventually single cells (Vasil and Vasil, 1972) leading to the *in vitro* regeneration of whole plants.

The basic steps in the procedure involved the induction of rapid cell division in suitable explants leading to the production of a more or less uniform mass of callus tissue (Vasil 1977) on a support media providing all nutrient requirements, with correct auxin/cytokinin balance; organogenesis of callus by alteration of hormone balance normally aimed at the production of shoots; initiation of roots either *in vitro* via a hormonal shift/reduced nutrient status or via very controlled horticultural techniques (paying particular attention to humidity levels) *in vivo*.

The plants produced following these techniques for many species have been demonstrated to be different to the original explant source. Thus, so called 'somaclonal' variation has been demonstrated for many species to be the result of a range of cytological changes such as aneuploidy/polyploidy, inversions/translocations etc. (Larkin, 1987). Of 98 number of species listed by Ogura 1990, which were characterised cytologically, 6 showed the development of haploid cell lines, although this did not necessarily reflect the final number of species which produced free living haploid plants by this route.

High incidences of variant plants are particularly prevalent in somaclones derived from protoplasts, where every single cell clone may differ (Shep ard et al., 1981). This variation encompasses the whole range of qualitative (albinism) traits to quantitative traits (yield) (Karp and Bright, 1985). The somaclonal variation occurs due to genotype (Larkin, 1987), ploidy of source material (Fish and Karp, 1986), tissue culture procedures (Orton, 1980; Mecoy et al., 1982), tissue source (Murashige and Nakano, 1967) and media composition (Karp and Bright, 1985).

2.3.1 Requirements for Haploid/Aneuploid Production from Callus

Cultured plant tissues require a continuous supply of certain inorganic chemicals. Aside from carbon, hydrogen and oxygen, the essential elements required in relatively large amounts are the macro elements: nitrogen, phosphorous, potassium, calcium, magnesium and sulphur (Dodds and Roberts, 1985). The composition of the culture medium is an important factor in the successful establishment of a tissue culture. A standard medium consists of a balanced mixture of macro and micro elements. The media of Gamborg, et al., (1968) and Hildebrandt (1973) support the growth of a wide variety of tissue. The medium developed by Murashige and Skoog (1962) fulfills the requirements of both micro as well as macro elements and is the most commonly used. Iron is added in the form of ferric citrate or better known as chelated EDTA in order to ensure its availability over a wide range of pH of the medium (Ferguson, Street and David, 1958; Sheat, Fletcher and Street, 1959).

The standard carbon source is sucrose or glucose. Fructose can be used but generally has been found to be much less suitable. Other carbohydrates which have been tested include lactose, maltose and starch, but these

compounds are generally much inferior to sucrose or glucose as a carbon source (Gamborg and Shyluk, 1981). Sucrose is generally used at a concentration of 2-3% (Dodds and Roberts, 1985). In early work, some workers (White, 1963) maintained that use of media with complex natural extract was advantageous for initiation of callus. Some workers suggested that mineral salt mixture plus sucrose and 10% (v/v) coconut water was difficult to surpass (Straus and La Rue, 1954). Sometimes coconut water was replaced by yeast extract (Torrey and Shigomura, 1957) or even 5% tomato juice (La Rue, 1949). However, to-day most workers tend not to use such ill defined media additives preferring standardised media for effective comparison.

The growth regulator requirements for most callus cultures are auxin (NAA, IAA, IBA and 2, 4-D) and cytokinin (BAP, BA and Zeatin). Auxins and cytokinins which promote cell division and cell elongation also cause chromasomal abnormalities leading to chromasome number variation in cultured tissue (Bayliss, 1973; Ogura, 1982). GA3 causes stimulation of cambial activity in stem segments and cell division in free cell suspension (Digby and Wareing, 1966). Monocotyledonous tissues were inhibited by GA3 without exception (Butenko, 1968).

The maintenance of callus culture may require the presence of selected amino acids such as Glycine or a mixture of amino acids usually supplied as casein hydrolysate (Steward and Caplin, 1952). Vitamins (B-B6, nicotinic acid have also been shown to be beneficial in callus culture (Reinert and White, 1956).

Callus cultures require regulated environmental conditions, i.e. temperature, light and humidity. Precise environmental conditions vary

depending on the species and purpose of the experiment and considerations have been given to diurnal temperature variation, light intensity, light quality and photoperiod (Wetherell, 1982). An optimum light intensity of 1,000 lux is required during culture initiation and proliferation. A high optimum of 3,000 - 10,000 lux with 16 hours day-light regime with constant temperature set at 25-27°c ± has frequently been found to be essential for the establishment of plantlets (Murashige, 1974).

2.4 EXPLOITATION OF GAMETOPHYTE FOR THE PRODUCTION OF HAPLOIDS

Great advances have been made in the production of haploid and diploid homozygous plants by anther culture (Bajaj, 1990). The low yield of haploids has been a recurring common problem in many species. However, progress has been made in a number of crop species, resulting in acceptable levels of production of haploid green plants (Quyang, 1986). In the past, the potential of using the gametophyte generations for haploid production was limited by the high percentage of albino plants obtained. As research workers have defined culture needs more precisely, then the number of species which successfully produce green haploid plants has increased (Bajaj, 1990 b).

Success in the use of the gametophytes to produce haploids may be determined in part, by the breeding system of individual species. It might be expected that an out breeding species, carrying a load of deleterious genes masked by dominancy, would result in a lower level of success in terms of haploid plants, than say, an in-breeding species which has had such genes selectively removed. Evidence for such a hypothesis is difficult to find being confused in the past, by other features, such as whether or not the species is monocot or dicot each with their specific and perhaps as yet, ill

defined requirements. Interestingly in maize, it has been found that haploid production is much more difficult in the inbred parental lines rather than the F1 hybrid (Genovesi and Collins, 1982; Dieu and Beckert, 1986). Furthermore, the commercial demands of breeding may also obscure any underlying link between success and species nature, with economically important crop species attracting more attention and more opportunity for achieving the goal of haploidy.

2.4.1 General History of Gametophyte Culture

Progress in the field of plant cell and tissue culture has made this area of research into one of the most dynamic and promising in experimental biology. *In vitro* cultures are now being used as tools for the study of various basic problems not only in plant physiology, cell biology and genetics, but also in agriculture, forestry and horticulture. The introduction and development of these techniques has allowed the study of problems previously inaccessible and has in a few instances turned dreams into realities.

The idea to make use of the simple gametophytic genome in basic and applied science of higher plants is almost 70 years old, when Blakeslee, *et al.*, in 1922 while working with *Datura stramonium*, reported the occurrence of haploids. This was followed in the *Solanaceae*, by the production of haploids in *Nicotiana tabacum* (Clausen and Mann, 1924). Publications about these haploids created a tremendous amount of interest and activity in research workers which subsequently resulted in the reporting of haploidy in some other field crop species such as *Triticum compactum* (Gaines and Aase, 1926), *Oryza sativa* (Morinaga and Fukushima, 1931) and *Hordeum vulgare* (Johanson, 1934). In 1928 haploids were reported in the *Compositae* and

again in the Solanaceae, in Crepis capillaris (Hollingshead, 1928) and Solanum nigrum (Jorgensen, 1928). Soon after, haploids were detected in Oenothera rubricalyx (Gates, 1929), Zea mays (Randolph, 1932a) and Gossypium barbadense (Harland, 1936).

Haploidy was first reviewed by Gates and Goodwin (1930) when they described new haploidy in Oenothera. Subsequently, Ivanov (1938) described the attempts to initiate artificial haploidy production. By the early 1950's, 56 species were reported to demonstrate haploidy (Kehr, 1951). Angiosperm plants contain a large amount of haploid pollen grains in their flowers. If this pollen is stored in a desiccator at about 4°c it can remain viable for several years (Tuleke, 1954). These pollen grains could be triggered into an active growth by the simple expedient of culturing with an anther (Guha and Maheshwari, 1964).

Callus was first initiated by culturing pollen grains on a medium containing either yeast extract or casein-hydrolysate (Guha and Maheshwari, 1966). This callus then underwent differentiation leading to the formation of haploid plants (Guha and Maheshwari 1967). A further improvement in this approach was achieved when it was realised that the stage of pollen development at the time of culture is the most important factor leading to the formation of embryoids (Sunderland and Dunwell, 1971). It was quickly accepted that this optimum stage of culture may be different for different species (Kasperbauer and Wilson, 1979). However, usually the highest frequencies of callus induction can be obtained by culturing the anthers at the uninucleate stage (Tsay and Su, 1985). The yield of this callus could be increased with pretreatment of anthers (Sunderland et al., 1981).

To-day, haploids have been produced in more than 50 genera , but the greatest efforts have shifted to economically important field crop species such as the cereals and vegetables. However, low yields of haploids are still a serious drawback. The low yield may be due to some as yet undefined limitation in anther culture. In cereals, particularly in the anther culture of barley there were until quite recently major obstacles to be solved before the anther culture or microspore culture of barley could be as effectively handled for haploid production as those of Solanaceae (Sunderland, 1974; Bajaj, 1976). These limitations were the result of lack of understanding of three main areas: the physiological conditions, the growth conditions of donor plants and the induction of haploids via callus. Non-synchronous development of microspores at the culture stage in Graminae (Wenzel et al., 1975), and differential behaviour of genoptypes (Gresshoff and Doy, 1972b) lead to low frequency and variability in embryogenesis and organogenesis from callus and the formation of chlorophyll deficient and karyotypically abnormal plants. Many of these limitations have now been overcome for barley and rice, and anther/pollen culture is now becoming one of the standard methods available to breeders of these crops (Nitzsche and Wenzel, 1977).

2.4.2 General Requirements for Haploid Production via Gametophyte Culture

In vitro induction of haploids using anthers/microspores and ovules has been, and is being applied on a large scale in relatively few crops (Jensen, 1983). Success requires the use of correct growth media and knowledge of the optimal stage of gametophyte development at the start of experimentation. It has become increasingly apparent that donor genotype, physical conditions during culture, and pretreatment of donor plant or of

anthers prior to culture may also influence the successful outcome of any experiment for the production of haploids in many species. Workers have attempted to improve efficiency by using a diagnostic which would indicate potential for successful culture. It has been suggested that the proportion of non-staining (i.e. taking up only very little acetocarmine in the cytoplasm) pollen may be linked positively to the subsequent development of the pollen once cultured in vitro (Dale, 1975). Evidence to support this proposition has been collected for barley, but evidence for other species is conflicting (Finnie, 1990).

2.4.2.1 Media Successful anther, pollen and ovule culture depends upon many factors, but the key is often the nature of the culture medium used. The medium must supply all requirements for major nutrients such as nitrogen, phosphate, potassium, calcium, magnesium and sulphur; and minor but essential elements like cobalt, zinc, copper, boron, etc. Many workers have exploited the basal media of White (1943) or Murashige and Skoog (1962) with slight modification such as the addition of growth regulators for the culture of excised anthers. The normal level of sucrose is 2.4%, however, with barley (Clapham, 1971), tomato (Sharp et al., 1971a) and wheat (Ouyang et al., 1973) anthers have been observed to grow better on media with 6-12% sucrose. This seems to be an osmotic effect rather than a need for a higher carbohydrate level (Matsubayashi and Kuranuki, 1975).

Other workers have examined the suitability of other carbon sources for this type of tissue culture. Glucose was found to be the best source of carbon for maintaining long term culture of viable microspores of cotton (Barrow 1986). Indeed all cotton cells in culture responded better to glucose than sucrose (Davis et al., 1974; Price et al., 1977 and Barrow et al., 1978). Maltose was found to be superior as a carbon source for anther culture in

petunia (Raquin, 1983). Similarly, the number of anthers of barley responding was much higher on media containing maltose than sucrose (Finnie, 1990). This improvement resulted in a substantial increase in the development of embryoids and eventually plantlets directly on induction media (Finnie, et al., 1989).

Iron in the medium plays a very important role and appears to be indispensable. Although androgenesis can be initiated in tobacco without any iron, the proembryos produced do not develop beyond the globular stage. The nutritional requirements of excised anthers are simpler than those of isolated microspores. It is obvious that certain factors that are responsible for the induction of androgenesis, which might have been provided by the anther wall, have to be included in the medium for microspore culture. Excised anthers of tobacco can be successfully cultured on a simple basal medium while the isolated microspores require higher amounts of nitrogen in the form of amino acids (Reinert et al., 1975).

Provision of a basal medium with the correct proportion of nutrients does not necessarily lead to successful haploid initiation. Complete androgenesis in the case of tobacco (Nitsch, 1969) and atropa (Rashid and Street, 1973) can be achieved on simple media, but in most cases a certain balance of auxin, cytokinin or other additives such as casein hydrolysate, yeast extract, coconut milk have to be added to the medium. The requirement for auxin/cytokinin depends on endogenous levels in the anther. Media rich in growth regulators, encourage the proliferation of tissues other than microspores (i.e. anther wall, connective tissues and filament) and should be avoided, because in such cases mixed calli with the cells of different ploidy level are obtained (Nishi and Mitsuoka, 1969). The range

and diversity of additives included into media for successful initiation of haploid development would create a very long list. It would appear from many workers dealing with diverse species that the precise balance of additives is specific, certainly to individual species and may even be specific to individual genotypes within a species. Other features or changes to the media which have led to successful haploid induction include the use of charcoal and manipulation of the pH of the media. Incorporation of activated charcoal into the medium has stimulated the induction of androgenesis in Nicotiana anthers (Anagnostakis, 1974). In Nicotiana addition of activated charcoal (2%) in media can raise the androgenetic potential of anthers from 41 to 91% (Bajaj et al., 1976). Although charcoal has highly increased the efficiency of androgenesis in Nicotiana anthers, it seems less effective for the anthers of Atropa belladonna and Petunia hybrid.

The pH of the medium is adjusted during the preparation stage and is usually arranged between 5.0 to 6.5. This is an important factor as pH can effect the availability of nutrients, gelling of agar, and growth of plant tissues.

2.4.2.2 Hormones (Plant Growth Regulators) Hormones are compounds which are generally active at very low concentration but which have a profound effect on growth and development. The actual amount of hormones which must be added to the medium depends on genotype and stage of development of culture. Species can be considered as hormone-dependent or hormone-independent in respect of exogenously supplied compounds. Hormone independent species are all in the bi-cellular group and include Datura innoxia, Nicotiana tabacum, N. sylvestris, N. knightiana, Paeonia hybrida and Hyoscyamus niger. Hormones utilised in this 'independant' class must come from the anther wall or the pollen itself. Hormone dependent species

belong to either bicellular or tri-cellular groups. All genera in which the emergent product is callus (Hordeum, Oryza, Triticum, Triticale) are included together with some of the embryo producing species (e.g. Brassica campestris, Asparagus officinalis). There are two important classes of hormones : auxins and cytokinins.

Auxins These are widely used in micropropagation/tissue culture and are also incorporated into nutrient media to promote the growth of callus, cell suspension and organ culture and regulate morphogenesis especially in conjunction with cytokinins. The auxin requirement will vary and there are a number of different kinds of auxins available for this use. In the rooting phase, the culture will require auxin for initiation of roots. Too high a concentration or long exposure period can inhibit initiation or the subsequent growth of any root initiated. Indole-3-acetic acid (IAA) is a naturally occurring auxin, but it is least active and stable. The more stable analogues, Indole-3-butyric acid (IBA) and 1-napthaline-acetic-acid (NAA) are usually preferred. The highly active auxin 2, 4-D (2, 4-dichlorophenoxy acetic acid) is the most effective in promoting callus but strongly inhibits shoot initiation and has been linked to certain chromosomal variation (Gamborg et al., 1976).

Cytokinins Little or no cytokinin in the medium is a normal requirement to induce shoot development from callus in some species whilst in others, quite a high level is required. Generally the higher the concentration the greater the number of shoots that are produced up to a point at which further increases produce an adverse effect (Pennel, 1987). Cytokinin is not used alone for callus formation, mostly it is used with auxin, either 2, 4-D or NAA. Kinetin, a cytokinin is usually combined with NAA or IAA for plant regeneration (Gresshoff, 1978). The two main side-effects of

increasing cytokinin levels are the induction of a very large number of shoots which are difficult to handle and increasing the incidence of growth disorders. This is an indication that genetic instability increases in plant tissue growth on a high level of cytokinins (Pennel, 1987).

2.4.2.3 Pretreatment For the development of pollen into embryoids and plants in vitro it is very important to take and use only healthy pollen cells. It is necessary to take pollen from plants grown under optimum environmental conditions. The donor plants should be well fed and placed in an optimum light regime. If the plant is to be grown in the greenhouse, it is advisable that natural daylight should be supplemented with artificial light (Nitsch, 1981). In order to minimise stress during pollen development it is advisable to give nutrient support to the donor plant for a few days before the removal of the anther/pollen sample (Nitsch, 1975). Success with anther culture is frequently dependent on the genotype of the parent plant (Guhua Mukherjee (1973) on rice; Bajaj (1976) on wheat). It may also be possible to exploit a genotype that responds favourably to anther culture by using it as one of the parents in a cross with the hope that haploids are more easily obtained from the hybrid as well. In addition to the above pretreatment of pollen mother plants pretreatment of anthers before placing in culture have been shown to enhance the frequency of anther induction. A cold shock treatment (3-5°C for 48-72 hours) resulted in an increased number of embryos per anther in Nicotiana and Datura (Nitsch, 1974b; Nitsch and Norreel, 1972). Cold shocks to the wheat spike before anther culture, promoted nonpolarised mitosis and formation of pollen callus (Picard, 1973). An alternative approach is to spray plant growth substances before anther culture (Pandey, 1973). Ethrel (2-chloroethyl phosphoric acid) for 48 hours at 10°C induce mitosis in wheat (Bennett and Hughes, 1972), rice (Wang et al., 1974) and Petunia (Bajaj, 1975) pollen in

vivo. Other types of pretreatment include soaking the detached inflorescences in water for several days (Wilson, Mix and Foroughtwehr, 1978) and centrifugation of anthers at 3-5°c for approximately 30 minutes (Sangwan-Norreel, 1977). The frequency of embryogenesis from anthers can also be enhanced by the pretreatment of anthers under reduced atmospheric pressure and/or anaerobic conditions. Significant positive results were obtained by treating the anthers of Nicotiana tabacum with reduced atmospheric pressure for 10, 20 and 60 minutes. However, any further extension of the treatment resulted in the inhibition of plantlet formation. Short treatments of anthers under anaerobic conditions (2.5 or 5% O_2) also significantly increased the frequency of anther embryogenesis (Harada et al., 1988).

2.4.2.4 Culture Period The most usually considered variables during the culture period are those of temperature and light. In general, relatively high temperatures of 25-30°c have been found to be optimal for anther culture and pollen culture. Light is a very important factor which induces pollen to undergo androgenesis. High intensity of white light of 5,000 lux is inhibitory for androgenesis, whereas darkness and blue light are not. However, low intensity (500 lux) white light or red light shortened the time necessary to produce plants from pollen by about 20% (Nitsch, 1981). In Nicotiana tabacum light is beneficial for post induction growth and enhances plantlet yields (Sunderland, 1971). Photoperiods of 16 hours from fluorescent tubes (1,500 lux) at 28°c alternating with 8 hours darkness at 20°c gave the highest plantlet yields (Corduan, 1975; Sopory and Maheshwari, 1976a). Other incubation parameters found to influence pollen response include position of anther as it is placed on the surface of agar media (D. annoxia; Sopory and Maheshwari, 1976b), the density of anthers per culture vessel (Oryza sativa; Fouletier, 1974) and the atmosphere

volume of the culture vessel. Inclusion of 2-chloroethyl phosphonic acid in the culture medium increased the response of Oryza sativa anthers approximately threefold (Wang et al., 1974).

2.5 PROGRESS IN THE PRODUCTION OF HAPLOIDS IN HELIANTHUS ANNUUS L.

The development of tissue and anther culture technology, leading eventually to homozygous diploid lines in Helianthus annuus L. could be valuable in speeding up the improvement process. Success depends upon the ability to manipulate explants in vitro in such a way that maximum regeneration frequency of haploid plants may be obtained. Unfortunately, such skills and requirements do not exist as yet for this species. However, some success has been achieved towards understanding the requirements for tissue and gametophytic culture in Helianthus annuus L.

2.5.1 Tissue Culture

Plantlets produced via callus followed by organogenesis may be haploid/aneuploid/polyploid as well as diploid. Many factors are known to influence the development of callus and subsequent differentiation from a wide range of crop plants (Jensen, 1983). Although the number of studies on sunflower tissue culture are limited (Trifi et al., 1981), some workers have reported some success with callus production from normal Helianthus tissue (Paterson and Everett, 1985). Plant regeneration was obtained from Helianthus pith cultured on a modified White's medium with 1 mg/l 1AA (Sadhu, 1974). Shoots were also regenerated from protoplasts isolated from cultured shoot tips (Binding, et al., 1981). In 1984 Greco et al., reported regeneration of plants from nodular callus induced from synthetic variety 'Sannac'. The genetic status of this material is not known.

2.5.2 Gametophyte Culture

Using Murashige and Skoog (MS) medium with the addition of 2, 4-D and kinetin it is possible to obtain callus from anthers of Helianthus annuus L. and interspecific hybrids. Direct androgenesis was induced with MS media enriched with 5 mg/l zeatin only for hybrid Helianthus annuus x H. decapetalus (Bohorova, Atanassov and Antonova, 1980). Anthers and unpollinated ovaries of four French and four Hungarian cultivars were also cultured on MS medium supplemented with auxin and cytokinins. The percentage of anthers forming callus ranged from 15% for Jaszberenyi to 80% for 'INRA'. Six plantlets, two of which were haploids, were regenerated from anther callus of 'INRA', and a single haploid plantlet was obtained from Luciole. The percentage of ovary forming callus ranged from 10% for Jaszberenyi to 85% for INRA, and diploid plantlets were obtained from callus of INRA (12), Clairesole (2), and Luciole (5), (Mix, 1985). Callus organogenesis and anther embryogenesis in H. annuus L. was greatest when anthers were cultured on MS medium with NAA and BAP at a late stage of meiosis. However, at their premitotic stage 95% of the anthers formed callus, but did not differentiate. Whereas at uninucleate stage 40% of the anthers turned brown within 2 or 3 weeks and died. Anther embryogenesis improved with increased temperature in the absence of light. However, these modifications did not produce a large number of haploids. Three diploids and one haploid plants were produced when anthers were cultured at 35°c and 12 days of darkness (Mezzarobba and Jonard, 1986)

SECTION 3

ANTHER/GAMETOPHYTE CULTURE

INTRODUCTION

Although domesticated and grown for many years, Helianthus species have only relatively recently attracted major interest as an oil crop worthy of greater exploitation (Harry and Dotty, 1978). Considerable reserves of genetic variability still exist but require characterisation (Bohorova et al., 1980). Using traditional approaches towards crop improvement, it should be possible to produce hybrid cultivars with increased seed and oil percentage, combined with better disease/pest resistance. However, this goal demands considerable efforts and long periods in order to identify useful parental lines. These are usually produced via forced in-breeding (to increase homozygosity) with selection. Doubled haploids offer one possibility for speeding up the production of potential parental lines. To date there has been relatively little progress in the production of haploids in Helianthus species and what little knowledge that has been accumulated does not give any clear leads as to what might be the obstacles which require to be overcome before this technique can be of value in the improvement of Helianthus annuus L. (Bohorova and Atanassov, 1990). Therefore, it was decided to investigate the potential of various plant growth regulators (known to be used with advantages in other species) and other media additives including complex organic compounds as well as charcoal and alternative carbon sources, on haploid production in Helianthus species.

3.1 EXPERIMENTAL MATERIALS

The seven cultivars of Helianthus annuus L. of different origin used in this study are given in Table 3.1.1.

TABLE 3.1.1 Source of Helianthus annuus L. used in Experimental Studies

Cultivar	Source	Agric./Horti.	Country of Origin
Giant yellow	Suttons seeds	Agric.	U.K.
Sunburst	" "	Horti.	U.K.
Autumn beauty	" "	"	U.K.
Holiday	" "	"	U.K.
Uniflorous giganteus	" "	"	U.K.
Twyford A (Hybrid)	Twyford seeds Ltd	Agric.	U.S.A.
Twyford C (Hybrid)	" "	"	U.S.A.

TABLE 3.1.2 Characteristics of Helianthus annuus L. Cultivars (Data based on examination of a typical plant)

Cultivar	Height (cm)	Leaves/ plant	Branches/ plant	Petiole length (cm)	Inter. length (cm)	Leaf area (sq. cm)
Giant yellow	161.0	22	-	10.66	12.33	333.66
Sunburst	137.0	27	25	14.00	18.00	297.66
Autumn beauty	159.0	26	-	15.33	12.66	243.16
Holiday	97.0	21	17	16.66	12.33	350.00
Uniflorous giganteus	180.0	26	-	12.00	11.66	290.00
Twyford A	39.0	14	-	4.90	4.16	71.66
Twyford C	52.0	17	-	7.50	6.50	129.62

A mixture of ornamental and agricultural cultivars was examined primarily to ensure that it was possible to have flowers at suitable growth stages throughout the year as well as to ensure that the genetic base of the material under examination was not too narrow. Information with regard to

cultivar characteristics was collected (Table 3.1.2) to facilitate comparison with any regenerated plants obtained in these investigations.

3.2 IN VIVO GROWTH OF HELIANTHUS ANNUUS L.

Although possible to grow Helianthus annuus L. under field conditions at Edinburgh, it was decided to utilize only glasshouse grown material to ensure a constant supply of material at the correct stage of growth. Plantings were carried out every 4 weeks. Seeds were sown in pots (18 cm diameter) containing moss peat with the composition, ammonium nitrate 120 gms, filtered trace elements 120 gms, potassium nitrate 120 gms, superphosphate 450 gms, dolomited lime 675 gms and garden lime 675 gms. The glasshouse compartment was not allowed to go below 12°c at night, and was maintained at a minimum of 18°c during the day. Supplementary lighting (1,200 lux) was also supplied throughout the growing period. After an initial period of 6 weeks growth, plants received supplementary feeding of potassium nitrate 55g/l, mono ammonium phosphate 20g/l, and ammonium nitrate 45g/l at dilution rate 1/500. All cultivars became infested with white fly, aphids and occasionally red spidermite. Control was affected by the application of Pirimor supplied by Active Ingredient Pirimicarb ICI Agrochemicals, Nicotine supplied by Campbell's Nicosoap M+M Agrochemicals and smoke (Pirimiphos-methyl) supplied by Smoke Generator Octavios Hunt Ltd.

3.3 PLATING AND INCUBATION OF CULTURES

From the initial plant material raised under controlled environmental conditions, flower buds at the stage of development considered by most workers in the area (Dunwell, 1985 and Bohorova et al., 1980) to be appropriate (one nuclear microspore stage) were selected. The stage was

determined by observing bud size in relation to pollen development, observed by squashing anthers in a drop of acetocarmine and examined microscopically. Suitable buds were surface sterilized with 2% sodium hypochlorite solution for three minutes with three-fold(3) washings with sterilized distilled water to remove the last traces of sterilant. Anthers were dissected out under a dissecting microscope and 15 anthers were plated in each petri dish (5 cm diameter) containing media. Each medium was repeated 5 times giving 75 explants per treatment. The cultured petri dishes were sealed with parafilm and incubated at a temperature of 25°c-27°c, illumination of 500 lux and photoperiod of 16 hours daylength regime.

3.3.1 Estimation of Pollen Fertility in Helianthus annuus L.

1. A mature undehisced anther was squashed in a drop of acetocarmine. The debris was removed and a coverslip gently placed over the stain. Alternatively, pollen from the dehisced anther was dusted in a drop of acetocarmine and a coverslip applied.

2. The slide was heated gently over the flame of a burner.

3. The slide was observed microscopically under low magnification. The stained and unstained pollen grains were counted. The pollen fertility recorded in different cultivars of Helianthus annuus L. is given for a single typical recording period March, 1991.

(See Table 3.3.1.1)

All the cultivars of Helianthus annuus L. showed the same level of pollen fertility whether it was observed before anthesis or after anthesis. However, cultivar Giant yellow showed a lower level of fertility before anthesis.

TABLE 3.3.1.1. Pollen Fertility Percentage (Mean of four replications)

Cultivar	Before Anthesis	After Anthesis
Sunburst	94.86	97.95
Giant yellow	88.84	98.53
Twyford A	94.25	94.03
Twyford C	93.64	91.29
Autumn beauty	91.84	97.55
Holiday	97.38	98.92
Uniflorous giganteus	95.56	98.39

3.4 BASAL MEDIA USED FOR ANTHER CULTURE

Many nutrient media have been used in tissue and anther culture. Pioneering work during the 1950's and early 1960's was usually carried out on White's (1943) medium with various modifications. Other media such as those of Gautheret (1957), Heller (1953), Nitsch (1951), and Tukey (1933) were also used. The success of these media was generally limited to the continued development of structure already initiated *in vivo* and to the limited proliferation of unorganized tissues. It appears that limited somatic embryogenesis did occur in nucellar explants in which some natural nucellar embryos were already present. The immature embryos generally failed to develop into plantlets. With the application of Murashige and Skoog (MS) (1962) medium, progress was stimulated greatly, particularly in respect to organogenesis and embryogenesis.

The mineral elements of Murashige and Skoog are generally adequate, although probably not entirely optimum for tissue cultures. The main differences between MS medium with the other formulations is in the concentrations and source of nitrogen. Most media contain only nitrate, while MS contains a relatively high concentration of ammonium in addition to a higher nitrate level. It appears possible that embryogenesis in unorganized tissues may be enhanced by NH_4NO_3 (Reinert and Tazawa, 1969). The calcium

concentrations is also somewhat higher in MS formulation than others. There appears to be no necessity to modify the concentrations of inorganic constituents of MS medium for the culture of tissues. The nitrogen status of the donor plants has been shown to influence the production of microspore derived embryos (Sunderland, 1978). Previous work carried out in Helianthus annuus L. showed the extensive use of MS nutrient package as basal medium. Therefore, it was decided to use Murashige and Skoog (1962) (Appendix 1) supplied by Flow Laboratories Ltd., P.O. Box 17, Second Avenue Industrial Estate, Irvine, Ayrshire, U.K., as basal medium for anther culture.

3.4.1 Media Preparation

The required amount of MS medium (supplied by Flow Laboratories Ltd.) was weighed and placed in Erlenmayer volumetric flasks. The nutrients were dissolved in distilled water in each flask. While stirring the solution, the pH of the medium was adjusted to 5.5 - 5.7 by delivering droplets of 1NaOH and 1HCL with separate pipettes. The required quantity of agar (supplied by Davis Gelatin, Leamington Spa, Warwickshire, CU32 5AN) was weighed and added to the MS nutrient solution. The mouths of the flasks were sealed with aluminium foil caps and sterilized with wet heat for 15 minutes at a temperature of 121°c (250°F) under 10/15 psi (0.70-1.05 kg.cm) pressure. While the media was in the autoclave, the interior of the Laminar Flow cabinet was cleaned with 70% (v/v) ethanol and tissue paper. The sterile petri dishes were arranged to receive the autoclaved media.

After the sterilized media was removed from the autoclave, the hot media was permitted to cool in the water heated bath for 10-15 minutes maintained at 60°c before adding other sterile additives. The plant growth regulators

and other vitamin preparations, sterilized by an ultrafilt-ration unit attached to the end of syringes was added to each flask. The flasks were swirled for a few minutes to ensure the dissolution and mixture of additives and agar with the remainder to the medium prior to pouring into the culture petri dishes. After the agar solidified, the petri dishes were wrapped in parafilm and placed in storage for further use.

3.5 PLANT GROWTH REGULATORS/OTHER ADDITIVES USED

From: Sigma Chemical Company Limited, Fancy Road, Poole, Dorset, England, BH17 7NH.

Auxins:

1-naphthalene acetic acid	(NAA)	(Cat. No. 1641)
Indole-3-acetic acid	(IAA)	(Cat. No. 1.1250)
Indole-3-butyric acid	(IBA)	(Cat. No. 1.1875)
2, 4-Dichlorophenoxy acetic acid	(2, 4-D)	(Cat. No. D 2128)

Cytokinin:

6-benzyl amino purine	(BAP)	(Cat. No. 1.1875)
Kinetin	(Kin)	(Cat. No. K 3378)

Aminoacids:

L. Asparagin	(Cat. No. A 0884)
L. Glutamine	(Cat. No. G 3216)

Others:

Adenine Sulphate	(Cat. No. A 9126)

3.6 EXPERIMENTAL APPROACH

Anther culture can result in subsequent development via two routes; direct embryogenesis or callus production followed by organogenesis or

embryogenesis. Direct embryogenesis is generally the preferred route, giving rise to haploid plantlets. The former, callus production and subsequent organogenesis tends to leave open the question of origin of the callus material; either from the pollen or from other anther parts which could initially be diploid. Callus growth itself, even if initiated from a haploid pollen source can undergo abnormal development leading to the production of a population of plants with varying ploidy levels (Bohorova and Landjeva, 1987).

Previous work in this area in Helianthus annuus L. has resulted in the development of friable, light green and granular callus (70-100%) in all species and hybrids, but which was difficult to differentiate (Plotnikov, 1975). Direct androgenesis was induced in nutrient medium MS supplemented with 5mg/l Zeatin. A nutrient medium containing MS, 2mg/l BAP, 0.2mg/l 1AA and 20mg/l adenine was found to be most suitable for obtaining compact callus with numerous green meristmoid sections (Bohorova et al., 1980). Then the supplementation of 800mg/l L. Glutamine and 800mg/l L. Asparagine resulted in a total of 126 regenerants over a series of subsequent sub cultures. These regenerants were variable in their morphological characteristics. The cytological behaviour of these regenerants showed that 33.35% of them were diploids and 65% aneuploids. In another study 2 haploid plants were created with the use of MS + IAA, NAA, IBA and BAP (Mix, 1985). Regeneration of plants was also observed from the induction media MS and other additives like NAA, IAA and BAP in different concentrations (Alissa Asad et al., 1985).

As a result of previous work carried out in Helianthus annuus L. it was decided to concentrate efforts in essentially three main areas; investigation of the effects of the plant growth regulators

(IAA/NAA/IBA/BAP) on the subsequent development of plated anthers; investigation of the possible role of other media additives such as complex organic, charcoal and other carbon sources as well as mother plant pretreatment on subsequent development of plated anthers and finally, investigation of the needs of callus produced, for differentiation and regeneration.

3.6.1 Effect of NAA, IBA, IAA, 2, 4-D and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

There are only sporadic reports of the production of haploid plants via anther culture in Helianthus annuus L. Alissa Asad, et al., (1985) reported the regeneration of plantlets either directly or through callus from anthers of a number of Helianthus species and interspecific hybrids using modified MS medium with NAA and BAP, IAA and kinetin or NAA and zeatin, 3% sucrose and 0.8% agar. Haploid and diploid plants were regenerated but did not survive. Similarly, callus was obtained from three cultivars of Helianthus annuus L. using NAA, 2, 4-D and kinetin media additives, but regeneration was not observed (Tzen and Lin, 1975). Because of this previous work, it was decided to investigate the use of auxins NAA, IBA, IAA, and 2, 4-D and cytokinin BAP on the subsequent development of anthers containing pollen at the one nuclear stage of development. BAP was chosen, because previous experience using kinetin had failed to give positive results. Similarly, workers had success when using a combination of auxin and cytokinin rather than either independently. Exploratory work in this study supported this finding and so by and large only the effect of a combination of both types of plant growth regulators were investigated. The concentrations/combinations of plant growth regulators were chosen to encompass those used by other workers in this field (Bohorova, et al., 1985;

Tzen and Lin, 1975; Mix, 1985; Alissa Assad et al., 1985 and Plotnikov, 1975). The results obtained in terms of embryogenesis, callus production/growt and regenerations are given in Tables 3.6.1.1-3.6.1.14.

TABLE 3.6.1.1 Effect of NAA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

Callus Initiation (Arcsin transformed data) (After 4 weeks)

	Medium			
Cultivar	**M1**	**M2**	**M3**	**Direct Embryogensis**
Sunburst	90.0	70.6	69.7	0
Giant yellow	90.0	80.0	73.6	0
Twyford A	80.0	74.7	84.3	0
Twyford C	90.0	90.0	90.0	0
Autumn beauty	80.0	78.5	73.6	0
Holiday	90.0	67.2	70.6	0
Uniflorous giganteus	44.4	55.6	64.2	0
S.E.	6.276			

TABLE 3.6.1.2 Effect of NAA and BAP on the subsequent growth of anther callus of Helianthus annuus L. in culture

Mean Callus Growth (mm) (Increase in size over original anther structure after 4 weeks)

	Medium		
Cultivar	**M1**	**M2**	**M3**
Sunburst	5.87	5.13	5.31
Giant yellow	4.26	3.80	2.73
Twyford A	6.80	1.73	1.86
Twyford C	6.53	2.67	3.53
Autumn beauty	3.86	3.33	2.93
Holiday	4.13	1.66	2.13
Uniflorous giganteus	2.93	3.13	3.13
S.E.	0.658		

TABLE 3.6.1.3 Effect of NAA and BAP on shoot organogenesis on primary anther callus of Helianthus annuus L.

Shoot Organogenesis on Primary anther Callus

Cultivar	Medium M1	M2	M3
Sunburst	0	2	0
Giant yellow	0	0	0
Twyford A	0	0	0
Twyford C	0	3	0
Autumn beauty	0	0	0
Holiday	21	0	0
Uniflorous giganteus	0	0	0

M1. MS + NAA 1mg/l + BAP 1mg/l
M2. MS + NAA 5mg/l + BAP 5mg/l
M3. MS + NAA 10mg/l + BAP 10mg/l

No direct embryogenesis was observed. Development occurred through callus production followed by some organogenesis. The combinations of media chosen did not discriminate between any genotypes with respect to callus initiation (See Appendix 3.1), subsequent growth of callus, measured only by increase in size over the original anther structure was influenced significantly (Appendix 3.2) by plant growth regulators levels in the media. Individual genotypes responded differently to the media examined but overall there were no significant differences between the cultivars. With most cultivars there was no further development from the callus phase on the media. However, shoot organogenesis occurred commonly with only cv. "Holiday" and sporadically with two others. No roots were observed on these media. Transfer of these shoots to half strength MS with no plant regulators did not stimulate root development except for the odd plant. The only plant obtained from this experiment originated from cv. "Twyford C" (Fig. I) and was found to be cytologically abnormal with haploid/diploid cells (method described in appendix 2).

TABLE 3.6.1.4 Effect of IBA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

Callus Initiation (Arcsin transformed data)
(After 4 weeks)

	Medium			
Cultivar	M1	M2	M3	Direct Embryogenesis
Sunburst	50.8	90.0	90.0	0
Giant yellow	46.1	84.3	90.0	0
Twyford A	90.0	84.3	90.0	0
Twyford C	90.0	90.0	90.0	0
Autumn beauty	58.0	80.0	68.9	0
Holiday	73.6	90.0	84.3	0
Uniflorous giganteus	43.3	80.0	90.0	0
S.E.	5.272			

TABLE 3.6.1.5. Effect of IBA and BAP on the subsequent growth of anther callus of Helianthus annuus L. in culture

Mean Callus Growth (mm)
(Increase in size over original anther structure after 4 weeks)

Cultivar	M1	M2	M3
Sunburst	3.27	6.53	7.49
Giant yellow	1.87	4.20	4.51
Twyford A	6.33	4.13	4.67
Twyford C	7.20	3.59	2.33
Autumn beauty	2.80	4.60	4.06
Holiday	2.53	5.46	4.60
Uniflorous giganteus	1.27	3.13	3.60
S.E.	0.860		

TABLE 3.6.1.6 Effect of IBA and BAP on shoot organogenesis on primary anther callus of Helianthus annuus L.

	Shoot Organogenesis on Primary Anther Callus		
	Medium		
Cultivar	M1	M2	M3
Sunburst	0	1	1
Giant yellow	0	0	0
Twyford A	0	0	0
Twyford C	0	0	0
Autumn beauty	0	0	0
Holiday	3	22	7
Uniflorous giganteus	0	0	0

M1. MS + IBA 1mg/l + BAP 1mg/l
M2. MS + IBA 5mg/l + BAP 5mg/l
M3. MS + IBA 10mg/l + BAP 10mg/l

Again no direct embryogenesis occurred. Callus development was a common occurrence with increasing concentrations of IBA/BAP in the media resulting in a significantly (Appendix 4.1) greater frequency of callus initiation. This was different to the results observed with NAA/BAP media additions, where lower levels of auxin/cytokinin resulted in the initiation of callus. Subsequent growth of callus as measured by increase in size over the original anther structure was not influenced significantly (Appendix 4.2) by the concentrations/combinations of IBA/BAP in the media. Shoot organogenesis occurred commonly in only one cv. "Holiday" but again root initiation was not observed. Subsequently transfer to half strength MS media gave rise to only one plant from cv. "Sunburst". This was short lived (Fig. 2), flowered and died before cytological analysis could be carried out.

TABLE 3.6.1.7 Effect of IAA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

Callus Initiation (Arcsin transformed data)
(after 4 weeks)

	Medium		
Cultivar	M1	M2	M3
Sunburst	74.7	80.0	61.3
Giant yellow	44.4	78.5	53.7
Twyford A	90.0	90.0	72.5
Twyford C	90.0	90.0	90.0
Autumn beauty	84.3	78.5	77.1
Holiday	90.0	90.0	90.0
Uniflorous giganteus	50.2	69.7	70.6
S.E.	7.536		

TABLE 3.6.1.8 Effect of IAA and BAP on the subsequent growth of anther callus of Helianthus annuus L. in culture

Mean Callus growth (mm)
(Increase in size over original anther structure after 4 weeks)

	Medium		
Cultivar	M1	M2	M3
Sunburst	2.81	5.53	5.73
Giant yellow	1.66	2.80	2.00
Twyford A	6.46	6.33	4.86
Twyford C	4.26	6.33	3.60
Autumn beauty	6.53	5.80	5.93
Holiday	4.80	5.73	5.13
Uniflorous giganteus	1.80	3.20	4.06
S.E.	0.884		

TABLE 3.6.1.9 **Effect of IAA and BAP on shoot organogenesis on primary anther callus of Helianthus annuus L.**

Shoot organogenesis on primary anther callus

	Medium		
Cultivar	**M1**	**M2**	**M3**
Sunburst	4	0	0
Giant yellow	0	0	0
Twyford A	0	0	0
Twyford C	0	0	0
Autumn beauty	0	0	0
Holiday	21	5	0
Uniflorous giganteus	0	0	0

M1. MS + IAA 1mg/l + BAP 1mg/l
M2. MS + IAA 5mg/l + BAP 5mg/l
M3. MS + IAA 10mg/l + BAP 10mg/l

As with NAA/IBA combinations with BAP, IAA/BAP combinations did not result in direct embryogenesis, only callus was formed. However, there was no significant (Appendix 5.1) effect of plant growth regulators on the initiation of callus in this case. Similarly, subsequent growth of callus as measured by the increase in size over the original anther structure was not influenced significantly (Appendix 5.2) by the concentrations/combinations of IAA/BAP in the media. Again, cv. "Holiday" was the only cultivar to undergo subsequent shoot organogenesis on any scale, most frequently at lower concentrations of IAA/BAP. As with previous experiments there were no root initiation on these media, transfer to half MS gave rise to only one plant from cv. "Sunburst" (Fig. 3) and this was found to be cytologically abnormal with root squash analysis demonstrating the presence of haploid and diploid cells.

As shoot organogenesis only occurred with 3 cultivars (Sunburst, Twyford C and Holiday), it was decided to examine in greater detail the influence of

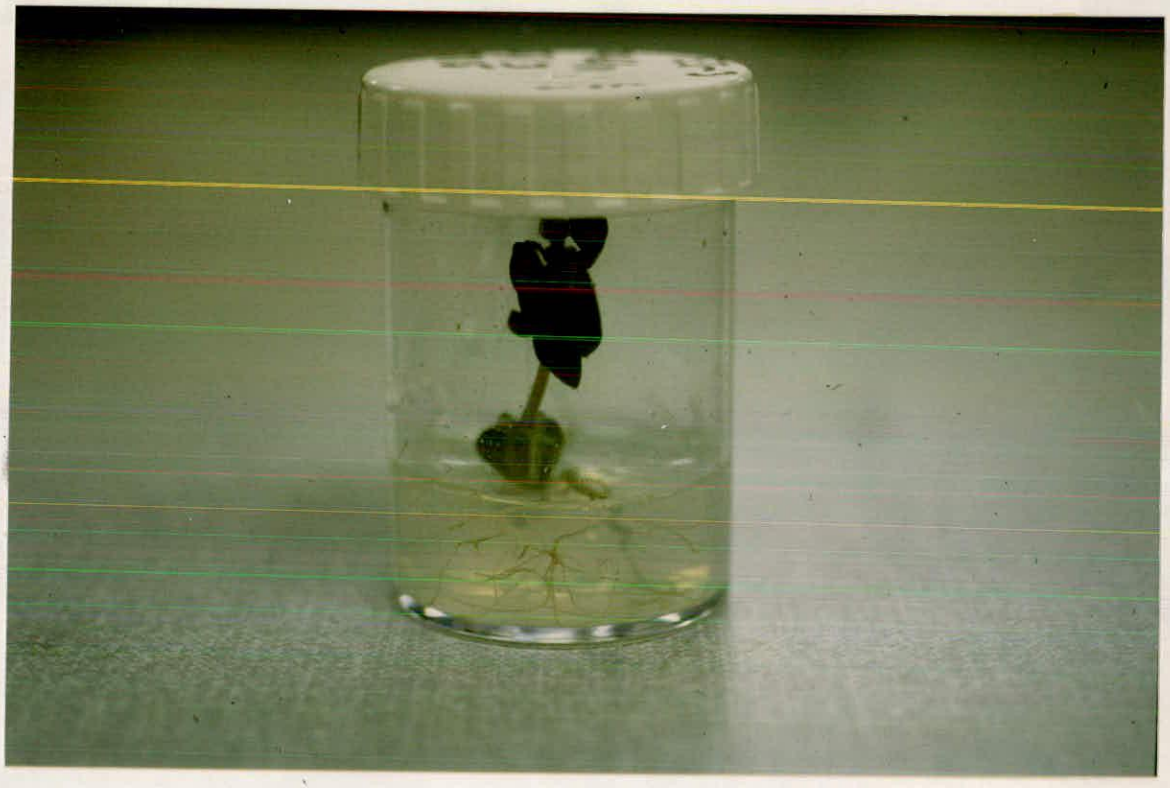

Figure 1: Plant regenerated on primary anther callus of cv. "Twyford C" with media components MS + NAA 5mg/l + BAP 5mg/l and roots regenerated on ½ strength MS media.

Figure 2: Plant regenerated on primary anther callus of cv. "Sunburst" with media components MS + IBA 5mg/l + BAP 5mg/l and roots regenerated on ½ strength MS media.

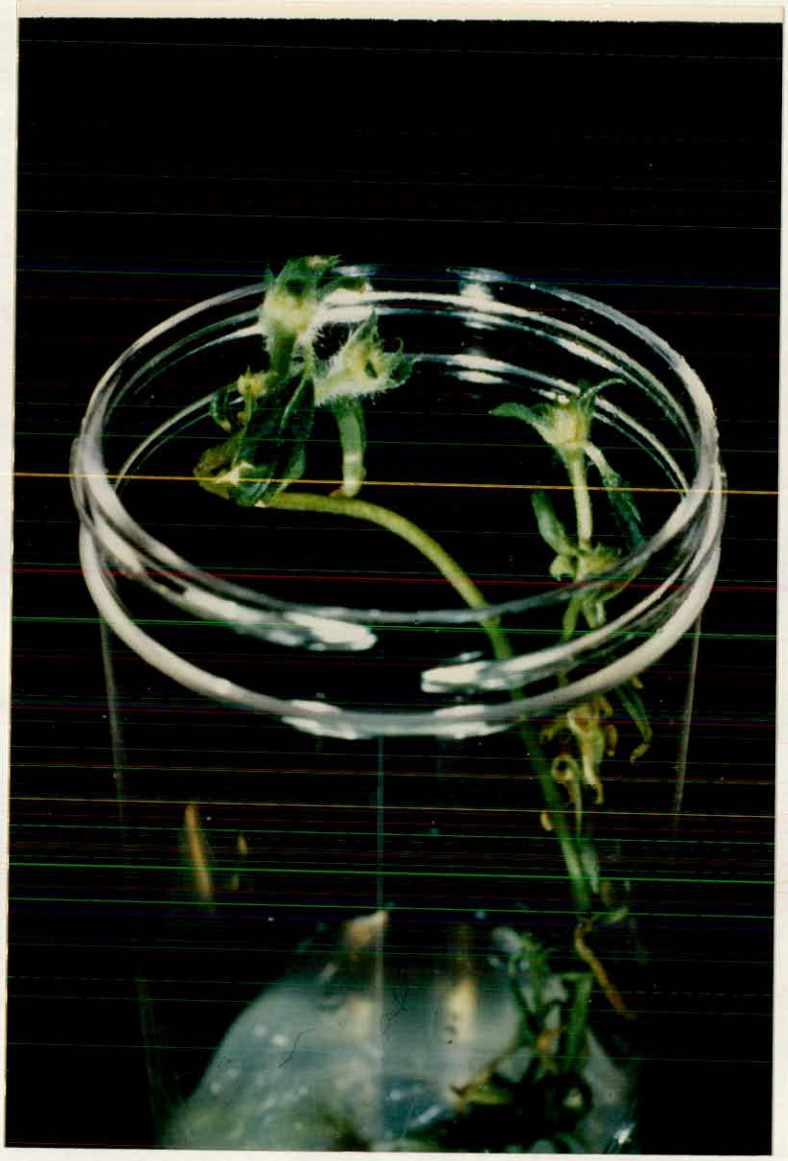

Figure 3: Plant regenerated on primary anther callus of cv. "Sunburst" with media components MS + IAA 1mg/l + BAP 1mg/l and roots regenerated on ½ strength MS media.

auxin/cytokinin combinations. All previous work had examined equal levels of the auxin and cytokinin. As most success in terms of shoot organogenesis tended to occur at lower levels of auxin and cytokinin additives, it was decided to examine 3 levels of the auxin IAA (1, 2, 5mg/l) with 3 levels of BAP (1, 2, 5mg/l).

TABLE 3.6.1.10 Effect of IAA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

Callus initiation (Arcsin transformed data)
(After 4 weeks)

	Cultivar			
Medium	Twyford C	Sunburst	Holiday	Direct Embryogenesis
MS+IAA 1mg/l +BAP 1mg/l	90.0	80.0	90.0	0
MS+IAA 1mg/l +BAP 2mg/l	90.0	90.0	90.0	0
MS+IAA 1mg/l +BAP 5mg/l	90.0	90.0	90.0	0
MS+IAA 2mg/l +BAP 1mg/l	80.0	80.0	90.0	0
MS+IAA 2mg/l +BAP 2mg/l	90.0	84.3	90.0	0
MS+IAA 2mg/l +BAP 5mg/l	90.0	77.1	90.0	0
MS+IAA 5mg/l +BAP 1mg/l	90.0	65.7	90.0	0
MS+IAA 5mg/l +BAP 2mg/l	90.0	80.0	90.0	0
MS+IAA 5mg/l +BAP 5mg/l	90.0	84.3	90.0	0
S.E.	2.063			

TABLE 3.6.1.11 Effect of IAA and BAP on the subsequent growth of anther callus of Helianthus annuus L. in culture

Mean Callus growth (mm)
(Increase in size over original anther structure after 4 weeks)

	Cultivar		
Medium	Twyford C	Sunburst	Holiday
MS+IAA 1mg/l +BAP 1mg/l	4.00	4.10	4.25
MS+IAA 1mg/l +BAP 2mg/l	4.07	4.76	4.66
MS+IAA 1mg/l +BAP 5mg/l	5.33	4.76	5.73
MS+IAA 2mg/l +BAP 1mg/l	4.53	3.43	5.06
MS+IAA 2mg/l +BAP 2mg/l	5.53	2.83	4.76
MS+IAA 2mg/l +BAP 5mg/l	4.80	4.10	5.06
MS+IAA 5mg/l +BAP 1mg/l	4.73	2.30	4.53
MS+IAA 5mg/l +BAP 2mg/l	3.93	4.96	4.86
MS+IAA 5mg/l BAP 5mg/l	5.86	4.90	3.86
S.E.	0.348		

TABLE 3.6.1.12 Effect of IAA and BAP on shoot organogenesis on primary anther callus of Helianthus annuus L.

Shoot organogenesis on primary anther callus

Medium	Cultivar		
	Twyford C	Sunburst	Holiday
MS+IAA 1mg/l +BAP 1mg/l	0	0	6
MS+IAA 1mg/l +BAP 2mg/l	0	0	12
MS+IAA 1mg/l +BAP 5mg/l	0	0	3
MS+IAA 2mg/l +BAP 1mg/l	0	0	6
MS+IAA 2mg/l +BAP 2mg/l	0	0	1
MS+IAA 2mg/l +BAP 5mg/l	0	0	2
MS+IAA 5mg/l +BAP 1mg/l	0	0	3
MS+IAA 5mg/l +BAP 2mg/l	0	0	4
MS+IAA 5mg/l +BAP 5mg/l	0	0	2

As with the previous studies, no direct embryogenesis occurred with any media. There was a significant (Appendix 6.1) difference in genotype response with respect to callus initiation with cv. "Sunburst" showing reduced levels of initiation. Once initiated, subsequent growth of callus as measured by increase in size over the original anther structure, was significantly (Appendix 6.2) influenced by media composition in terms of plant growth regulator additives. At lower levels of IAA, the influence of BAP on callus growth appears to be rather constant, with increasing callus growth being associated with increased levels of BAP. At the higher levels of IAA, the influence of BAP does not appear to be as consistent.

The influence of IAA on callus growth does not follow any simple pattern and appears to be influenced by genotype, although overall in this experiment genotype effects were non significant. As in the previous studies cv. "Holiday" was the most prolific cultivar with respect to shoot organogenesis. However, difficulties were still experienced in obtaining rooted plants from such shoots. Overall, from 39 shoots, only two plants were finally grown *in vivo* following an intermediary stage of culture on half strength MS. These two plants found to have mixed cell types (haploids and diploids) in root tip squashes.

Some studies have exploited the active plant growth regulators 2, 4-D for anther culture of *Helianthus annuus* L. (Bohorova *et al.*, 1980). It was decided to examine the potential of this plant growth regulator on three cultivars of *Helianthus annuus* L. both alone and in combination with BAP.

TABLE 3.6.1.13 Effect of 2, 4-D and BAP on the subsequent development of anthers of *Helianthus annuus* L. in liquid/solid culture

	Callus Initiation (After 4 weeks)		
	Cultivar		
Medium	**Twyford A**	**Twyford C**	**Direct Embryogenesis**
MS basic medium	0	0	0
MS + 2, 4-D 0.1mg/l + BAP 0.1mg/l	0	0	0
MS + 2, 4-D 0.5mg/l + BAP 0.5mg/l	0	0	0
MS + 2, 4-D 1.0mg/l + BAP 1.0mg/l	0	0	0
MS + 2, 4-D 2.0mg/l + BAP 2.0mg/l	0	0	0

* Results identical for solid + liquid cultures

TABLE 3.6.1.14 Effect of 2, 4-D on the subsequent development of anthers of Helianthus annuus L. in liquid/solid culture

Medium	Callus Initiation (After 4 weeks)	Direct Embryogenesis
MS basic medium	0	0
MS + 2, 4-D 0.1mg/l	0	0
MS + 2, 4-D 0.5mg/l	0	0
MS + 2, 4-D 1.0mg/l	0	0
MS + 2, 4-D 2.0mg/l	0	0

As with other plant growth regulator studies, there was no direct embryogenesis in any media containing 2, 4-D. However, unlike all previous experiments there was no subsequent callus initiation for any cultivar either grown in a combination 2, 4-D/BAP or for cv. "Sunburst" when grown in a media containing 2, 4-D alone. These experiments were carried out using both solid and liquid media. Both approaches failed to initiate any further development from the anthers.

GENERAL DISCUSSION

Most workers attempting to produce haploid plants by exploiting anther/gametophyte culture have indicated that direct embryogenesis is the preferred route (Berlyn et al., 1986). Plants created by this approach are considered more stable, genetically, than those regenerated via organogenesis. Media supplementation with auxins and cytokinins has been shown to be successful for embryogenic development following anther culture for a wide range of species (Nitsch, 1969 and Rashid and Street, 1973). In this study, media modifications did not lead to direct embryogenesis but rather to callus formation. This is a feature of other studies aiming to produce haploids via anther culture in Helianthus annuus L. However, Bohorova et al., (1980) did manage to produce a very small number of plants directly when utilising zeatin as the cytokinin. In these studies only BAP

was used as the cytokinin and most effort was centred on examining the possible role of the different auxins currently exploited in tissue culture.

Subsequent callus growth was influenced by the plant growth regulators included in the media. Low concentrations of NAA + BAP showed maximum callus growth as compared to IBA + BAP and IAA + BAP, where higher concentrations gave better results. However, overall with the exception of 2, 4-D, there appears to be no real difference between success rate in terms of initiation, subsequent growth and shoot regeneration between any of the auxin type plant growth regulators used in the experiments. Similar results were obtained by Plotnikov (1975) who was able to produce regenerants with the modification of the MS medium with NAA, IBA, IAA and kinetin, although these plantlets did not survive to maturity.

Shoot organogenesis tended to occur at low levels of auxins/cytokinins with only particular cultivars giving consistent production, and only one, cv. "Holiday" giving substantial numbers. Shoot organogenesis, however, did not necessarily lead to the production of plants capable of growing in vivo. Considerable difficulties were experienced in encouraging root growth on these shoots. Half strength MS medium with no plant growth regulator was used, not particularly successfully, but primarily to ensure that these shoots were not engulfed by subsequent callus growth when placed on regeneration media containing an altered hormonal balance.

Of the total of 129 shoots produced only 5 regenerated a root system. Cytologically the results obtained from these 5 plants were not encouraging and showed a lack of consistency with respect to ploidy status. So it was not possible to indicate whether these plants were the product of haploid or diploid callus sources.

3.6.2 Effect of complex organic additives, alternative carbon sources, charcoal and pretreatment on the subsequent development of cultured anthers of Helianthus annuus L.

In addition to the use of auxins and cytokinins, normally as highly refined products, as media additives to improve response in culture, workers have also used a wide range of alternative approaches similarly aimed to give greater efficiency in culture.

Early workers in this area, often used rather undefined additives to media. Coconut water and casein hydrolysate have been used to improve the frequency of embryoid formation from anthers of a number of species (Guhua and Maheshwari, 1964). Most success from this aproach has been observed in species which produces binuclear pollen (Guhua and Maheshwari, 1967). Helianthus annuus L., like many other trinuclear species tends to produce callus rather than direct embryogenesis following anther culture (Sunderland and Dunwell, 1977). However, some workers have noted improved efficiency of embryoid formation after the addition of such undefined media additives even in tricellular species (Marburger et al., 1987).

Charcoal has been used by a number of workers to stimulate improved response in anther culture. It is thought that charcoal works as sink. It absorbs the 5-hydromethylfurfural compound that is produced during the autoclaving of sucrose and which is thought to have an inhibitory effect. Moreover, charcoal is also thought to absorb other inhibitory substances produced during the culture phase and thereby reduces the number of potential pollen embryos that would normally have been aborted (Weatherhead et al., 1978). Drastic improvements in efficiency following the incorporation of charcoal into the media have observed in Nicotiana (Anagnostakis, 1974), rye (Wenzel et al., 1977) and potato (Sopory et al., 1978).

A number of recent studies, examining embryoid formation in anther culture, have indicated that the carbon source used in the culture media can have a significant effect on the efficiency of embryogenic development. Most notably Finnie, et al., (1989) working with barley, observed a dramatic improvement in embryogenic development. Other workers have noted that sucrose may have an inhibitory effect on embryogenic development (Hunter, et al., 1989). Most workers examining embryogenic development from Helianthus annuus L. pollen/anthers have used sucrose as a carbon source and no knowledge is available on whether it is most suitable for use in this type of culture for this species.

A number of studies have demonstrated the efficiency of pretreatment of excised flower buds prior to culture. Most of these relate to temperature stress as a stimulus for subsequent desired development in culture. Notable success has been observed in Nicotiana (Sunderland and Roberts, 1979), and Hordeum vulgare (Huang and Sunderland, 1982). Most work in this area has been carried out using species which tend to develop via direct embryogenesis rather than by callus initiation and subsequent organogenesis. There are no reports on the efficacy of this approach in Helianthus, to see whether embryogenic development is enhanced or whether there is an effect on callus initiation and differentiation.

A series of experiments were carried out to investigate whether these approaches, complex organic additives (Coconut water), charcoal, alternative carbon source and flower bud pretreatment, would have a stimulatory effect on pollen/anther development in culture.

TABLE 3.6.2.1. Influence of coconut water on the subsequent development of anthers of Helianthus annuus L. in culture

Medium	Callus Initiation (After 4 weeks)	Direct Embryogenesis
MS basic medium	0	0
MS + 5% C.W.	0	0
MS + 10% C.W.	0	0
MS + 15% C.W	0	0
MS + 20% C.W.	0	0
MS + 25% C.W.	0	0
MS + 30% C.W.	0	0

The addition of coconut water did not have any stimulatory effect on embryogenesis. Indeed no callus formed either. The experiment was repeated with coconut water added using microfilter to ensure that autoclaving of the media plus coconut water did not cause the destruction/modification of the coconut water such that it was inhibitory to subsequent development. Regardless of the method of incorporation, the results were the same.

TABLE 3.6.2.2. Effect of charcoal on the subsequent development of anthers of Helianthus annuus L. in culture

Cultivar	Callus Initiation (After 4 weeks)	Direct Embryogenesis
MS basic medium	0	0
MS + 2% charcoal	0	0

As with the addition of coconut water, charcoal did not appear to have any effect on subsequent development. Obviously the anthers required other externally applied stimulants in order to develop, but these, particularly in the form of auxin and cytokinin were not included in the experiment since experience had shown these tended to lead to callus development rather than embryogenesis.

TABLE 3.6.2.3 Embyrogenesis/callus initiation of anthers of 3 cultivars of Helianthus annuus L. in culture on media containing maltose plus a range of auxin/cytokinin additions

(Arcsin transformed data, measured after 4 weeks)

	Twyford A				Twyford C				Sunburst			
	Sucrose		Maltose		Sucrose		Maltose		Sucrose		Maltose	
MS Basic Medium	D.E.	C.I.	D.E.	C.I.	D.E.	C.I.	D.E.	C.I.	D.E.	C.I.	D.E.	C.I.
MS + IAA 1mg/l	0	0	0	0	0	0	0	0	0	0	0	0
MS + IAA 5mg/l	0	0	0	0	0	0	0	0	0	0	0	0
MS + IAA 10mg/l	0	0	0	0	0	0	0	0	0	0	0	0
MS + BAP 1mg/l	0	0	0	0	0	0	0	0	0	0	0	0
MS + BAP 5mg/l	0	0	0	0	0	0	0	0	0	0	0	0
MS + BAP 10mg/l	0	0	0	0	0	0	0	0	0	0	0	0
MS + BAP 1mg/l + IAA 1mg/l	0	90.0	0	0	0	90.0	0	0	0	80.0	0	26.60
MS + BAP 1mg/l + IAA 5mg/l	0	0	0	0	0	90.0	0	0	0	67.5	0	17.5
MS + BAP 1mg/l + IAA 10mg/l	0	0	0	0	0	0	0	0	0	0	0	26.6
MS + BAP 5mg/l + IAA 1mg/l	0	0	0	0	0	90.0	0	0	0	90.0	0	48.5
MS + BAP 5mg/l + IAA 5mg/l	0	90.00	0	0	0	90.0	0	0	0	84.3	0	23.6
MS + BAP 5mg/l + IAA 10mg/l	0	0	0	0	0	0	0	0	0	0	0	22.8
MS + BAP 10mg/l + IAA 1mg/l	0	0	0	0	0	0	0	0	0	0	0	68.9
MS + BAP 10mg/l + IAA 5mg/l	0	0	0	0	0	0	0	0	0	0	0	55.6
MS + BAP 10mg/l + IAA 10mg/l	0	0	0	0	0	0	0	0	0	0	0	46.1

D.E. Direct embryogenesis
C.I. Callus initiation

There was no embryogenic development apparent in any media. For cv. (Sunburst) on media containing single hormone additives, the presence of maltose instead of sucrose, did not show any stimulatory effect with respect to callus initiation. Indeed, overall where comparison was possible, maltose appears to have an inhibitory effect on callus initiation. This callus material did not undergo any shoot organogenesis, even after an extended growth period.

TABLE 3.6.2.4 Pretreatment temperature-stress (4°c) on the subsequent development of anthers of Helianthus annuus L. in culture

Medium	Callus Initiation (Arcsin transformed data) (After 4 weeks) T1(Control)	T2(Pretreated)	Direct Embryogenesis
MS basic medium	00.00	00.00	0
MS + NAA 1mg/l + BAP 1mg/l	83.88	90.00	0
MS + NAA 5mg/l + BAP 5mg/l	80.82	90.00	0
MS + NAA 10mg/l + BAP 10mg/l	80.82	71.90	0
MS + IBA 1mg/l + BAP 1mg/l	90.00	90.00	0
MS + IBA 5mg/l BAP 5mg/l	86.94	81.62	0
MS + IBA 10mg/l + BAP 10mg/l	90.00	85.60	0
MS + 1AA 1mg/l + BAP 1mg/l	79.66	64.30	0
MS + IAA 5mg/l + BAP 5mg/l	83.88	62.62	0
MS + 1AA 10mg/l + BAP 10mg/l	83.88	75.76	0
S.E.	3.361		

TABLE 3.6.2.5 Pretreatment temperature stress (4°c) on the subsequent anther callus growth of Helianthus annuus L. in culture

Mean Callus Growth (mm)
(Increase in size over original anther structure after 4 weeks)

Medium	T1(Control)	T2(Pretreated)
MS basic medium	0.00	0.00
MS + NAA 1mg/l + BAP 1mg/l	4.92	4.83
MS + NAA 5mg/l + BAP 5mg/l	2.16	4.83
MS + NAA 10mg/l + BAP 10mg/l	1.27	2.63
MS + IBA 1mg/l + BAP 1mg/l	5.16	5.30
MS + IBA 5mg/l + BAP 5mg/l	4.10	7.63
MS + IBA 10mg/l + BAP 10mg/l	3.43	5.76
MS + IAA 1mg/l + BAP 1mg/l	1.35	2.76
MS + IAA 5mg/l + BAP 5mg/l	3.39	2.96
MS + IAA 10mg/l + BAP 10mg/l	3.36	4.86
S.E.	0.386	

As with the other approaches aimed at stimulating embryogenenic development, pretreatment of flower bud at the correct stage of anther of plating, did not give any positive results. The pretreatment did have a significant (Appendix 7.1, 7.2) effect for this range of media/auxin-cytokinin balance on cv. "Sunburst". The initiation of callus was reduced as a result of the pretreatment. However, subsequent growth of the callus as measured by the increase in size over the original anther structure appears to be

enhanced as a result of this pretreatment of the buds. Overall the callus produced on different media grew faster when chilling pretreatment was applied.

GENERAL DISCUSSION

As possible approaches to stimulate embryogenic development, or to result in improvement of shoot organogenesis following callus initiation, these were not a success. None of the approaches resulted in embryogenic development. Indeed, the inclusion of maltose was inhibitory on callus initiation and subsequent shoot organogenesis. Pretreatment appear to have a stimulatory effect on callus growth, but which did not then lead to greater shoot organogenesis. Thus, it would appear that sucrose is the better carbon source, but whether it is the best, requires further experimentation. Similarly, whether the incorporation of charcoal into the media has any advantages to offer requires experiments to investigate the use of charcoal combined with auxin/cytokinin inclusion in the media. The value or otherwise of pretreatments for anther culture in Helianthus annuus L. is not confirmed from the results obtained in this study. The length of pretreatment at low temperature may be significant. The arbitrary choice to use a period of 30 days may not be optimum. Further experiments would need to be carried out to determine whether the length of time of pretreatment had any determinant effect.

In retrospect many of these exploratory experiments would be better repeated using cultivars which were known to give consistent and repeatable results. The initial choice of cultivars was arbitrary. In other experiments, the cv. "Holiday" was found to be the most positive to work with, based on the results obtained examining the role of auxins and cytokinin on embryogenic development or callus initiation and subsequent shoot organogenesis.

However, by the time this was determined, it was rather too late to initiate investigations of alternative stimuli for embryogenic development using cv. "Holiday".

3.6.3. Regeneration from Callus

The previous studies examining the role of auxin/cytokinin and other treatments revealed that callus often formed but did not undergo any further development or development was sporadic. Initiation of callus is thought to involve a complex relationship between the plant material used to initiate the callus, the composition of the medium and environmental conditions during the incubation period (Dodds and Roberts, 1985). Media requirements for organogenesis may be different from media designed to promote callus initiation. Organogenesis is thought to be controlled by a balance between auxin and cytokinin (Skoog and Tsui, 1948). Root organogenesis was observed to occur after the culture tissue had produced buds. Shoot development undoubtedly alters the endogeneous hormones within the culture (Gresshoff, 1978). Shoot initiation is thought to be induced by the appropriate balance of exogeneous auxin and cytokinin, and in some cases, either one or other of these growth regulators must be omitted from the medium in order to produce buds (Street, 1977). For some species, reduced availability of nutrient requirements may be a stimulus to organogenesis.

Regeneration from barley callus frequently led to the production of albino plants (Malepszy and Grunewaldt, 1974). Alteration of media, particularly by reducing to half strength MS plus 1mg/l IAA, and 2mg/l BA (Benzyl adenine) led to the improvement in the frequency of green shoots (up to 40% buds). Subsequent transfer of these green buds to half strength MS agar medium without hormone additives led to root organogenesis Sunderland *et al.*, 1981).

Bohorova et al., (1980) investigated the requirements for media aimed at stimulating shoot organogenesis in Helianthus annuus L. Their study indicated that additional aminoacids in particular adenine, aspergine and glutamine were also necessary for shoot organogenesis in addition to auxins/cytokinins.

The aim of this investigation was to determine whether callus could be further stimulated to undergo differentiation leading to shoot production. Callus from 4 cultivars (Sunburst, Giant yellow, Holiday and Uniflorous giganteus) produced on media containing auxin such as NAA, IBA and IAA and cytokinin BAP were examined for subsequent development on 9 possible regeneration media. The regeneration media investigated included:

Regeneration Medium	**Cultivar**
RM1.MS + NAA 0.1mg/ + BAP 0.1mg/l	Sunburst Giant yellow
RM2.MS + NAA 0.5mg/l + BAP 0.5mg/l	
RM3.MS + NAA 1.0mg/l + BAP 1.0mg/l	
RM4.MS + NAA 2.0mg/l + BAP 2.0mg/l	
RM5.MS + NAA 5.0mg/l + BAP 5.0mg/l	
RM6.MS + NAA 1.0mg/l + Kin 1.0mg/l	Giant yellow
RM7.MS + NAA 5.0mg/l + Kin 5.0mg/l	
RM8.MS + NAA 10.0mg/l + Kin 10.0mg/l	
RM9.MS + 1AA 0.2mg/l + BAP 2.0mg/l + Adenine 20mg/l + L. Aspergine 800mg/l + L. Glutamine 800mg/l	Holiday Uniflorous giganteus

Experiments to examine regeneration were dictated, in part, by the availability of a suitable quantity of nodular callus.

TABLE 3.6.3.1. Root/shoot organogenesis of callus of Sunburst/ Giant yellow grown on media with reduced levels of NAA/BAP

Initial Medium	Regeneration Medium	Root Organogenesis	Shoot Organogenesis
MS + NAA 1mg/l + BAP 1mg/l	MS + NAA 0.1mg/l + BAP 0.1mg/l	0	0
MS + NAA 5mg/l + BAP 5mg/l	-	0	0
MS + NAA 10mg/l + BAP 10mg/l	-	0	0
MS + NAA 1mg/l + BAP 1mg/l	MS + NAA 0.5mg/l + BAP 0.5mg/l	0	0
MS + NAA 5mg/l + BAP 5mg/l	-	0	0
MS + NAA 10mg/l + BAP 10mg/l	-	0	0
MS + NAA 1mg/l + BAP 1mg/l	MS + NAA 1.0mg/l + BAP 1.0mg/l	0	0
MS + NAA 5mg/l + BAP 5mg/l	-	0	0
MS + NAA 10mg/l + BAP 10mg/l	-	0	0
MS + NAA 1mg/l + BAP 1mg/l	MS + NAA 2.0mg/l + BAP 2.0mg/l	0	0
MS + NAA 5mg/l + BAP 5mg/l	-	0	0
MS + NAA 10mg/l + BAP 10mg/l	-	0	0
MS + NAA 1mg/l + BAP 1mg/l	MS + NAA 5.0mg/l + BAP 5.0mg/l	0	0
MS + NAA 5mg/l + BAP 5mg/l	-	0	0
MS + NAA 10mg/l + BAP 10mg/l	-	0	0

MS + IBA 1mg/l + BAP 1mg/l	MS + NAA 0.1mg/l + BAP 0.1mg/l	0	0
MS + IBA 5mg/l + BAP 5mg/l	-	0	0
MS + IBA 10mg/l + BAP 10mg/l	-	0	0
MS + IBA 1mg/l + BAP 1mg/l	MS + NAA 0.5mg/l + BAP 0.5mg/l	0	0
MS + IBA 1mg/l + BAP 1mg/l	MS + NAA 0.5mg/l + BAP 0.5mg/l	0	0
MS + IBA 5mg/l + BAP 5mg/l	-	0	1 S
MS + IBA 10mg/l + BAP 10mg/l	-	0	0
MS + IBA 1mg/l + BAP 1mg/l	MS + NAA 1.0mg/l + BAP 1.0mg/l	0	0
MS + IBA 5mg/l + BAP 5mg/l	-	0	0
MS + IBA 10mg/l + BAP 10mg/l	-	0	0
MS + IBA 1mg/l + BAP 1mg/l	MS + NAA 2.0mg/l + BAP 2.0mg/l	0	0
MS + IBA 5mg/l + BAP 5mg/l	-	0	0
MS + IBA 10mg/l + BAP 10mg/l	-	0	0
MS + IBA 1mg/l + BAP 1mg/l	MS + NAA 5.0mg/l + BAP 5.0mg/l	0	0
MS + IBA 5mg/l + BAP 5mg/l	-	0	0
MS + IBA 10mg/l + BAP 10mg/l	-	0	0
MS + IAA 1mg/l + BAP 1mg/l	MS + NAA 0.1mg/l + BAP 0.1mg/l	0	0
MS + IAA 5mg/l + BAP 5mg/l	-	0	0
MS + IAA 10mg/l + BAP 10mg/l	-	0	0

MS + IAA 1mg/l + BAP 1mg/l	MS + NAA 0.5mg/l + BAP 0.5mg/l	0	0
MS + IAA 5mg/l + BAP 5mg/l	-	0	0
MS + IAA 10mg/l + BAP 10mg/l	-	0	0
MS + IAA 1mg/l + BAP 1mg/l	MS + NAA 1.0mg/l + BAP 1.0mg/l	0	0
MS + IAA 5mg/l + BAP 5mg/l	-	0	0
MS + IAA 10mg/l + BAP 10mg/l	-	0	0
MS + IAA 1mg/l + BAP 1mg/l	MS + NAA 2.0mg/l + BAP 2.0mg/l	0	0
MS + IAA 5mg/l + BAP 5mg/l	-	0	0
MS + IAA 10mg/l + BAP 10mg/l	-	0	0
MS + IAA 1mg/l + BAP 1mg/l	MS + NAA 5.0mg/l + BAP 5.0mg/l	0	0
MS + IAA 5mg/l + BAP 5mg/l	-	0	1 S
MS + IAA 10mg/l + BAP 10mg/l	-	0	0

Most results were disappointing. Regeneration of single shoots only occurred on two media, but these shoots failed to survive. Thus, it would appear that a reduction in hormonal constituents of the media did not stimulate further regeneration. These experiments were repeated, substituting kinetin for BAP in the regeneration media. The results obtained (not presented) were entirely consistent with the earlier attempt at regeneration giving no further organogenesis.

Bohorova et al., (1980) found that MS media containing IAA 0.2 mg/l, BAP 2.0 mg/l. Adenine 20 mg/l, L. Asparagine 800 mg/l and L. Glutamine 800 mg/l

stimulated regeneration in the materials included in their study. It was decided to investigate whether the particular media would have a stimulatory effect for calli of cv's. "Holiday" amd "Uniflorous giganteus". The regeneration media did not stimulate buds on any extensive scale (results not presented). Indeed, on only 2 calli of cultivar "Holiday" buds initiated and these did not develop further. It may be that the genotype of the initial source of callus may have a major influence on the subsequent ability of the callus to differentiate and that the source of callus used in this study was inappropriate.

3.6.4 General Conclusions

The number of possible variables to be investigated in a study of this kind can be enormous. Permutations may include construction of the media, particularly major/minor elements constituent levels/plant growth regulators levels/carbon sources/aminoacid supplementation, as well as pretreatment growth conditions, and culture environment in terms of light/temperature. It was not possible in the time and resources available to examine all the variables. Efforts concentrated on plant growth regulators complex organic supplements and donor plant pretreatment. Overall the results were extremely disappointing in terms of encouraging direct embryogenesis. All the investigations carried out resulted in either no further development, or the development of callus. Therefore, the precise requirements for a media which promotes direct embryogenesis requires much further investigation. Such approaches would include examination of other plant growth regulators, particularly alternative cytokinin (zeatin) type hormones, either in suitable combinations with an auxin or in combination perhaps with other media additives.

The callus produced, whether from haploid or diploid sources, in these studies was difficult to exploit in terms of regeneration. Results obtained for the initiation of organogenesis, were somewhat variable. No single media and combination of plant growth regulators gave consistently better results in terms of shoot organogenesis. Again, it was not possible in the time available to investigate all the possible culture variables which might influence the rate of organogenesis. Additional variables which might be worthy of further investigation include alternative plant growth regulators, again particularly for the cytokinin and amino acid supplementation. Even where success was achieved in terms of shoot organogenesis as in cv. "Holiday", difficulties were experienced in transferring the shoots produced on primary callus to in vivo conditions. Root organogenesis was not observed at all on primary callus from anther sources. The approach adopted to encourage root development, transfer to half strength MS media was not particularly successful. Further experimentation should include examination of hormonal addition to the subculture transfer media as well as other possible horticultural techiques for the direct transfer of shoots to in vivo conditions without prior root organogenesis.

As this work progressed, it became apparent that from the range of cultivars used, only two cv's "Sunburst" and "Holiday" gave positive results, as represented by shoot organogenesis. Both of these ornamental cultivars are based on open pollinated populations and can be described as having multi-branched characteristics with small individual flower heads. Future work may concentrate using these responsive cultivars, particularly in relation to determining requirements for the initiation of organogenesis from anther callus, and possibly for determining the requirements for subsequent root initiation or direct transfer to in vivo conditions. For studies on direct

embryogenesis, these cultivars do not appear to have any particular advantage over any other cultivar examined. Therefore, unless there is a link between callus shoot organogenesis and direct embryogenesis under different but suitable conditions, there is no advantage in using these particular cultivars for studies on direct embryogenesis. Indeed, it may be that insufficient genotypes/populations were examined and that future studies should widen the genetic range of material to be examined.

Exploitation of haploid production in a breeding programme is really only justified if the ease of production of homozygous individuals by this route is favourable as compared to traditional inbreeding approaches. This comparison lies some way off in the future. A reliable protocol for the production of haploids by direct embryogenesis still awaits definition. The evidence accumulated in the study does not encourage the view that these conditions are going to be defined quickly.

SECTION 4

TISSUE CULTURE

INTRODUCTION

Genetic variability is the basis of crop improvement. Traditionally, this variability was found occurring spontaneously in crops or related species (Hockett and Nilan, 1985). Induced variability using chemicals/physical mutagens has been exploited with mixed success for some species over the last 40-50 years (Nilan, 1981b). More recently, many plant breeders have sought to exploit the variability commonly found arising from tissue culture (Evans et al, 1984). This somaclonal variation, may be thought as variation arising via some ill-defined mutagenic treatment as a result of culture or to the culture releasing the variability pre-existing between cells of some plants (Moseman and Smith, 1985). One of the attractions of this approach for plant breeders lies in the possibility of exposing plant materials to selection pressures whilst in the culture phase. Before any such system can be utilised it is necessary to have an efficient protocol for callus initiation followed by the regeneration of free living plants from this callus. Plantlets produced via callus followed by organogenesis may be cytologically variable. Factors known to influence the level of callus and subsequent differentiation have been investigated for many plant species. However, relatively little work has been published on this subject for Helianthus annuus L. Plant growth regulators supplementation has, however, been found to be important for the initiation of callus in Helianthus annuus L. (Greco et al., 1984). The aim of this study was to establish an efficient plant tissue technique which represents a basic step in non conventional improvement of crop plants. Plant regeneration from

multicellular calli has been obtained in numerous crop species while attempts at regeneration from single cells and protoplast have been relatively unsuccessful (Vasil et al., 1977). The overall aim of this study was to observe the effect of different plant growth regulators and aminoacids at different concentrations, alone or in combination on callus initiation of hypocotyl explants of Helianthus annuus L.

4.1 EXPERIMENTAL MATERIALS

The four cultivars of Helianthus annuus L. of different origin used in this study are given in Table 4.1.1.

TABLE 4.1.1 Source of Helianthus annuus L. used in experimental studies

Cultivar	Source	Agric/Hort.	Origin
Giant yellow	Suttons seeds	Agric.	U.K.
Twyford A	Twyford seeds	"	U.S.A.
Twyford B	"	"	U.S.A.
Twyford C	"	"	U.S.A.

4.2 RAISING OF EXPLANT MATERIAL

Achenes were surface sterilized by treating them with 70% ethanol for two minutes, followed by 30% solution of sodium hypochlorite with a few drops of Tween 20 as wetting agent and finally washed twice with distilled water to remove the last traces of sterilant. The achenes were then sown in sterilized jars containing water agar media with tilt fungicide (10µg/l), penicillin (50mg/l) and streptomycin (100mg/l) to avoid contamination from seed born fungus and bacteria respectively. Earlier studies have indicated that surface sterilization alone was not sufficient to eradicate fungal and bacteria contamination. Extended periods of surface sterilization were not beneficial with respect to seed germination.

4.3 PLATING AND INCUBATION OF CULTURES

Hypocotyl segments of 4mm average length were used from these seedlings as initial explant material. Four explants were placed on the medium in each petri dish (5cm dia.) Each medium was replicated 5 times giving 20 explants per treatment. The cultured petri dishes were sealed with parafilm and incubated at a temperature of 20°C-27°C and light of 500 lux for 16 hours daylength regime.

4.4 BASAL MEDIA USED FOR TISSUE CULTURE

See Section 3.4.

4.4.1 Media Preparation

See Section 3.4.1

4.5 PLANT GROWTH REGULATORS/OTHER ADDITIVES USED

Auxin

1-naphthalene acetic acid (NAA) (Cat. No. 1641)

Cytokinin

6-benzylaminopurine (BAP) (Cat. No. 1.1875)

Gibberellin

Gibberellic acid (GA3) (Cat. No. G 3250)

From: Sigma Chemicals Co. Ltd., Fancy Road, Poole, Dorset, England, BH17 7NH.

Aminoacid

Casamino acids (Cat. No. B 230)

From: Difco Laboratories Ltd., P.O. Box, Central Avenue Estate Morsey, Surrey, U.K. KT. Bose.

Others

Adenine sulphate	(Cat. No. A.9126)
Penicillin	(Cat. No. PEN-P)
Streptomycin	(Cat. No. S.6501)

From: Sigma Chemicals Co.Ltd.P.O.Box, Central Avenue Estate, Morsey, Surrey, U.K. KT. Bose.

Tilt

Ciba-Geigy Agrochemicals, Whittlesford, Cambridge, CB2 4QT.

4.6 EXPERIMENTAL APPROACH

An appraisal of the literature on all aspects of tissue culture of Helianthus annuus L. indicated subsequent development in culture normally lead to the production of callus (Patterson and Everett, 1985). Exploitation of callus of Helianthus annuus L. has been limited due to the difficulties of obtaining regenerated plants (Freyssinet and Freyssinet, 1988). The overall aim of this study was the production of haploid plants via embryogenesis of gametophyte material. However, it was decided that an appreciation of the problems of callus initiation and subsequent regeneration from explant sources other than anthers would be of benefit to the overall aim, as well as giving a small insight into the possible exploitation of callus for the generation of variation of value to plant breeders.

Many previous studies both with Helianthus and other species have indicated the importance of correct auxin/cytokinin balance in the media, both for the initiation of callus and for the subsequent differentiation (Skoog and Tsui, 1948). Similarly, some workers have indicated that supplementation with certain amino acid, particularly casamino acids have a beneficial effect on the regeneration of Helianthus annuus L. callus (Paterson and Everett, 1985). As a result of this previous work, it

was decided to concentrate efforts on three areas; investigation of the effects of plant growth regulators, NAA, BAP, and GA_3 alone or in combination on callus initiation, subsequent growth and differentiation; investigating the effects of casamino acids, alone or in combination with plant growth regulators on callus initiation and subsequent growth, and finally the examination of the plants regenerated.

4.6.1. Effect of NAA, BAP and GA_3 on Callus initiation, Callus growth and subsequent organogenesis of roots and shoots in culture

There have been few investigations of the tissue culture requirements of Helianthus annuus L. (Greco, et al., 1984). Most workers used a range of plant growth regulator additions to the basic media (Paterson and Everett, 1985). Rather than investigate a wide range of auxin/cytokinin type plant growth regulators, efforts were concentrated on examining the effects of only 3, (NAA/GA_3/BAP) on callus initiation/callus growth and the subsequent regeneration of roots/shoots. The results obtained are given in Tables 4.6.1.1 - 4.6.1.4.

TABLE 4.6.1.1 Effect of plant growth regulators on the subsequent callus growth of hypocotyl of Helianthus annuus L. in culture

Callus Growth (mm) (Mean of 20 explants)

(Increase in size over the original explant after 4 weeks)

	Cultivar			
Treatment	**Giant yellow**	**Twyford A**	**Twyford B**	**Twyford C**
MS basic media	3.93	4.20	2.80	4.65
MS + BAP 1mg/l	6.25	7.45	8.25	6.80
MS + NAA 1mg/l	4.80	8.20	9.50	4.60
MS + GA_3 0.1mg/l	6.45	6.15	5.40	5.45
MS + NAA 1mg/l + BAP 1mg/l	4.60	7.35	6.90	7.50
MS + BAP 1mg/l + GA_3 0.1mg/l	11.00	5.35	6.85	6.75
MS + NAA 1mg/l + GA_3 0.1mg/l	4.95	6.85	8.50	5.65
S.E.	0.726			

TABLE 4.6.1.2 Effect of plant growth regulators on root organogenesis on Primary hypocotyl callus of Helianthus annuus L. after 4 weeks

Root organogenesis on primary hypocotyl callus (Arcsin transformed data)

	Cultivar			
Treatment	**Giant yellow**	**Tywford A**	**Twyford B**	**Twyford C**
MS basic media	67.2	18.4	12.9	-
MS + BAP 1mg/l	12.9	22.8	12.9	18.4
MS + NAA 1mg/l	90.0	90.0	12.9	90.0
MS + GA_3 0.1mg/l	-	30.0	-	-
MS + BAP 1mg/l + NAA 1mg/l	30.0	36.3	-	-
MS + BAP 1mg/l + GA_3 0.1mg/l	22.8	12.9	-	-
MS + NAA 1mg/l + GA_3 0.1mg/l	39.2	42.1	-	71.6
S.E.	9.011			

N.B. In all Tables in section 4, - represents 0.0

TABLE 4.6.1.3 Effect of plant growth regulators on shoot organogenesis on primary hypocotyl callus of Helianthus annuus L. after 4 weeks

Shoot organogenesis on primary hypocotyl callus (Arcsin transformed data)

	Cultivar			
Treatment	**Giant yellow**	**Twyford A**	**Twyford B**	**Twyford C**
MS basic media	18.4	22.8	-	-
M-S + BAP 1mg/l	12.9	30.0	30.0	18.4
MS + NAA 1mg/l	12.9	-	12.9	-
MS + GA_3 0.1mg/l	18.4	12.9	18.4	-
MS + BAP 1mg/l + NAA 1mg/l	-	-	-	
MS + BAP 1mg/l + GA_3 0.1mg/l	-	12.9	22.8	12.9
MS + NAA 1mg/l + GA_3 0.1mg/l	39.2	12.9	12.9	12.9
S.E.	4.115			

Results described in Tables 4.6.1.3 and 4.6.2.3. were originally obtained in a single large experiment. The data has been presented as tables with the same control data.

TABLE 4.6.1.4 Effect of Plant growth regulators on subsequent callus weight of hypocotyl of Helianthus annuus L. (with Twyford B) in culture

Initiation Media	Subculture Media	Mean Callus Weight (gms)
MS basic media	+ BAP 2mg/l	4.16
MS + BAP 1mg/l	+ BAP 2mg/l	3.31
MS + NAA 1mg/l	+ BAP 2mg/l	4.67
MS + GA_3 0.1mg/l	+ BAP 2mg/l	3.30
MS + BAP 1mg/l + NAA 1mg/l	+ BAP 2mg/l	4.90
MS + BAP 1mg/l + GA_3 0.1mg/l	+ BAP 2mg/l	3.09
MS + NAA 1mg/l + GA_3 0.1mg/l	+ BAP 2mg/l	6.20
S.E.		0.368

Callus was initiated on all the media examined, even when plant growth regulators were not present. Subsequent growth and differentiation of callus into roots and shoots were variously influenced by genotype origin and particular treatment. Callus growth as measured by increase in size (Table 4.6.1.1) was influenced significantly (Appendix 8.1) by the genotype origin and the particular combination of plant growth regulators. Certainly BAP alone or in combination with GA_3 produced the most callus as measured simply as increase in size over original explant for all cultivars. Of the varieties, Twyford B proved to be the most responsive over all treatments. However, taking the individual responses of Twyford B did not necessarily accord with the overall mean treatment effects and tended to show maximum growth of callus to be associated with NAA inclusion in the media. Callus growth recorded as increase in size masks the different type of callus which can be produced by a particular genotype/treatment combination. When callus weight after a fixed period was used as an indicator of growth (for cultivar Twyford B only, Table 4.6.1.4) BAP inclusion alone or in combination with GA_3 resulted in significantly (Appendix 8.4) less callus weight than in media in which NAA was included.

This can be interpreted in terms of the different type of callus initiated with the different plant growth regulators, with NAA alone or in combination with GA_3 tending for this cultivar, to lead to the fast production of friable callus, whereas BAP led to the production of compact nodular callus.

Hormone addition to the media influenced significantly (Appendix 8.2) the incidence of root organogenesis from calli of all cultivars. NAA alone gave the best results for most cultivars with the single exception of Twyford B, which was significantly non typical for the incidence of root organogenesis. Shoot organogenesis on primary callus occurred on many of the media examined, but no significant (Appendix 8.3) trend was found in relation to hormone additions or cultivars. The shoots produced on primary callus were encouraged to continue development and some were transferred to soil in order to facilitate the morphological examination of and comparison with source mother plants (see 4.6.4.1).

GENERAL DISCUSSION

In order to exploit the possible variations arising through organogenesis of callus, it is essential to have a well developed protocol for the production of shoots. The treatments examined in this study were not particularly satisfactory, giving fairly low levels of occurrence of shoot organogenesis. BAP inclusion with basic media gave the most shoots, although not significantly more than other treatments. Follow up studies will be needed in order to define further the media requirements for shoot organogenesis. In particular, alternative sources and different levels of cytokinin need to be investigated with a view to establishing a media which will encourage shoot organogenesis on primary callus at a rate which will allow exploitation of the callus phase for selection purposes.

4.6.2 Effect of Casamino acids supplementation alone and in combination with plant growth regulators on callus initiation, callus growth and subsequent organogenesis of roots and shoots in culture.

In addition to the use of auxins and cytokinins as media additions to improve response in culture, researchers have investigated a wide range of other additives with the same overall aim in mind. Paterson and Everett (1985) examining regeneration from callus of a series of inbred lines of Helianthus annuus L. indicated that organic acid supplementation was beneficial. In particular, casamino acids and adenine sulphate were investigated but only casamino acids incorporation led to the improvement in regeneration.

A series of experiments were carried out to investigate whether casamino acids would have a stimulatory effect on callus initiation, growth and subsequent regeneration from a range of sunflower cultivars. The results obtained are presented in Tables 4.6.2.1 - 4.6.2.4.

TABLE 4.6.2.1 Effect of Casamino acids and plant growth regulators on the subsequent growth of hypocotyl callus of Helianthus annuus L. in culture

Callus growth (mm) (Mean of 20 explants)

(Increase in size over the original explant after 4 weeks)

	Cultivar			
Treatment	**Giant yellow**	**Twyford A**	**Twyford B**	**Twyford C**
MS basic media	3.93	4.20	2.80	4.65
MS + Casamino acids 500 mg/l	3.70	4.80	5.35	6.40
MS + BAP 1mg/l + casamino acids 500mg/l	1.20	7.80	7.50	10.10
MS + NAA 1mg/l + casamino acids 500mg/l	4.10	3.15	9.85	5.65
MS + GA_3 0.1mg/l + casamino acids 500mg/l	2.85	9.65	3.85	7.70
S.E.	0.529			

TABLE 4.6.2.2 Effect of casamino acids and plant growth regulators on root organogenesis on primary hypocotyl callus of Helianthus annuus L. after 4 weeks

Root organogenesis on primary hypocotyl callus

(Arcsin transformed data)

Treatment	Cultivar			
	Giant yellow	Twyford A	Twyford B	Twyford C
MS basic media	67.2	18.4	12.9	-
MS + Casamino acids 500mg/l	26.6	39.2	30.0	26.6
MS + BAP 1mg/l + casamino acids 500mg/l	53.7	22.8	-	-
MS + NAA + casamino acids 500mg/l	67.2	50.8	39.2	90.00
MS+ GA_3 + casamino acids 500mg/l	22.8	12.9	-	-
S.E.	7.962			

TABLE 4.6.2.3. Effect of casamino acids and plant growth regulators on shoot organogenesis on primary hypocotyl callus of Helianthus annuus L. after 4 weeks

Shoot organogenesis on primary hypocotyl callus

(Arcsin transformed data)

Treatment	Cultivar			
	Giant yellow	Tywford A	Twyford B	Twyford C
MS basic media	18.4	22.8	-	-
MS + casamino acids 500mg/l	22.8	26.6	12.9	18.4
MS + BAP 1mg/l + casamino acids 500 mg/l	-	30.0	26.6	12.9
MS+ NAA 1mg/l + casamino acids 500mg/l	-	-	12.9	-
MS + GA_3 0.1mg/l + casamino acids 500mg/l	18.4	12.9	18.4	-
S.E.	4.219			

TABLE 4.6.2.4 **Effect of casamino acids and plant growth regulators on the subsequent hypocotyl callus weight of Helianthus annuus L. (with Twyford B) in culture**

Initiation media		Subculture media	Mean callus weight gms
MS basic media	+	BAP 2mg/l	4.16
MS + casamino acids 500 mg/l	+	BAP 2mg/l	3.96
MS + BAP 1mg/l + casamino acids 500 mg/l	+	BAP 2mg/l	3.76
MS + NAA 1 mg/l + casamino acids 500 mg/l	+	BAP 2mg/l	5.78
MS + GA_3 0.1 mg/l + casamino acids 500 mg/l	+	BAP 2mg/l	2.09
S.E.	0.369		

Callus was produced on all media including basal media as control. However, the inclusion of casamino acids stimulated callus growth as measured by increase in size across all cultivars. The combination of casamino acids with hormones further stimulated growth. Varieties differed significantly (Appendix 9.1) with respect to responsiveness to media treatment as measured by increase in size with Giant yellow showing substantially less growth than the other cultivars examined. Twyford B showed maximum response with the inclusion of NAA in addition to casamino acids. This was confirmed when callus weight was examined as the indicator of growth. However, the results (Appendix 9.4) of the experiment examining callus weight also indicates the weaknesses of using increase in size as a measure of growth since the ranking order of the treatments differed with the different indicators of growth.

Roots were found to occur on many media. The inclusion of casamino acids alone does appear to alter significantly (Appendix 9.2) the incidence of root organogenesis in culture. However, the combination of NAA with

casamino acids led to maximum incidence of root organogenesis. Overall it does appear that the inclusion of casamino acids in the media does have a stimulating effect as NAA alone, without casamino acids also gave maximum results, (see Table 4.6.1.2).

Shoot organogenesis for each cultivar occurred sporadically over a range of media with no significant (Appendix 9.3) trends apparent. However, it was interesting to note in this experiment, the addition of casamino acids did lead to increase in shoot initiation across all cultivars when compared with the level occurring on the basal media alone.

Refinement of media requirement for hypocotyl culture of Helianthus annuus L.

As maximum incidence of shoot organogenesis occurred with either casamino acids alone or in combination with BAP, a further experiment was carried out to examine whether refinement in terms of levels of each of these media components could lead to increased callus growth and subsequent organogenesis (Table 4.6.2.5).

(See Table 4.6.2.5)

There were significant (Appendix 9.5) differences between the treatments. Increasing BAP levels resulted in greater callus growth as measured by increase in size, up to 5 mg/l level. Increasing level of casamino acids did not show any consistent trend, with maximum levels of growth (by size) occurring at 250 mg/l. Root and shoot organogenesis was disappointing with no clear pattern of occurrence. Most shoot organogenesis occurred with low levels of BAP (1 mg/l). With increasing level of casamino acids resulting in more organogenesis but not consistently.

TABLE 4.6.2.5 Effect of casamino acids and BAP on the callus growth, root and shoot organogenesis of Helianthus annuus, L (with Twyford B) in culture

Treatment	Callus growth (mm) Mean of 20 explants	Shoot Organogenesis	Root Organogenesis
MS basic media	3.25	-	-
MS + BAP 1mg/l	10.20	3	-
MS + BAP 5mg/l	9.30	-	-
MS + BAP 10 mg/l	10.90	-	-
MS + casamino acids 250 mg/l	5.15	-	-
MS + casamino acids 500 mg/l	4.55	1	1
MS + casamino acids 1000 mg/l	4.40	-	-
MS + casamino acids 250 mg/l + BAP 1mg/l	10.75	5	-
MS + casamino acids 250 mg/l + BAP 5mg/l	13.80	-	-
MS + casamino acids 250 mg/l + BAP 10 mg/l	12.40	1	-
MS + casamino acids 500 mg/l + BAP 1 mg/l	9.15	-	-
MS + casamino acids 500 mg/l + BAP 5 mg/l	11.70	2	-
MS + casamino acids 500 mg/l + BAP 10 mg/l	12.10	-	-
MS + casamino acids 1000 mg/l + BAP 1 mg/l	9.00	6	-
MS + casamino acids 1000 mg/l + BAP 5 mg/l	14.10	-	-
MS + casamino acids 1000 mg/l + BAP 10 mg/l	11.85	-	-
S.E.	0.6		

GENERAL DISCUSSION

From these experiments it appears that the inclusion of casamino acids as a component of the media can lead to greater levels of shoot organogenesis, although this conclusion cannot be supported by statistical interpretation. Combining casamino acids with other hormones did not give any enhancement over casamino acids alone. These experiments illustrate some of the difficulties of determining whether single component addition to the media have beneficial or otherwise effects. Further

experimentation is required to fully clarify whether casamino acids inclusion has any significant beneficial effect.

4.6.3 Regeneration Requirements

Many of the treatments examined and presented in 4.6.1 and 4.6.2 produced primary callus which did not undergo shoot/root organogenesis. With other species, researchers have been able to stimulate development by sub-culturing on media with altered hormonal levels (Street, 1977). Primary callus from a range of treatments were examined for subsequent regeneration on 3 further media (Table 4.6.3.1).

TABLE 4.6.3.1 Regeneration media examined for callus sub-culturing

RM1	=	MS + NAA	1mg/l	+ BAP	1mg/l
RM2	=	MS + NAA	1mg/l	+ BAP	2mg/l
RM3	=	MS + NAA	1mg/l	+ BAP	5mg/l

None of the callus material tested in this way, gave rise to any subsequent development. Only a relatively few media options for regeneration were examined, but it does appear that if development has not been initiated on primary callus, then sub-culturing to stimulate development has very little effect.

4.6.4 Morphological characteristics of regenerated plants

All the cultivars of Helianthus produced shoots (differing in number) in different media treatments. Some shoots developed a root system in the same induction media and others had to be transferred into half strength MS medium before a root system developed. All the shoots which were transferred into half MS strength medium, particularly from Giant yellow,

did not develop a root system. However, these shoots which regenerated roots were transferred into pots containing peat media. The plants in the pots were kept moist and protected with plastic domes, in order to reduce dessication. Despite all the protection measures employed, some plants failed to adjust to the altered environment and died. Those that adjusted to the new environment, survived and flourished to maturity, but were stunted and flowered much earlier than the normal (Figs. 4-8). These plants were studied in detail with respect to their morphological characters to compare them with their parental lines.

TABLE 4.6.4.1 Morphological characteristics of regenerated plants along with their parents

Particular		Height (cms)	Branches/ Plant	Leaves Plant	Petiole Length (cms)	Internode Length (cms)	Leaf area (sq cms)
Twyford A (Parent)		42.00	-	14	5.11	4.33	79.74
Regenerated Plants							
T7	P1	5.00	-	17	0.93	1.33	1.83
	P2	11.50	-	10	1.66	2.50	10.00
	P3	7.50	4	32	1.50	4.33	9.48
	P4	7.00	3	22	0.97	2.16	6.42
	P5	7.00	-	7	0.43	2.50	2.66
T4	P1	7.00	2	10	1.00	1.00	4.42
Twyford B (Parent)		179.00	-	35	8.49	10.65	237.14
Regenerated Plants							
T8	P1	30.00	-	24	1.83	4.00	29.56
	P2	20.00	2	15	2.50	3.05	10.33
T1	P1	50.00	2	16	3.00	6.33	43.50
Twyford C (Parent)		52.00	-	16	6.91	7.32	116.74
Regenerated Plants							
T2	P1	15.00	-	17	1.16	4.67	5.80
T4	P1	15.50	-	20	2.97	3.83	23.00
	P2	11.00	2	18	1.13	2.66	6.68

Figure 4: Hypocotyl derived plant from cultivar Twyford A on MS media with BAP 1mg/l + casamino acids 500mg/l. The regenerated plant was raised in a pot of 13cms diameter and mother plant in a pot of 18cms diameter.

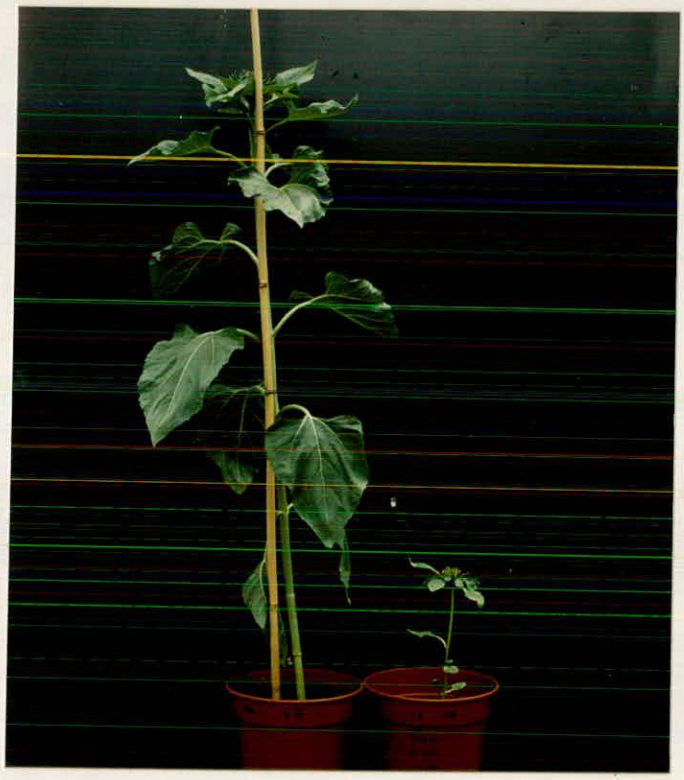

Figure 5: Hypocotyl derived plant from cultivar Twyford B on media MS with BAP 1mg/l + GA_3 0.1mg/l. The regenerated plant was raised in a pot of 18cms diameter and mother plant in a pot of 18 cms diameter.

Figure 6: Hypocotyl derived plant from cultivar Twyford C on MS media with casamino acids 500mg/l. The regenerated plant was raised in a pot of 13cms diameter and mother plant in a pot of 18cms diameter.

Figure 7: Hypocotyl derived plant from cultivar Twyford A on media MS with BAP 1mg/l + casamino acids 500mg/l. The regenerated plant was raised in a pot of 13cms diameter.

Figure 8: Hypocotyl derived plant from cultivar Twyford C on MS media with casamino acids 500mg/l. The regenerated plant was raised in a pot of 13cms diameter.

The stunting and premature maturity observed are a common feature of regeneration of sunflower. Paterson and Everett (1985) suggested that this may be an epigenetic effect since cultured sunflower tips (not regenerated from callus) also flower prematurely. Seed from these shoot tip derived regenerants showed normal morphology when grown to maturity. Seeds were produced in the callus derived regenerants produced in this study. However, the seed was shrivelled and failed to germinate.

4.6.5 General Discussion

A reliable system of root/shoot organogenesis is a prerequisite for the exploitation of callus as a source of potentially useful variation. This approach to breeding, allowing selection to occur under defined conditions in the laboratory, is not suitable for all desired characteristics. Only those characters which are expressed at the callus level may be selected, for example, herbicide resistance.

Previous studies of regeneration from callus of sunflower has led to mixed success, indeed some workers have described this species as 'recalcitrant' (Paterson and Everett, 1985). The results obtained in these studies examining the possible effects of plant growth regulators and casamino acids supplementation certainly support that view. No clear protocol for the regeneration of plants from callus was determined although BAP addition to the media generally resulted in greater callus growth and shoot organogenesis. Similarly, NAA tended to lead to the production of roots. Further considerable study will be required to determine the precise levels of each needed to give satisfactory production of shoots/plants before this system can be explained to produce lines which have novel characteristics of values.

SECTION 5

GENERAL DISCUSSION

Pakistan at present has to import the seed of all hybrid sunflower varieties. This type of variety is preferred, partially because of the yield levels attainable and partially because all the crop reaches maturity simultaneously. Breeding approaches currently exploited, involve repeated cycles of inbreeding and selection to produce inbred lines with good specific combining ability. This is very time-consuming, taking many years.

This study examined some aspects of two possible breeding approaches which might have value for speeding up the breeding and selection of inbred lines and improving the intensity of selection. For some crops, the exploitation of the gametophyte for haploid production can substantially reduce the time taken to produce inbred lines. Similarly, selection operating on callus cells, allows a level of selection pressure unobtainable with field grown material.

The results obtained in these studies does not engender much optimism that these techniques will be easily applied to the improvement of sunflowers. This may be because sunflower, being cross pollinated, and therefore, highly heterozygous, may not tolerate the expression of deleterious recessive genes in the haploid state when stimulated to undergo embryogenesis. If this is the case, it may be only possible to produce inbred lines by selfing, and selection to eliminate those which express such genes. Similarly, the exploitation of techniques to facilitate *in vitro* selection require substantial numbers of plants to be obtained

from callus in order to make this approach more efficient than traditional approaches to the creation of variability, such as mutagenesis.

The requirements for the culture of anthers leading to the production of haploid plants via embryogenesis still have to be determined. All the approaches adapted in this study led to callus initiation rather than embryogenesis. This callus of uncertain origin (from haploid/diploid material) was slow to undergo organogenesis as was callus derived from hypocotyl material. The factors determining the pattern of development towards embryogenesis or callus initiation, require much further experimentation. Similarly, the requirements for organogenesis have yet to be defined.

It could be argued that it was inappropriate to examine the potential of these two approaches to crop improvement, for a species which has attracted so little experimentation along these lines in the past. Indeed, many workers have concentrated on examining the potential of these techniques on so called "Model Species". The difficulties experienced and found in this study may actually support this argument. Nevertheless, it is only by undertaking these experimental approaches, that the value of them will be determined for the improvement of sunflower.

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APPENDIX 1

	mg/litre
$CaCl_2.2H_2O$	439.80
$Ca(NO_3)_2$ (anhyd)	-
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_2BO_3	6.20
KCI	-
KH_2PO_4	170.0
KI	0.83
KNO_3	1900
$MgSO_4.7H_2O$	370.60
$MnSO_4.4H_2O$	22.30
MoO_3	-
Na_2SO_4	-
$NaH_2PO_4.2H_2O$	-
$Na_2MoO_4.2H_2O$	0.25
NH_4NO_3	1650
$(NH_4)_2SO_4$	-
$ZnSO_4.7H_2O$	8.60
Sucrose	30000
Inositol	100.0
Folic acid	-
Nicotinic acid	1.0
Thiamine HCI	1.0
Pyridoxine HCI	1.0
Glycine	2.0
Biotin	-
Adenine $SO_4.H_2O$	71.59
IAA	-
NAA	-
Kinetin	-
L-Tyrosine disodium salt	-
2-i-P	-
Agar	(8000)

APPENDIX 2

Protocol for mitotic analysis of root tips derived from regenerated plants.

1. Young, actively growing roots were chosen, cut 1 cm from the tip.
2. The root tips were placed in an aqueaus solution of paradichlorobenzene for 4-6 hours at room temperature.
3. The root tips were removed from paradichlorobenzene and placed for 5 minutes in a test tube containing fixative of composition as follows:

Ethanol	5 parts
Formalin	1 part
Acetic acid	1 part
Chloroform	1 part

4. The root tips were transferred to 1NHcL for 5 minutes and maintained at 60°C to soften the tissues.
5. The root tips were washed with distilled water.
6. The root tips were placed in fulgen stain for 2 hours.
7. 1-2 mm of root tips were excised and squashed with a brass rod in a drop of acetic orcein.
8. The large debris was removed, and the remainder covered with a coverslip and pressure applied.
9. Slide examined under microscope.

APPENDIX 3

Effect of NAA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

3.1 Callus initiation

Analysis of Variance

Source	df	ss	ms	vr	sig
Var	6	484	81	0.411	ns
Med	2	183	92	0.467	ns
Error	12	2368	197		
Total	20	3036			

3.2 Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Var	6	8.34	1.39	0.640	ns
Med	2	76.22	38.11	17.562	**
Interaction	12	26.70	2.23	1.027	ns
Error	84	182.29	2.17		
Total	104	293.56			

APPENDIX 4

Effect of IBA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

4.1 Callus initiation

Analysis of Variance

Source	df	ss	ms	vr	sig
Var	6	1237	206	1.482	ns
Med	2	2119	1059	7.618	**
Error	12	1663	139		
Total	20	5019			

4.2 Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Var	6	8.85	1.47	0.397	ns
Med	2	18.30	9.15	2.472	ns
Interaction	12	9.01	0.75	0.202	ns
Error	84	310.96	3.70		
Total	104	347.12			

APPENDIX 5

Effect of IAA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

5.1 Callus initiation

Analysis of Variance

Source	df	ss	ms	vr	sig
Var	6	386	64	0.225	ns
Med	2	318	159	0.559	ns
Error	12	3405	284		
Total	20	4109			

5.2 Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Var	6	5.72	0.95	0.242	ns
Med	2	19.76	9.88	2.526	ns
Interaction	12	27.25	2.27	0.580	ns
Error	84	328.09	3.91		
Total	104	380.82			

APPENDIX 6

Effect of IAA and BAP on the subsequent development of anthers of Helianthus annuus L. in culture

6.1 Callus initiation

Analysis of Variance

Source	df	ss	ms	vr	sig
Var	2	406.8	203.4	9.549	**
Med	8	183.2	22.9	1.075	ns
Error	16	341.1	21.3		
Total	26	931.1			

6.2 Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Var	2	0.046	0.023	0.037	ns
Med	8	19.166	2.396	3.947	**
Interaction	16	57.013	3.563	5.869	**
Error	108	65.516	0.607		
Total	134	141.741			

APPENDIX 7

Pretreatment-stress on the subsequent development of anthers of Helianthus annuus L. in culture

7.1. Callus initiation

Analysis of Variance

Source	df	ss	ms	vr	sig
Med	9	63650.0	7072.2	125.171	**
Trt	1	589.0	589.0	10.424	**
Interaction	9	1936.2	215.1	3.807	**
Error	80	4519.0	56.5		
Total	99	70694.3			

7.2. Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Med	9	288.469	32.052	42.850	**
Trt	1	38.502	38.502	51.473	**
Interaction	9	39.706	4.412	5.898	**
Error	80	59.879	0.748		
Total	99	426.556			

APPENDIX 8

Effect of NAA, BAP and GA_3 on callus growth, subsequent organogenesis of roots, shoots and callus weight in culture

8.1 Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	6	170.24	28.37	10.746	**
Var	3	21.83	7.28	2.757	*
Interaction	18	245.94	13.66	5.174	**
Error	112	295.42	2.64		
Total	139	733.44			

8.2 Root organogenesis

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	6	11838	1973	4.859	**
Var	3	4560	1520	3.743	*
Error	18	7315	406		
Total	27	23713			

8.3 Shoot organogenesis

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	6	1403.7	234.0	2.762	ns
Var	3	303.7	101.2	1.194	ns
Error	18	1524.8	84.7		
Total	27	3232.2			

8.4 Callus weight (gms)

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	6	22.534	3.756	9.19	**
Error	14	5.718	0.408		
Total	20	28.252			

APPENDIX 9

Effect of casamino acids supplementation alone and in combination with plant growth regulators on callus growth and subsequent organogenesis of roots, shoots and callus weight in culture

9.1 Callus growth (mm)

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	4	87.78	21.94	15.671	**
Var	3	193.56	64.52	46.085	**
Interaction	12	340.07	28.34	20.242	**
Error	80	112.17	1.40		
Total	99	733.58			

9.2 Root organogenesis

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	4	6392	1598	5.041	*
Var	3	2664	888	2.801	ns
Error	12	3802	317		
Total	19	12858			

9.3 Shoot organogenesis

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	4	693.4	173.3	1.947	ns
Var	3	387.0	129.0	1.449	ns
Error	12	1068.6	89.0		
Total	19	2149.0			

9.4 Callus weight (gms)

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	4	21.203	5.301	12.96	**
Error	10	4.093	0.409		
Total	14	25.296			

9.5 Refinement of media requirement

Analysis of variance

Source	df	ss	ms	vr	sig
Trt	15	890.41	59.36	32.97	**
Error	64	115.47	1.80		
Total	79	1005.89			