The Institute of Biology's
Studies in Biology no. 65

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General Preface to the Series

It is no longer possible for one textbook to cover the whole field of Biology and to remain sufficiently up to date. At the same time teachers and students at school, college or university need to keep abreast of recent trends and know where the most significant developments are taking place.

To meet the need for this progressive approach the Institute of Biology has for some years sponsored this series of booklets dealing with subjects specially selected by a panel of editors. The enthusiastic acceptance of the series by teachers and students at school, college and university shows the usefulness of the books in providing a clear and up-to-date coverage of topics, particularly in areas of research and changing views.

Among features of the series are the attention given to methods, the inclusion of a selected list of books for further reading and, wherever possible, suggestions for practical work.

Readers' comments will be welcomed by the authors or the Education Officer of the Institute.

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Preface

Plant tissue culture methods have advanced considerably in recent years and are now firmly established in the repertoire of biological techniques. Originally organs and tissues were cultured in order to study fundamental problems of plant morphogenesis. However, it is becoming increasingly clear that such cultures, grown under precisely controlled conditions and in the absence of contaminant micro-organisms, provide excellent experimental materials in many other aspects of plant biology.

We have presented the basic concepts of plant tissue culture with an emphasis on practical procedures. We suggest that problems associated with tissue cultures are very suitable for sixth form projects and similar research topics since they provide an excellent training in experimentation. With this in mind we have designed practical exercises which illustrate basic principles, and involve a minimum expenditure on apparatus.

We wish to thank Professor P. W. Brian, F.R.S., for encouragement, and Mr. A. Sogeke and Mrs. A. Hand for trying out the practical exercises. We also wish to thank Professor H. E. Street and Dr. P. King for reading the manuscript and making helpful suggestions.

Cambridge, 1974

D. N. B. and D. S. I.

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1 Introduction—Why Culture Plant Cells?

The reasons for wishing to culture cells in isolation from the plant were first clearly stated by the German botanist Gottlieb Haberlandt in 1902. Although he was unsuccessful in his attempts to culture cells he foresaw that they could provide an elegant means of studying morphogenesis. The principal rationale for using cell cultures for this purpose is the belief that a knowledge of the properties of cells isolated from the plant will give important information regarding the properties and interrelationships of cells within the plant.

There was little progress towards culturing isolated cells or tissues until the early 1930s when technical developments introduced by a number of investigators led to the successful aseptic culture of isolated roots of several species. The next significant advances came in 1938 with the culture of wound callus of carrot and tumour tissues from a tobacco hybrid. These successes were followed by a period of development when culture media and technical methods were improved, permitting the culture of a wide range of organ and callus cultures from many different species. Since 1960 methods have progressively become more advanced and have culminated in highly specialized techniques for culturing single cells, cell suspensions and naked protoplasts.

During the last decade it has been realized that some of the special properties of plant tissue cultures make them highly suitable as experimental material for many fields of study in addition to morphogenesis. This has led to a complete change in the status of tissue culture methods. Whereas, ten years ago, tissue cultures were the prerogative of a few investigators primarily interested in growth and development, they are now used by workers in a wide range of disciplines including biochemists, geneticists, plant breeders and plant pathologists.

In the past the terms used in studies of plant tissue culture have been poorly defined and often confusing. For the most part we will use the terms as they have been defined by STREET (1973) in the volume entitled *Plant Tissue and Cell Culture*. The following types of aseptic culture of plants may be distinguished:

Organ cultures—these are isolated organs, including cultures derived from root tips, stem tips, leaf primordia, primordia or immature parts of flowers and immature fruits.

Embryo cultures—these are cultures of isolated mature or immature embryos.

Callus (or tissue) cultures—these are tissues arising from the disorganized

proliferation of cells from segments (explants) of plant organs. Callus cultures are usually grown as a mass of cells on a solid medium.

Suspension cultures—these consist of isolated cells and very small cell aggregates remaining dispersed as they grow in agitated liquid media. Suspension cultures are sometimes called cell cultures on the grounds that they represent a lower level of organization than callus cultures.

Like intact plants, cultured cells, tissues and organs require for growth the elements N, P, Ca, Fe, Mg, Mn, Cu, Zn, B, Mo, S and K. These are usually added to culture media in the form of mineral salts. There are also requirements for oxygen and hydrogen in the form of water, and for oxygen as a gas. Unlike most intact plants growing in the light, plant cultures also need carbon to be supplied in an organic form, usually as a sugar. In addition amino acids, B vitamins and growth hormones or complex extracts such as coconut milk are often required. In other words, while the majority of intact plants have an autotrophic nutrition, plant cultures are heterotrophic.

In the first part of the book we describe the various kinds of plant tissue cultures and give an account of their respective contributions to studies of growth and development. In addition we consider the uses of tissue culture in other areas of plant science. In the second part we deal with practical aspects of growing plant tissue cultures in the hope that it will encourage beginners to try their hand.

2 Organs and Embryos

2.1 Introduction

An essential feature of organ and embryo cultures is that they retain their characteristic structures and continue to grow in a manner comparable to that of their intact counterparts. In this respect organ and embryo cultures are clearly distinguishable from callus and suspension cultures where the organization and development of the intact tissues are lost. Organ cultures provide excellent experimental material in that they allow the properties and functions of the individual organs to be studied in isolation. They have been particularly valuable in studies of the interdependence of organs for growth hormones and other growth factors.

2.2 Roots

Isolated roots have a significant place in the history of culture methods since they were the first aseptic cultures to be maintained for extended periods by serial transfer. The pioneers in this field were Kotte and Robbins who in 1922 reported a limited amount of growth of root tips from aseptically germinated wheat seedlings. However, it was not until 1934 that White succeeded in culturing isolated roots of tomato for indefinite periods in a liquid medium containing inorganic salts, sucrose and yeast extract.

Detailed instructions for initiating and maintaining excised root cultures of tomato are given in Chapter 8, but briefly the procedures used by White were as follows. Tomato seeds were surface sterilized and allowed to germinate in aseptic conditions. The radicle tips, 10 mm long, were then excised and transferred to flasks containing nutrient medium. These 'tip cultures' grew at about 10 mm per day and lateral roots developed after 4 days. After 7 days (Fig. 2–1) they were used to initiate

Fig. 2–1 Tracing of a 7-day-old isolated root of tomato.

new cultures by excising the tips of lateral roots and transferring them to fresh medium. The laterals continued to grow at the same rate as the original tip to provide several roots which, after 7 days, were used to initiate stock or experimental cultures. Thus root material derived from a single radicle could be multiplied and maintained in continuous culture. Such genetically uniform root cultures are referred to as a clone of isolated roots. When the procedures outlined above were carefully standardized, large numbers of uniform root tips were available to initiate experiments which quickly established that the requirement for yeast extract could be replaced by the addition of three B vitamins, namely thiamine, pyridoxine and nicotinic acid, and the amino acid glycine.

After the basic techniques had been worked out for tomato roots, attention was directed towards other species. The results were mixed. Species can be divided into three groups according to the way their roots respond in culture. There are those such as tomato, clover and *Datura* which have high growth rates and produce numerous vigorous laterals. These can be grown indefinitely in culture. There are others such as pea, flax and wheat which can be grown for prolonged periods in culture, but not indefinitely. Here failure is due either to a declining growth rate or to insufficient or weak lateral roots. Finally there are roots, particularly of woody species, which hardly grow at all. Presumably they require growth factors which so far have not been identified.

Studies of the nutrition of isolated roots have provided basic information regarding the dependence of roots on the shoot for growth factors. A survey of the effectiveness of different carbohydrate sources, for example, has revealed that sucrose is far better than the others tested. It has also been shown that roots of most species require the vitamin thiamine for prolonged growth. Pyridoxine and nicotinic acid are beneficial, but not essential. It has therefore been concluded that intact roots normally depend on the shoot for adequate supplies of vitamins. However, a word of caution is necessary since it is possible that the limited biosynthetic capacity of cultured roots results from the culture conditions rather than the physical isolation from the plant.

Further experiments have suggested that excised roots of tomato and several other species are self-sufficient for the hormones, auxins, gibberellins and cytokinins, since under optimal growing conditions the addition of these substances either has no effect or is inhibitory. This interpretation has been supported by the extraction of significant quantities of auxins, gibberellins and cytokinins from excised tomato roots. In cases such as groundsel, where auxins promote growth, the endogenous auxins are presumed to be produced in sub-optimal amounts.

Isolated roots have also been used for studying the factors which are responsible for the initiation and development of secondary vascular

tissues. Excised roots of many species do not develop a vascular cambium and therefore do not form secondary vascular tissues. This indicates that materials which are translocated from the shoot are responsible for the initiation and development of a vascular cambium in the root. This hypothesis was tested by Torrey and co-workers in an elegant series of experiments in which isolated roots of pea and radish were first allowed to grow in a medium where they did not usually form secondary vascular tissues; then substances thought likely to stimulate development were fed into the root system via the basal cut end (Fig. 2–2). When the bases of pea

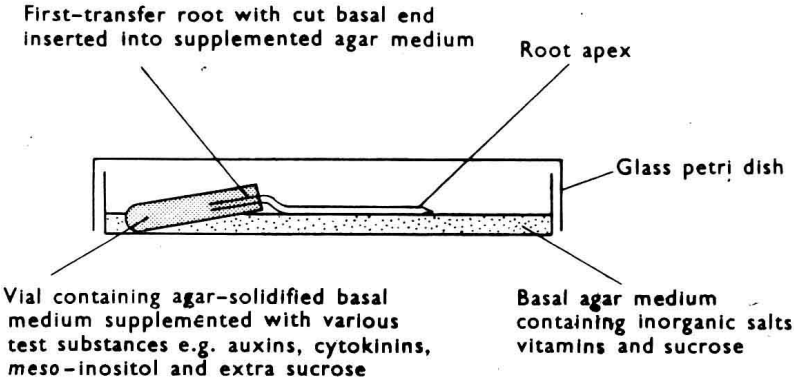

Fig. 2–2 A method used for initiating cambial activity in excised roots.

roots were fed with extra sucrose (8%) and IAA (10^{-5}M) a vascular cambium was initiated. In the case of radish roots, in addition to sucrose and IAA a cytokinin was essential and *meso*-inositol was beneficial. The treatment caused a twenty-fold increase in diameter and thus appeared to simulate the effects of the shoot system. These experiments have suggested that auxins, cytokinins and *meso*-inositol may have an important role in cambial development, although other factors are likely to be involved.

2.3 Leaves

Leaves may be detached from shoots, surface sterilized and placed on a medium solidified by agar where they will often remain in a healthy condition for long periods. Since leaves have a limited growth potential the amount of growth in culture depends very much on their stage of maturity at excision, young leaves having more growth potential than nearly mature ones.

Sussex and his co-workers have successfully dissected leaf primordia

from the underground buds of the fern *Osmunda* and found that even extremely small primordia of about 1.2 mm are capable of developing normally on a simple medium containing inorganic salts and sucrose (Fig. 2–3). The only major difference between the intact and cultured

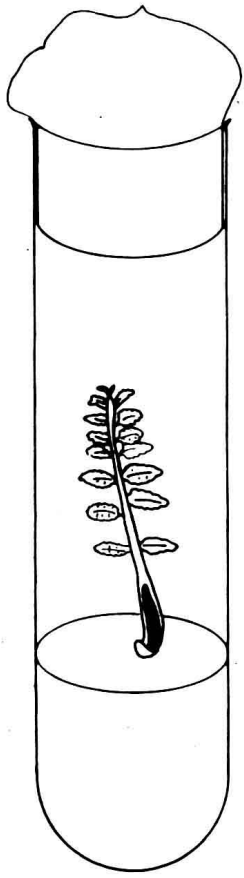

Fig. 2–3 Drawing of a cultured frond of *Osmunda cinnamomea* L. initiated from a young primordium. After 11 weeks on a medium containing inorganic salts and 2% sucrose a primordium would give rise to a frond 45 mm long with 10 pairs of pinnae.

leaves is that the growth of the latter is completed earlier, resulting in smaller leaves. Estimations of the numbers of cells in the intact and cultured leaves indicate that the differences in size are due to a reduced number of cells rather than a decrease in cell size.

Steeves has excised and cultured leaf primordia of different sizes from the stem apex of *Osmunda cinnamomea* in order to find out when the

primordia become irreversibly determined. He found that the smallest primordia, which were flat mounds about 300 μm high, usually gave rise to shoots. However, with increasing size of primordia at excision there was an increased tendency to form leaves, and finally primordia excised when 800 μm long always gave rise to leaves. Steeves concluded that the primordia did not become irreversibly determined as leaf primordia until a relatively late stage in development.

Young leaf primordia of Angiosperms such as sunflower and tobacco have also been cultured. As with ferns the primordia develop into leaves having a normal morphology except that they are much reduced in size.

2.4 Shoot tips

The excised shoot tips of many plant species can be cultured on relatively simple nutrient media and will often form roots and develop into whole plants. Such shoot tip cultures have not been widely used in studies of nutrition and morphogenesis, although they do have a number of commercial applications.

For example Loo has found that stem tips, 5 mm long, can be excised from aseptically grown seedlings of *Asparagus officinalis* and cultured on a medium consisting of inorganic salts and 2% sucrose. To sustain growth indefinitely the cultures must be exposed to light. These tip cultures form cladophylls which may be subcultured at regular intervals; these often form roots and thus become plantlets, providing a convenient way of propagating valuable asparagus plants vegetatively.

Morel discovered another important application when attempting to culture stem apices of the tropical orchid *Cymbidium* (Fig. 2–4). He observed that when placed on an agar medium containing inorganic salts and glucose, the excised stem tips first proliferated to form callus, but there then followed a period where growth was localized in discrete areas over the whole surface. These protuberances eventually grew out to form the organized juvenile structures known as protocorms, which were excised individually and transferred to fresh medium where they developed into normal plants. Subsequently he found that the rate of propagation was improved by growing the tips in agitated liquid media. Under these conditions the proliferating callus masses repeatedly broke up to form a large number of individual protocorms. Morel's work has been particularly rewarding since it has been possible to adapt it for the commercial production of orchids, resulting in marked reductions in price.

The technique of vegetative propagation from stem tips is not restricted to asparagus and orchids, since it has been found that cultured apices of several other plants such as cauliflower, carnation and tobacco give rise to numerous plantlets which can be nurtured to maturity.

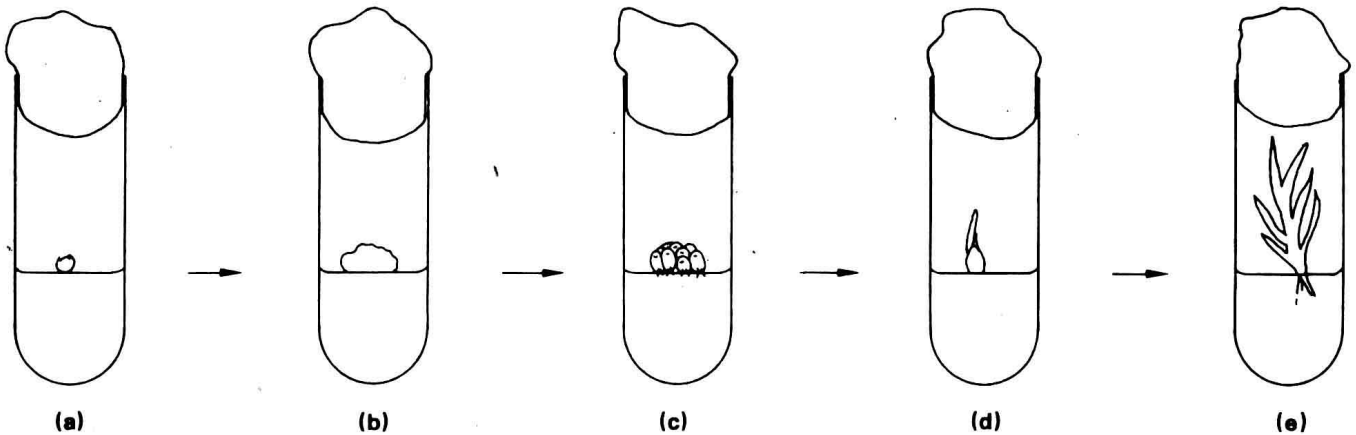

Fig. 2–4 Vegetative propagation of the orchid *Cymbidium*. (a) Apex excised and placed on a medium containing inorganic salts and glucose. (b) Proliferation of cells resulting in callus. (c) Development of a large number of protocorms bearing buds and rhizoids. (d) Protocorms separated and transplanted on to fresh medium. (e) A normal plantlet formed from a protocorm after 3 months in culture.

An important by-product of the culture of stem tips has resulted from the observation that the apices of some virus-infected plants become free of viruses when cultured on filter paper bridges standing in a liquid medium (Fig. 2–5). This has proved a very valuable method for obtaining virus-free stocks of plants where viruses have accumulated during prolonged periods of vegetative propagation, as in carnation, rhubarb, potatoes and geraniums. Its success arises partly from the fact that the apices of many virus-infected plants remain free of infection, although many other factors such as culture conditions may be involved. The effectiveness of the tissue culture procedures is enhanced when stem tips are taken from heat treated plants or when chemotherapeutants such as 2,4-D, malachite green or thiouracil are incorporated into culture media.

2.5 Complete flowers

Nitsch in 1951 reported the successful culture of the flowers of several dicotyledonous species. Not only did the flowers remain healthy in culture, but they developed normally to produce mature fruits. The procedure developed by Nitsch is as follows. Flowers, 2 days after pollination, are excised, sterilized by immersion in 5% calcium hypochlorite, washed with sterilized water and transferred to culture tubes containing an agar medium. The flowers of several species develop on media containing only inorganic salts and sucrose. Often the fruits which develop are smaller than their natural counterparts, but the size can be increased by supplementing the medium with an appropriate combination of growth hormones such as auxins, gibberellins and cytokinins. As might be expected, flowers put into culture before pollination do not usually produce fruits. Parthenocarpic fruit development has been observed, however, especially in the presence of synthetic auxins, which frequently induce parthenocarpy in intact plants.

The culture of young floral buds of cucumber has been employed to study sex determination. The young buds require a complex medium containing inorganic salts, B vitamins, tryptophan, casein hydrolysate and coconut milk. It has been shown that potentially male buds tend to develop ovaries in culture and that this tendency is enhanced by early excision or the addition of the auxin indole acetic acid (IAA). In contrast, gibberellic acid counteracts this trend towards femaleness. The development of potentially female or hermaphrodite buds seems to be more stable, since it is unaffected by the addition of IAA, gibberellic acid or other hormones.

2.6 Anthers and pollen

In order to culture anthers young flower buds are removed from the plant and surface sterilized. The anthers are then carefully excised and

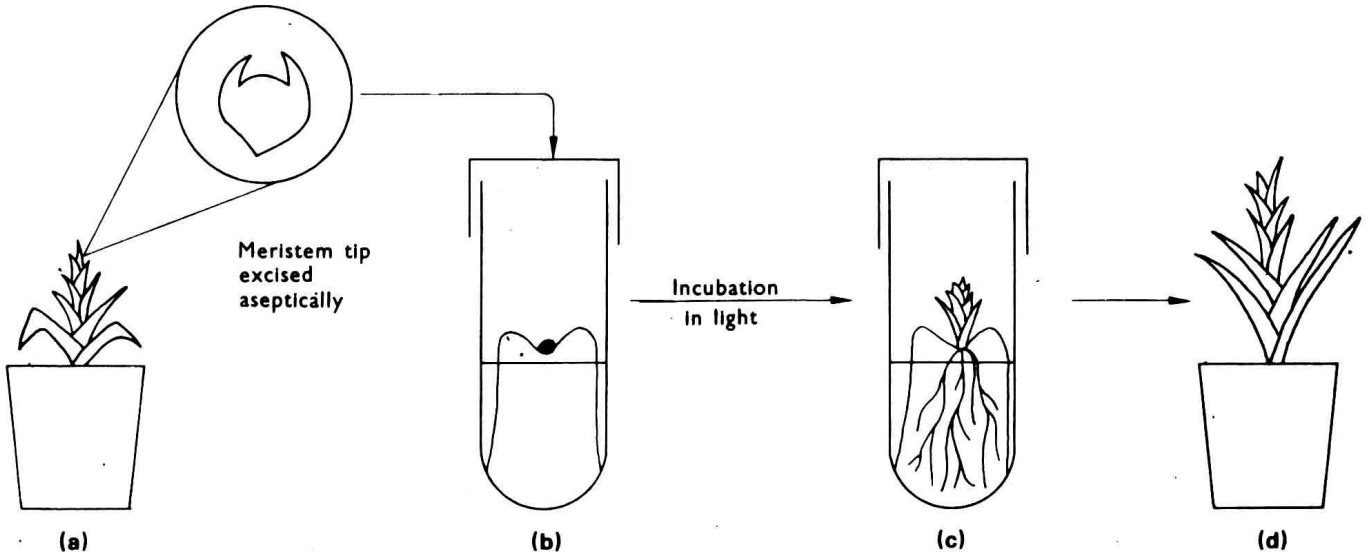

Fig. 2–5 Production of pathogen-free plants by the culture of meristem tips. (**a**) Stunted, virus-infected plant. (**b**) Meristem tip transferred to filter paper bridge in a tube of culture medium. (**c**) Rooted plantlet. (**d**) Rooted plantlet transferred to soil to produce a vigorous pathogen-free plant.

transferred to an appropriate nutrient medium. Those excised at an immature stage usually grow abnormally and there is no development of pollen grains from pollen mother cells.

Mature pollen grains (microspores) of several species of Gymnosperms can be induced to form callus by spreading them out on the surface of a suitable agar medium. Pollen of *Ginkgo biloba* proliferates on a medium containing inorganic salts, B vitamins, coconut milk and sucrose, while those of *Taxus brevifolia* and *Torreya nucifera* require a medium supplemented with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). The calluses consist of colourless parenchyma-like cells which can be subcultured indefinitely, but do not form roots, buds or embryo-like structures (see Chapter 3).

Mature pollen grains of Angiosperms do not usually form callus, although there are one or two exceptions. The pollen grains of *Brassica oleracea* may be induced to form small cell clusters if cultured by the hanging drop method. A drop of culture medium inoculated with pollen is put on a coverslip which is then inverted over the well of a cavity slide and sealed. Immature pollen of tomato can be induced to form callus colonies using a nurse culture technique. Anthers are placed on the surface of an agar medium and covered with a small disc of filter paper. A drop of pollen suspension containing about 10 grains is then pipetted on to the filter paper. Small colonies of green parenchyma-like cells develop within 28 days if the cultures are incubated in light at 25° C.

Recently Nitsch and Norreel have reported that pollen grains of tobacco and *Datura* can be induced to behave like zygotes instead of gametes. The pollen grains give rise to embryo-like structures (embryoids) which pass through the stages of embryogenesis to form plantlets and eventually flowering plants. They also found that a trauma such as a temperature shock given at the time of the first mitosis increases the number of cells following an embryogenic development.

Since anther cultures have been shown to have an abnormal development they have been of little value for studying the normal processes of microsporogenesis. However, anthers and pollen cultures have special features which make them very attractive for other purposes. Pollen grains cultured free or within cultured anthers give rise under certain conditions to haploid plants (plants having the same chromosome number as the gametes of a diploid plant). This discovery has aroused considerable interest among plant breeders. Its significance can be appreciated when it is realized that many cultivated plants do not breed true to type and inbreeding, even if possible, takes many years of patient work to produce a pure line. Sometimes this laborious procedure may be avoided by by-passing the sexual process and using haploid plants which possess only half the normal number of chromosomes. Haploid plants can arise naturally by the premature development of one or more cells in the embryo-sac, usually the egg cell. Obviously cells of haploid plants are

unable to undergo meiosis and are therefore infertile, but treatment with chemical agents such as colchicine causes a doubling up of chromosomes resulting in diploid plants with two identical sets of chromosomes (i.e. the plants are homozygous). Unfortunately, the frequency of occurrence of haploids in nature is very low. Hence the anther and pollen culture techniques, which increase the number of haploids available to the breeder, are potentially very valuable.

Although these studies are still in their infancy, haploid plants have already been produced from pollens of several species, mainly through the independent efforts of Maheshwari, Nitsch and Nitsch and Sunderland. The list includes the following genera: *Nicotiana* (tobacco), *Datura*, *Atropa*, *Oryza* (rice) and *Lolium* (rye-grass). The procedure for obtaining haploid plants from cultured anthers of tobacco is briefly as follows (Fig. 2–6). Closed flower buds of an appropriate age are excised from the plant and surface sterilized by immersion in a solution containing 1% calcium hypochlorite. The sepals and petals are then removed and the anthers excised and immediately placed on to an agar medium. With anthers of tobacco development proceeds on a normal tissue culture medium lacking plant hormones. Anthers of other species have complex hormonal requirements, needing both auxins and cytokinins for development. The cultures give rise to large numbers of embryoids which go through stages comparable with those of zygotic embryos to form plantlets. The cultured anthers split open after 4 to 5 weeks at 25° C revealing embryoids at the cotyledonary stage. The plantlets may then be teased apart and transferred individually to fresh culture medium. When an adequate root system has developed the young plants are transplanted to compost and raised to maturity.

The actual stage of development of the anther at the time of excision is very critical if haploid plants are required. In tobacco this critical time seems to coincide with the first unequal division of the pollen grains. Surprisingly it is the normally quiescent vegetative cell which usually gives rise to the haploid embryoid, whereas the generative cell does not appear to participate.

In one experiment Sunderland raised 400 plants from tobacco pollen. Chromosome counts on the root tips revealed that the vast majority were haploids, a few were chimaeras having both haploid and diploid roots and flowers, and 1 to 2% were homozygous diploids. In other experiments it was shown that treatment with colchicine increases the amount of chromosome doubling if given during the early stages of embryoid development.

An alternative way of producing haploid plants is to culture the anthers in a medium containing auxins which induce the proliferation of haploid callus instead of embryoid formation. Once the callus has been formed it is transferred to a medium with no auxins and a reduced sugar level. Shoots, and later roots, are thus induced to develop and numerous