

**Experimenting**

**with**

**Industry**

**13**

**Plant**

**Tissue**

**Culture**



**SCSST**

## About this document

This facsimile of the first edition of Plant Tissue Culture has been produced by the National Centre for Biotechnology Education (The University of Reading). The author, Tony Storr, and the Association for Science Education, the original publishers, gave their kind permission, as did Unilever plc, who supported the first work back in 1984.

We have tried as much as possible to reproduce the original document, complete with the 1984 prices and suppliers. Naturally costs and sources of materials change and most of the books referred to in the bibliography are now long out-of-print. Some safety guidelines are given below, and current suppliers are listed on the NCBE's Web site (<http://www.ncbe.reading.ac.uk>).

This booklet remains the only introduction to practical plant tissue culture for schools ever published in the United Kingdom. Its influence during the late 1980s, before the introduction of the National Curriculum in England and Wales, was considerable.

This publication is a testament to the dedication and creativity of the teachers and others who participated in the SCSST project nearly 20 years ago. We hope that it will provide inspiration for a new generation of teachers and their students.

## Safety

In the practical investigations described in this booklet, the author has tried to identify recognised hazards and to suggest suitable safety precautions. Where possible, the proposed procedures are in accordance with commonly-adopted general risk assessments. If special hazards are present, this has been indicated.

However, readers should be aware that errors and omissions can be made, and that different employers and educational authorities adopt different standards. Therefore, before doing any activity, users should always carry out their own risk assessment. In particular, any local rules issued by employers or educational authorities **MUST** be obeyed, whatever is suggested in the activities described here. Unless the context dictates otherwise, it is assumed that:

- practical work is carried out in a properly equipped and maintained science laboratory;
- any mains-operated or other equipment (such as autoclaves) are properly maintained;
- care is taken with normal laboratory operations such as heating substances (and especially when using ethanol near to naked flames);
- good laboratory practice is observed when chemicals (*e.g.* plant growth substances) are used;
- eye protection is worn whenever there is any recognised risk to the eyes (such as handling bleach solutions);
- pupils and / or students are taught safe techniques for activities such as handling and disposal of microorganisms (which may contaminate plant cell cultures).

# Experimenting with Industry

A series presenting industry-related science practicals for schools

## 13. Plant Tissue Culture

Devised by  
Tony Storr  
Sharnbrook Upper School, Sharnbrook, Bedford  
in association with  
Unilever PLC, Colworth House, Bedfordshire

Published for  
The Standing Conference on Schools' Science and Technology  
by  
The Association for Science Education,  
College Lane, Hatfield, Herts. AL10 9AA

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The *'Experimenting with Industry'* publications are the product of a 'teachers into industry' project organized by The Association for Science Education on behalf of The Standing Conference on Schools' Science and Technology.

### **The Standing Conference on Schools' Science and Technology**

The Standing Conference was established in 1971 to promote and encourage in the public interest the development of science and technology in schools. It is supported by the Department of Education and Science, the Department of Trade and Industry, the engineering profession, industry, and its member organizations.

### **The Association for Science Education**

The Association's activities are totally directed towards the advancement of science education. Its membership of over 17,000 is drawn from teachers of science and others interested in science education, both in the UK and overseas.

### **Project Working Party**

John Nellist

General Adviser, Cumbria  
Chairman-elect, The Association  
for Science Education

Brian Nicholl

NS Education Consultants

Richard Turner

Assistant Secretary, The Association  
for Science Education

Stuart Whitefoot

Development Manager, The Standing  
Conference on Schools' Science  
and Technology

### **Series Editors**

Brian Nicholl and Jenny Selfe, NS Education Consultants, 5 Dryden Street, London WC2.

### **Acknowledgements**

The SCSST and ASE wish to acknowledge the work of the Teacher Fellows and the support given by Head Teachers and LEA advisory staff. Thanks are also due to the participating companies and, in particular, to the many individuals within those companies who gave so generously of their time and knowledge.

# Foreword

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The need to provide a framework which relates to everyday life, for science taught in schools, is now widely accepted. Much existing science practical work relates only to an academic syllabus, expects a single 'right' answer and provides very limited opportunity for experimental design, problem-solving and group working.

The booklets in this series are based on the work of experienced science teachers who, during the summer of 1984, were linked with industrial companies as part of a project initiated by SCSST in collaboration with ASE. Their brief was to devise experiments drawn from industrial processes, which illustrate scientific concepts and show how they are applied in industry. This development was made possible by a generous donation from Lloyd's Register of Shipping.

Experiments described in the booklets aim to replace, within the existing curricula, some of the present school practical work. They allow opportunity for pupils to conduct challenging investigations which enhance their understanding, and there is a problem-solving and open-ended element.

The contribution of the teachers involved and the co-operation of the industrial firms who helped with the project is gratefully acknowledged. It is especially pleasing that medium-sized companies as well as major international groups were involved. The project has shown once again the value of effective links between schools and industry.

Sir Geoffrey Allen, FRS  
Chairman  
SCSST

# The Series

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Further 'Experimenting with Industry' titles will be published during 1985, the complete series being detailed below. The teacher/industry partnerships which developed this material were widely distributed throughout England and in Wales. The industrial participants ranged from multi-national concerns well-known for their support of educational initiatives to companies in highly specialized areas of technology with little experience of such activities.

<b>Title</b>	<b>Company</b>
1. Electrical Testing Bob Cheyne, St. John's School, Marlborough	Square D Ltd., Swindon, Wiltshire
2. Optical Fibres in School Physics Stephen Rutherford, Pimlico School, London	GEC Hirst Research Centre, Wembley, London
3. Industrial Use of Micro-organisms Steve Bowden, Whitchurch High School, Cardiff	Nipa Laboratories Ltd., Pontypridd, Mid-Glamorgan
4. Sugar Challenge Charles Dalleywater, Ramsey Ailwyn Community School, Ramsey	British Sugar plc, Peterborough, Cambridgeshire
5. Chemicals for Agriculture Lyn Bossons, Biddick School, Washington	ICI Agricultural Division, Billingham, Cleveland
6. Brake Fluid and School Science James Dawber, Sharples School, Bolton	Shell Chemicals, Carrington, Manchester
7. Safety in Gas Appliances Peter Hancock, Dame Alice Owen's School, Potters Bar	British Gas Eastern Region, Potters Bar, Hertfordshire
8. Extracting Metals from Scrap Bill Harrison, Pelsall School, Walsall	Elkington Copper Refiners, Walsall, West Midlands
9. Properties of Metals Hilary Laidler, Simon Balle School, Hertford	British Aerospace, Stevenage, Hertfordshire
10. Electronics of Control Systems Virginia Lavender, Collingwood School, Camberley	Marconi Command & Control Systems, Camberley, Surrey
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12. Physics of Fluid Flow Linda Scott, Pate's Grammar School for Girls, Cheltenham	Dowty Services Ltd., Cheltenham, Gloucestershire
13. Plant Tissue Culture Tony Storr, Sharnbrook Upper School, Sharnbrook	Unilever plc, Bedford

## Unilever PLC

Unilever was founded in 1930 when two businesses which were already established in many countries, Lever Brothers in the United Kingdom and the Margarine Unie in the Netherlands, came together.

Unilever is a major producer of consumer goods: mainly food and drinks, detergents and toilet preparations. Over 300 000 people are employed in more than 500 subsidiaries in some 75 countries.

Unilever's central research and engineering facilities are concentrated into three major laboratories: Colworth House, Bedfordshire, and Port Sunlight, Merseyside, in the UK; and Vlaardingen, near Rotterdam, in the Netherlands. Smaller research laboratories are located in India and the United States. Between them, they employ some 4000 people, of whom more than 1000 are graduates.

Unilever's Research and Engineering Division has two clearly defined tasks. The first is to improve existing products and processes, and to provide products which are safe, reliable and good value for money. The second is to identify and develop new opportunities arising from advances in science and technology.

# Contents

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## Teachers' guide

1. Industrial background	1
2. Educational scope	2
3. Notes on experiments	2
Experiment 1: Production of plantlets from floral organs of cauliflower	6
Experiment 2: Studies of carrot callus cultures	
a) Initiation and maintenance of carrot callus cultures	7
b) Effect of sucrose concentration on growth-rate of callus cultures	8
c) Embryogenesis in carrot callus cultures	9
d) Cytodifferentiation in carrot callus cultures	9
e) Nuclear division in callus cultures	9
Experiment 3: Control of organogenesis in cultures of <i>Nicotiana tabacum</i>	10
Experiment 4: Control of organogenesis in cultures of petals of <i>Saintpaulia ionatha</i> (African violet)	12
4. Appendix: Relationships to concepts and topics in A-level biology	13
5. Bibliography	14

## Students' notes

Introduction	15
Aseptic handling of cultures	17
Experiment 1: Production of plantlets from floral organs of cauliflower	19
Experiment 2: Studies on carrot callus cultures	
a) Initiation and maintenance of carrot callus cultures	23
b) Effect of sucrose concentration on growth-rate of callus cultures	26
c) Embryogenesis in carrot callus cultures	28
d) Cytodifferentiation in carrot callus cultures	29
e) Nuclear division in callus cultures	31
Experiment 3: Control of organogenesis in cultures of <i>Nicotiana tabacum</i>	32
Experiment 4: Control of organogenesis in cultures of petals of <i>Saintpaulia ionatha</i> (African violet)	37

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# Teacher's guide

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## 1. Industrial background

Apart from their use as tools for pure research, tissue culture techniques have, in recent years, become of major industrial importance in the following areas: plant propagation (micropropagation), disease elimination, plant improvement, and the production of secondary metabolites.

### Micropropagation

These techniques offer major advantages over traditional methods. Callus cultures from a single explant can give rise to hundreds of plantlets within a few months of callus initiation. These numbers can be increased if the callus initially produced is shaken in a liquid medium and grown rapidly as a suspension for a period of time. Such liquid suspension cultures will eventually form aggregates of cells which can be transferred to a solid medium to grow as callus. Both callus and aggregates of cells grown in suspension can produce roots and shoots or embryoids, although in many cases the ability to do so declines with time. The genetic instability associated with some callus cultures can be avoided if meristems, either isolated by dissection or as larger explants from buds, are cultured. The shoots grown from the meristem can then be further propagated by growing small sections of stem containing axillary buds (nodal cuttings) in tissue culture. The buds will give rise to further shoots which can be propagated in the same way. This has been applied commercially to the propagation of many crops including potatoes and strawberries. The genetic stability of meristem cultures has led to the establishment of *gene banks* for the conservation of plant genetic resources.

### Disease elimination

Active shoot meristems are often pathogen-free. For example, strawberry, cassava, poplar and many ornamentals have been cultured from meristems to produce disease-free stock.

Other tissues may be disease free and these, too, may be used as sources of clonal material. Heat treatment of the source plant is often used in conjunction with tissue culture to eliminate disease organisms.

### Plant improvement

Spontaneous variation in callus cultures has been employed as a source of strains of sugar cane that are resistant to downy mildew, mosaic disease and Fiji disease (a viral disease transmitted by a hemipteran vector). The reason for this destabilization is uncertain.

Gross karyotypic changes and chromosomal rearrangements including polyploidy and aneuploidy have been widely documented in long-established cultures lacking the ability to regenerate. However, *somaclonal variation* is now being industrially exploited in crop plants, e.g. tomato, maize, potato. Somaclonal variation arising from minor, sometimes extra-chromosomal, genetic changes appears to be induced by the culture process. In general, therefore, it would be unwise to assume that cells from the same clone are genetically identical.

New commercial varieties may in future be produced by the manipulation of haploid plants grown in tissue culture from anthers or isolated pollen cells. Techniques involving the fusion

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of protoplasts to produce intra- or interspecific hybrid cells may also become commercially significant. Fused and unfused protoplasts produced by this technique are then spread onto solid media and allowed to grow to form callus which may then give rise to plantlets. It would, of course, be hoped that the hybrid protoplasts would give rise to plantlets that possess the desirable characteristics of both types of plant.

### **Production of secondary metabolites**

It is unlikely that tissue culture will ever be able to compete economically with agricultural methods for the production of food substances. However, many plants produce pharmaceutically or pharmacologically valuable secondary metabolites, which are extremely expensive to obtain by extraction from the plant. Twenty-five per cent of all prescription medicines contain such plant extracts.

Shikonin derivatives are used in lipsticks and, in Japan, for the treatment of burns and skin diseases as well as for colouring silks. The natural product is extracted from mature shikon roots but callus cultures, from carefully selected cell lines, accumulate shikonin at 8–15 times the normal concentration and, therefore, offer a cheaper alternative source.

There can be little doubt that the techniques of plant tissue culture will be further exploited industrially in the future.

## **2. Educational scope**

These materials have been developed for use in A-level biology courses and assume some knowledge, but Experiments 1, 3 and 4 could readily be adapted for use with younger pupils — see ‘Apparatus and materials (general)’.

The experimental procedures that are given are fully detailed but need not be used totally prescriptively. They are, however, reliable and will encourage students to develop good working practices, for success requires careful preparation and organization as well as manipulative skills.

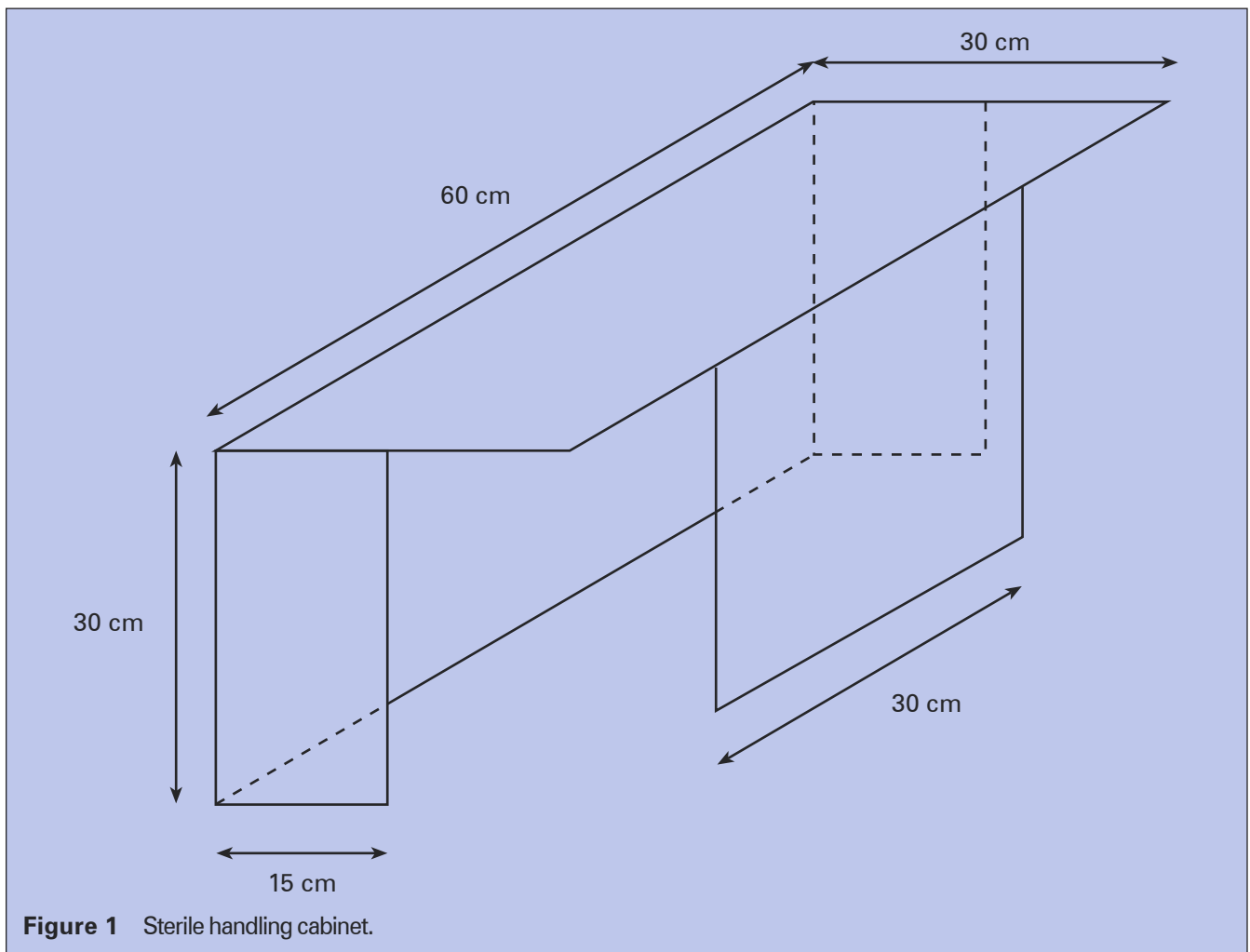
Suggestions for extension work or open-ended exercises (possibly projects) are made for three of the experiments.

## **3. Notes on experiments**

### **Apparatus and materials (general)**

Although this work can be carried out on the open bench, pre-sterilized with 70% ethanol, such a procedure is not recommended. Many schools will possess a commercially-produced sterile-handling cabinet which will be more than adequate for these experiments.

In the author's school, a very effective alternative has been used. It was produced for the sum of £5 (May 1984), using 2 mm transparent polystyrene sheeting bonded with polystyrene cement (see Figure 1).



After construction, the cabinet may be further strengthened at the joints (and any gaps filled) using silicone sealing compound. Construction of a small number of these cabinets would enable all members of practical classes to obtain some experience of these procedures. Alternatively, sterile explants might be prepared and dispensed by the teacher and/or by a small group of pupils. The explants might then be aseptically transferred by individuals to the required growth medium.

All procedures have been written assuming the use of petri dishes. These have several advantages, particularly for manipulation by inexperienced hands. However, as alternatives, any of the following may be used for the growth of cultures: glass jars, specimen tubes, conical flasks (100cm<sup>3</sup>), boiling tubes. The only requirements are that they must be shallow enough for easy handling of cultures with forceps, and must be fully autoclaveable.

They should be sealed with a double layer of aluminium foil or a bung of non-absorbent cotton wool covered with foil (to reduce water loss). If foil seals are used, they should be replaced with new sterile foils at each manipulation. It has been found that boiling tube and specimen tube cultures are easier to manipulate if the medium has been set at a slope.

'Parafilm' may be obtained from *Gallenkamp Ltd.*, but plastic insulating tape has been found to be perfectly adequate.

If possible, a stain jar, **with lid**, should be used to contain the ethanol and instruments, thus reducing the likelihood of ethanol fires.

It is recommended that surface sterilization of plant material is carried out in clean screw-top jars. Their internal surfaces will be sterilized by the surface-sterilization technique. However, accidental contamination of material will be less likely if the jars have been autoclave-sterilized before use.

Glass-distilled water is recommended for all purposes. However, if such water is in short supply, sterile tap water will suffice for all rinses of surface-sterilized plant materials. Sterile water should, if possible, be sterilized and distributed to students in aliquots of approximately 200cm<sup>3</sup>, e.g. in medical flats.

Incubation at 26°C in light can be achieved by the use of a plant propagator with a thermostatically controlled soil-warming cable. Light levels can be boosted with one or more 30 watt fluorescent tubes suspended in, or over, the propagator.

Alternatively, the cultures may be incubated in a 60 cm aquarium covered by glass. Both the temperature and the light intensity may be boosted by a 60 watt tungsten bulb in a metal hood. The bulb may be connected to the mains through an aquarium thermostat if finer temperature control is required.

In practice, these procedures work well at any temperature in the range 20°C–28°C, so an aquarium in a west-facing window might suffice in summer, However, wide variations in temperature must be avoided. If an artificial light source is used, a cycle of 16 hours light/eight hours dark is recommended.

## Preparation of media

The basal medium used in these experiments is based upon that of Murashige and Skoog (1962).

	Component	Final concentration (mg/dm <sup>3</sup> )
Inorganic macronutrients	NH <sub>4</sub> NO <sub>3</sub>	1650.0
	KNO <sub>3</sub>	1900.0
	CaCl <sub>2</sub> ·7H <sub>2</sub> O	440.0
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	370.0
	KH <sub>2</sub> PO <sub>4</sub>	170.0
Chelated iron	Na <sub>2</sub> EDTA	37.3
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8
Inorganic micronutrients	H <sub>3</sub> BO <sub>3</sub>	6.2
	MnSO <sub>4</sub> ·4H <sub>2</sub> O	16.9
	ZnSO <sub>4</sub> ·4H <sub>2</sub> O	8.6
	KI	0.83
	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
Organics	sucrose	20 000.0
	inositol	100.0
Vitamins	thiamine HCl	0.1

**Table 1**

The above medium ('M+S'), without sucrose, is available in packs containing the components for 1dm<sup>3</sup> of medium from: *Flow Laboratories Limited, PO Box 17, 2nd Avenue, Industrial Estate, Irvine, Ayrshire KA12 8NB (Tel: Irvine [0294] 74242); catalogue no. 26-100-20.*

Packs must be purchased in sets of ten (total cost, July 1984, including VAT and carriage, £10.20). Larger packs (for 10dm<sup>3</sup>) are available at lower cost/unit volume.

### **Growth substances used in tissue culture experiments (phytohormones)**

*Caution:* Some phytohormones are toxic, so care must be taken when preparing stock solutions, e.g. protective gloves should be worn. Students should not, of course, come into direct physical contact with media.

#### *Auxins*

Indol 3-yl acetic acid (IAA)  
1-Naphthyl acetic acid (NAA)  
2,4-dichlorophenoxy acetic acid (2,4-D)

#### *Cytokinins*

6-furfurylaminopurine (Kinetin)  
6-benzylaminopurine (BAP)

Suppliers of the above include:

*Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset BH17 7NH;  
BDH Chemical Ltd., Broom Road, Poole, Dorset BH12 4NN.*

Apart from the macronutrients, sucrose and inositol, the components of the medium are best prepared as concentrated stock solutions (Table 2).

Nutrient	Stock concentration	Volume added for 1 dm <sup>3</sup>
Chelated iron	200 × final	5 cm <sup>3</sup>
Micronutrients	100 × final	10 cm <sup>3</sup>
Vitamins	100 × final	10 cm <sup>3</sup>
Growth substances* (phytohormones)	10 mg/100 cm <sup>3</sup>	variable

**Table 2**

*\*IAA may require the addition of 5M NaOH in order to dissolve it.*

### **Procedure**

1. Dissolve the macronutrients in 100cm<sup>3</sup> of glass-distilled water. (This may require two or three drops of 5M HCl.)
2. Add stock solutions of inorganic nutrients.
3. Add organic solids and make up to 500cm<sup>3</sup> with glass-distilled water. Stir to dissolve.
4. Add the stock solution of vitamins, then add the required quantities of the growth substances.

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5. Adjust the mixture to pH 6.5 ( $\pm 0.3$ ).
  6. Add 9g Philip Harris or Difco Bacto Agar or 7.5g Oxoid Agar.
  7. Stir and boil the mixture to dissolve the agar. The container must be sealed with foil to reduce evaporation.
  8. Add glass-distilled water to make up to 1 dm<sup>3</sup>.
  9. Autoclave the medium at 15 p.s.i. for exactly 15 minutes.
  10. Recheck the pH. It should lie in the range pH 5.5–6.3.

If packaged 'M + S' medium is used, the pack contents should be dissolved as in Step 1. Step 2 is omitted and only sucrose and the growth substances need be added, as in Steps 3 and 4.

The medium is stable for several weeks at refrigerator temperatures.

Dispense the medium aseptically as required into sterile containers.

## Advance preparation

It is assumed throughout these exercises that students are familiar with the structure of angiosperms and have a knowledge of the basic properties of plant tissues.

The importance of aseptic handling cannot be over-emphasized (see 'Students' notes') but it is likely that students will have had little experience of aseptic techniques. It is, therefore, urged that, wherever possible, students are given the opportunity for a 'dry run' using non-sterile materials before trying aseptic manipulations.

The surface sterilization techniques suggested can only be guidelines, as plant material varies in cleanliness, surface wax and robustness. Particularly hairy or waxy material, e.g. *Saintpaulia* leaves, will require additional dewaxing for 30 seconds in 70% ethanol immediately before surface-sterilization. If possible, a range of different times of exposure to the sterilizing agent could be tried by different students or by the teacher.

*Caution:* Students should be expressly warned to handle all bleach solutions with care. The persistent smell of bleach on students' hands may be removed by rinsing in a dilute solution of sodium thiosulphate.

## Experiment 1: Production of plantlets from floral organs of cauliflower

This experiment gives a simple introduction to the micropropagation of plants using tissue culture techniques. An 80% success rate is to be expected.

<b>Time-scale (at 26 °C)</b>	<b>From initiation (weeks)</b>
Curd enlarges and produces chlorophyll	1–2
Leaves observed	2–3
Regenerated shoots	3
Separate shoots, transplant to hormone-free medium	3–6
Rooted plantlets ready for transfer	6–12

#### **Possible extension work**

Some authorities suggest the addition of IAA (at 8 mg/dm<sup>3</sup>) to the medium. The effects of this and other auxins/concentrations upon the rate of shoot production could be determined.

#### **Answers to students' questions**

1. It has been estimated that a typical curd contains 10000 meristematic apices. If the student assumes that the head is a hemisphere (approximate diameter 15cm) and makes a similar assumption for each explant, then the right order of magnitude will be achieved.

2. In addition to the numbers of clonal plantlets obtained, the method offers other advantages.

Leafy shoots produced on the stem or from the curd are undesirable traits in a crop plant of this sort. Propagation from a plant with these traits will, therefore, have harvesting and marketing disadvantages. Tissue culture methods, by their very nature, facilitate the elimination of disease-carrying or otherwise unsuitable clonal material because plantlets produced will not be subject to masking environmental effects. In this case, since floral meristems are used, clonal plantlets are more likely to be virus-free.

## **Experiment 2: Studies on carrot callus cultures**

### **a) Initiation and maintenance of carrot cultures**

These investigations illustrate a number of features of callus cultures. Here, we use an explant cultured directly on a solid medium but it is also of industrial importance in the production of plantlets from cells arising from manipulations in suspension.

Microscopic examination of the callus is employed to give an insight into the developmental processes occurring within it.

Mature, undamaged tap roots should be chosen for this exercise. A 70%–90% success rate is to be expected.

<b>Time-scale (at 25 °C)</b>	<b>From initiation (weeks)</b>
Explants swell	2–3
Callus overgrows explant	3–4
First subculture (xylogenesis)	4–5
Second subculture + ? embryogenesis	8–9*
Third subculture + ? embryogenesis	12–13

\* *Yields suitable material for Experiment 2(b).*

### Answers to students' questions

1. Cambium, phloem.
2. If explants are cut from secondary phloem tissue alone, they will dedifferentiate to give rise to the callus growth.
3. It is reversible if the cells are alive.

### Alternative material

Dormant Jerusalem artichoke (*Helianthus tuberosus*) tubers work very well as sources of explants. They may be grown on the same medium.

### b) Effect of sucrose concentration on growth-rate of callus cultures

To obtain the classic sigmoidal growth curve, without using large numbers of duplicate cultures to be sacrificed at intervals, calluses must be aseptically weighed and returned to precisely the same place on the original medium. This is virtually impossible to carry out repeatedly without contamination of the original culture dish. Alternatively, the growth of secondary callus cultures may be assessed by comparing the callus with hemispherical, Plasticine models, precalibrated by the teacher against calluses of known wet mass. Table 3 shows a typical calibration. The method is most accurate if the Plasticine is yellow, i.e. similar in colour to the callus.

Radius of hemispherical model (mm)	Wet mass of callus (mg)
2.5	50
3.0	80
4.0	108
4.5	160
5.0	217
6.0	310
7.0	482
7.5	628
9.0	920

**Table 3**

In this experiment, the callus cultures are maintained in the logarithmic phase of growth by repeated transfer onto fresh medium at weekly intervals.

It is suggested that different groups of students grow cultures using different sucrose concentrations in the range 0.75%–4.0%. At 0.5% sucrose, callus growth virtually ceases and an approximately linear response occurs above this value until, at approximately 3% sucrose, its concentration is no longer limiting.

It must be noted that the biomass growth rates observed do not exactly mirror changes in cell number.

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Students should be expected to summarize the data by determining the mean callus biomass at each weighing. If there are adequate replicates, the standard deviations of observed doubling times should be calculated.

#### **Answers to students' questions**

1. See above.
2. See above.
3. The exponential phase, B, although a lag phase of two or three days may be observed. An explanation based upon the depletion of the carbon source may be expected. Alternative explanations include autoinhibition, and depletion of other resources (particularly at the callus centre).
4. This will reduce the likelihood of a lag phase occurring on transfer of the callus.

#### **c) Embryogenesis in carrot callus cultures**

Embryogenesis is notoriously unreliable on solid media. It will occur spontaneously in regularly subcultured calluses eight or more weeks after initiation but its occurrence cannot be predicted nor stimulated by manipulation of the growth medium. The teacher must, therefore, be aware of the difficulties involved and, with the students, should regularly examine callus cultures for signs of embryogenesis.

'Globular' embryoids may be visible after eight weeks from initiation. 'Mature' embryoids may be visible after fifteen weeks from initiation. Some 'embryoids' observed in situ on the callus may, in fact, develop as root primordia.

#### **Answer to students' question**

1. Yes.

#### **d) Cytodifferentiation in carrot callus cultures**

In mature callus cultures, xylem elements may be readily observed using phloroglucinol or phase contrast. Phloem sieve tubes may also be present but, obviously, these will not be stained by this method. Callus cultures frequently exhibit the presence of more dispersed lignin which may give a background coloration.

The material may be chemically macerated before staining to facilitate observation of xylem elements but non-lignified tissue is unlikely to survive such a procedure.

#### **Answers to students' questions**

1. To eliminate, as far as possible, carry over of differentiated cells from the explant.
2. See above.

#### **e) Nuclear division in callus cultures**

Although mitotic activity is occurring in growing callus, it is limited to small meristematic centres in younger parts of the

callus. Cells in older calluses tend to become anucleate, so the material used must be regularly and frequently subcultured to ensure mitotic activity. Mitotic activity will be most likely to be observed in cells obtained from very pale cream or white regions from the edges of the callus. The teacher is urged to check the material before use by the class.

*Note:* Mitosis within a callus may be synchronized and occur at inconvenient times!

#### **Answer to students' question**

1. 5%–60% of cells at meristematic centres.

### **Experiment 3: Control of organogenesis in cultures of *Nicotiana tabacum***

The classic responses of wound callus from mature tobacco stem pith explants to various proportions of auxin and cytokinin are well documented. This exercise uses split-stem explants from seedlings, thus making considerable savings in the time and space required for the production of suitable starting material. However, seedlings are very variable in their response to surface sterilization with sodium chlorate(I) (hypochlorite), so it is recommended that, if possible, seedlings are germinated and grown aseptically.

#### **Apparatus**

Basic set of apparatus for aseptic handling (see p.17, 'Students' notes')

Aluminium foil

Autoclaveable glass containers with lips, e.g. jam jars (several)

Knops or Sachs solution

Agar sterile petri dish

Fine spatula

Muslin and thread

2% sodium chlorate(I) (hypochlorite) solution + 1 cm<sup>3</sup> detergent (or a 4% Domestos solution)

Clean screw-top jar

Sterile water, 500 cm<sup>3</sup>

Seeds of *Nicotiana tabacum*

#### **Aseptic germination of *Nicotiana* seeds**

##### **Procedure**

1. Dissolve agar in the mineral salts solution (at 9g/dm<sup>3</sup>) and transfer the medium to a number of glass containers to give a 2.5 cm depth.

2. Seal each container with a double layer of aluminium foil. Prepare an equal number of replacement foil covers and wrap them so that they will remain sterile after autoclaving.

3. Autoclave the containers and foil covers at 15 p.s.i. for 15 minutes.

*Surface sterilize the seeds as follows:*

4. Wrap the seeds in a muslin bag tied with strong thread.

(The following stages should be carried out aseptically in a sterile-handling cabinet.)

5. Totally immerse the bag for 30 minutes in the chlorate(I) solution in the screw-top jar. Shake the jar at frequent intervals.

6. Rinse the bag four times in sterile water in the sealed screw-top jar.

7. Cut open the bag with a flamed scalpel and transfer the contents with a little sterile water to a sterile petri dish.

8. Use a flamed fine spatula to transfer four seeds to each sterile container. Reseal each container with a fresh foil.

In good light the seedlings should be ready for use 4–5 weeks after germination.

Suggested types and proportions of auxin and cytokinin are listed below, with the expected growth responses.

Alternative media	Auxin + concentration	Cytokinin + concentration	Expected response
A {	(i) NAA 2.0 mg/dm <sup>3</sup>	Kinetin 0.02 mg/dm <sup>3</sup>	roots
	(ii) NAA 0.2 mg/dm <sup>3</sup>	Kinetin 2.00 mg/dm <sup>3</sup>	shoots
B {	(i) IAA 0.2 mg/dm <sup>3</sup>	Kinetin 0.02 mg/dm <sup>3</sup>	roots
	(ii) IAA 0.02 mg/dm <sup>3</sup>	Kinetin 1.00 mg/dm <sup>3</sup>	shoots

**Table 4**

As can be seen, media with high auxin levels and low cytokinin concentrations result in the production of roots, while high cytokinin levels stimulate shoot production.

The investigation may be extended by the use of intermediate concentrations of the two types of phytohormones. If the correct balance is achieved, callus production alone results, but the exact response to a particular intermediate proportion is unpredictable.

#### Alternative materials

The suggested varieties of *Nicotiana tabacum* are 'Xanthii' or 'White Burley' but other varieties and species of *Nicotiana* give similar results.

Petioles, leaf discs and stem pith sections from mature plants respond in a similar way to the stem explants suggested. They may be surface sterilized as follows:

1. Immerse for 30 seconds in a 70% ethanol solution (v/v).
2. Surface sterilize for 20 minutes in a 7% sodium chlorate(I) solution.
3. Rinse four times as normal.

Time-scale (at 26°C)	From initiation (weeks)
Callus visible (if present)	1–2
Roots and shoot primordia visible	2
Shoots with leaves visible	2–4
Shoots may be transferred to hormone-free medium	4–5
Plantlets may be transferred to compost	7–8

#### Answers to students' questions

1. —
2. See above.
3. Commercial preparations contain NAA and IBA (indolybutyric acid) as they are less readily translocated and, therefore, more local in their action. The cost and stability of the preparation are also important.

4. Such shoots will have arisen from lateral buds undetected during the production of explants.

## Experiment 4: Control of organogenesis in cultures of petals of *Saintpaulia ionatha* (African violet)

This may be regarded as an alternative exercise to the preceding experiment but the obviously differentiated nature of the source of the explants is worth emphasizing. It also illustrates that the role of cytokinins in controlling development is not clear cut.

The suggested phytohormone compositions are shown in Table 5.

Auxin + concentration		Cytokinin + concentration		Predicted response
NAA	1.0 mg/dm <sup>3</sup>	Kinetin	0.2 mg/dm <sup>3</sup>	roots + callus
NAA	1.0 mg/dm <sup>3</sup>	Kinetin	1.0 mg/dm <sup>3</sup>	roots + callus
NAA	0.2 mg/dm <sup>3</sup>	BAP	1.0 mg/dm <sup>3</sup>	shoots + callus
NAA	1.0 mg/dm <sup>3</sup>	BAP	1.0 mg/dm <sup>3</sup>	shoots + callus
*NAA	2.0 mg/dm <sup>3</sup>	BAP	0.2 mg/dm <sup>3</sup>	callus + ?

**Table 5**

\*Callus cultures using this medium, initiated and maintained in the dark, will not differentiate to produce organs until seven or eight weeks after initiation. If maintained in the light, many root and shoot primordia are produced within four or five weeks.

Vazquez *et al.* (*Acta Horticulturae* 78, 249–258, 1977) have shown that root and shoot primordia develop from cells derived from the epidermis of the petal.

Time-scale (at 26°C)	From initiation (weeks)
Swelling of explant tissue	1–2
Obvious callus formation	2–3
Roots and root hairs visible	3–5
Green shoot primordia visible	4–6
Leaves visible	6–7
Transplanting to hormone-free medium	8–9

Shoots may be encouraged to root, thus giving rise to plantlets which may be grown to maturity in potting compost, by transfer to hormone-free medium (see 'Organogenesis in *Nicotiana tabacum*').

These experiments may be further extended as proposed in Question 5.

### Answers to students' questions

1. See above.
2. See above.
3. Differentiation is reversible. The totipotence of the nuclei of these differentiated cells is confirmed.
4. This will obviously depend upon the original source of the explants. Very rarely, a different colour will result from mutation events (somaclonal variation) in the culture.
- 5(a) See above for predicted results.

## 4. Appendix

### Relationships to concepts and topics in A-level biology

Topic/concept	Experiment								
	1	2a	2b	2c	2d	2e	3	4	4 ext.
<b>Cell division</b>									
Process of mitosis*	-	-	-	-	-	✓	-	-	-
Significance of mitosis*	✓	-	-	✓	-	-	✓	✓	✓
<b>Increase in biomass</b>									
Biomass growth curve	-	-	✓	-	-	-	-	-	-
Limiting factors on biomass*	-	-	✓	-	-	-	-	-	-
<b>Differentiation and organization of cells</b>									
Plant tissue structure — xylem	-	-	-	-	✓	-	-	-	-
Plant organ structure	-	✓	-	-	-	-	✓	-	-
Development of embryos (embryoids in this case)	-	-	-	✓	-	-	-	-	-
Embryonic nature of meristems	✓	-	-	-	-	-	✓	-	-
Development of plant cells is plastic	✓	-	-	✓	✓	-	✓	✓	✓
Totipotence of somatic nuclei	✓	-	-	✓	-	-	✓	✓	✓
<b>Control of development</b>									
Plant hormones as growth regulators*	-	✓	-	-	-	-	-	-	-
Activation of genes by hormones	✓	-	-	-	✓	-	✓	✓	✓
Influence of environment on development	-	-	✓	-	-	-	-	-	✓
<b>Evolution</b>									
Artificial selection (crop breeding)*	✓	-	-	-	-	-	-	-	-
<b>Reproduction</b>									
Clone production by vegetative propagation*	✓	-	-	✓	-	-	✓	✓	-
<b>Use of statistics</b>									
	-	-	✓	-	-	-	✓	-	-

\*Indicates that the concept occurs in syllabuses at 16+ [The 16+ examination developed into the GCSE].

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## 5 Bibliography

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# Students' notes

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The following experiments were developed by a teacher working with biologists at Colworth House, Bedfordshire — one of two research establishments in the UK operated by Unilever.

The experiments are based on research being undertaken in the Plant Physiology Laboratory, and focus on the growth and development of plant tissues. Tissue culture techniques have, in recent years, become of major industrial importance in such areas as plant propagation, disease elimination, plant improvement and the production of plant extracts for use in pharmacology.

It is hoped that this practical work will help you to understand something about how the science you are learning in your studies is applied in the solution of real problems affecting our society.

## Introduction

It is well known that, given the right conditions, higher plants have the ability to regenerate from damaged tissue. This has obvious survival value for the plant and has long been exploited by both amateur and commercial growers for the vegetative propagation of desirable varieties.

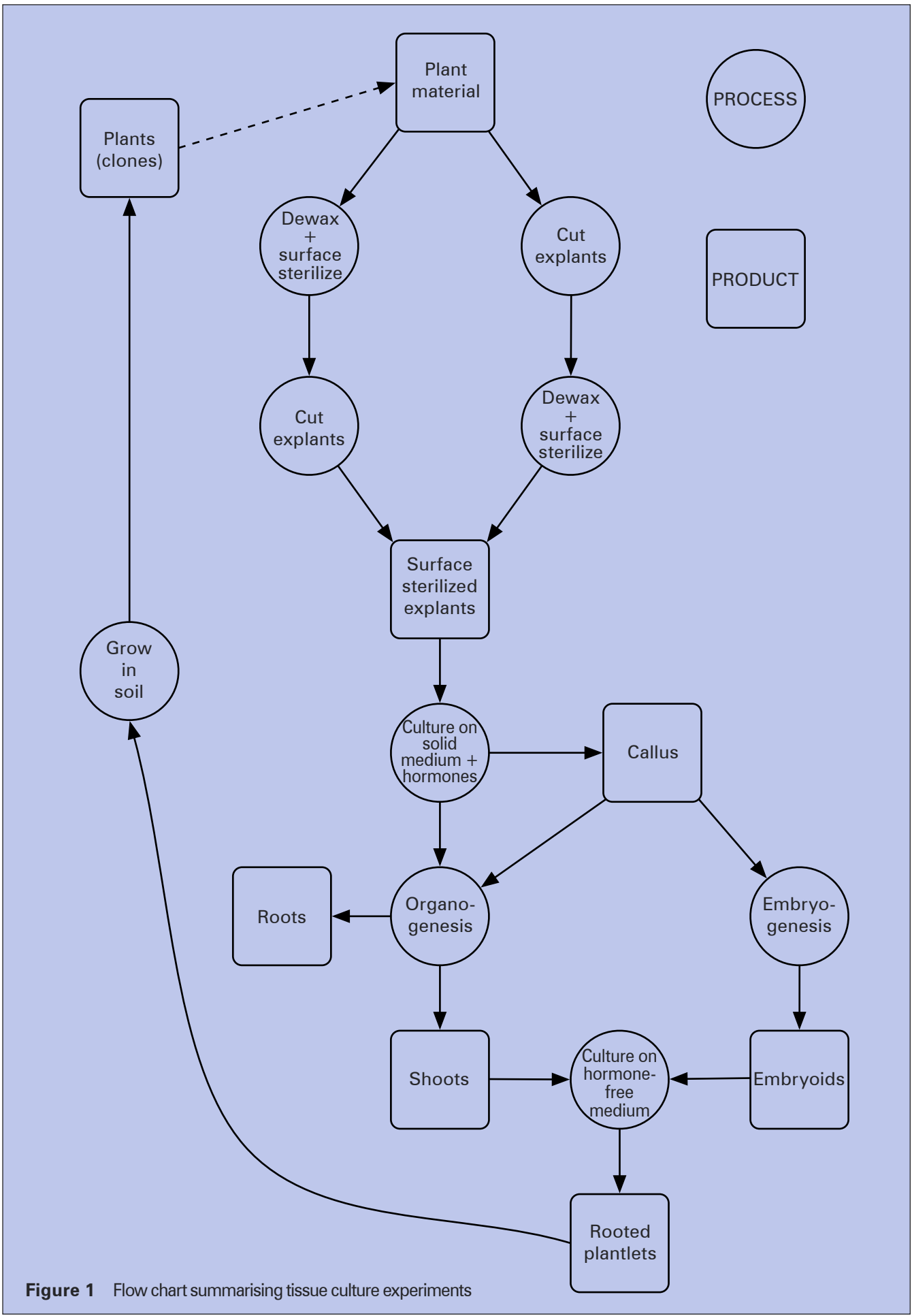
These experiments explore aspects of some of the relatively new industrial techniques that make use of plants' regenerative ability.

When higher plants are damaged, tissue is often stimulated to grow in a disorganized manner to produce a mass of largely undifferentiated tissue, called a *callus*. *Explants*, portions of tissue cut from higher plants, can be stimulated to produce callus by the provision of a suitable medium containing a mineral salts mixture, an energy source (sucrose), vitamins and plant hormones (*auxins and cytokinins*). It is often possible, by adjusting the hormone balance of the medium, to modify the pattern of growth of the tissue arising from the explant. Callus cultures may give rise to differentiated structures, e.g. phloem and xylem elements, or to small, embryo-like structures (*embryoids*) at the callus surface. *Organogenesis* may take place, thus roots and/or shoots may be produced from the callus or directly from the explant.

Embryoids or shoots produced by cultures can be grown on, using suitable media, to produce small rooted plantlets which can, with care, be successfully transplanted into soil. Such plants, unlike the products of sexual reproduction, are likely to be genetically identical *clones* of the parent plant.

The explant itself need not contain meristematic tissue for the above processes to occur because the nucleus of every living cell is *totipotent*, i.e. contains all the genetic information necessary for the growth and development of the whole organism.

The processes studied in the experiments which follow have been widely used industrially. They are summarized in the flow diagram (Figure 1).



**Figure 1** Flow chart summarising tissue culture experiments

Tissue culture techniques for the vegetative propagation of plants (*micropropagation*) have a number of advantages over other methods. They are rapid, independent of season, and can be applied to a number of species that are otherwise difficult to propagate. Apical meristems in infected plants often remain virus-free. Their use for tissue culture has, therefore, permitted the elimination of viruses from infected stocks of a range of species.

Certain types of callus culture give rise to clones that have inheritable characteristics different from those of the parent plant. Improved varieties arising in this way can be propagated and used commercially.

In the future, it is likely that new varieties and hybrids produced using other modern techniques will be commercially propagated by tissue culture methods.

A recent development has been the commercial use of callus cultures of the shikon plant to produce large quantities of shikonin, a medically valuable product found, in a lower concentration, in the shikon root. Plants are valuable sources of many such products, and the application of tissue culture techniques in this way may, therefore, become more economically important.

## Aseptic handling of cultures

### **Basic apparatus for aseptic handling**

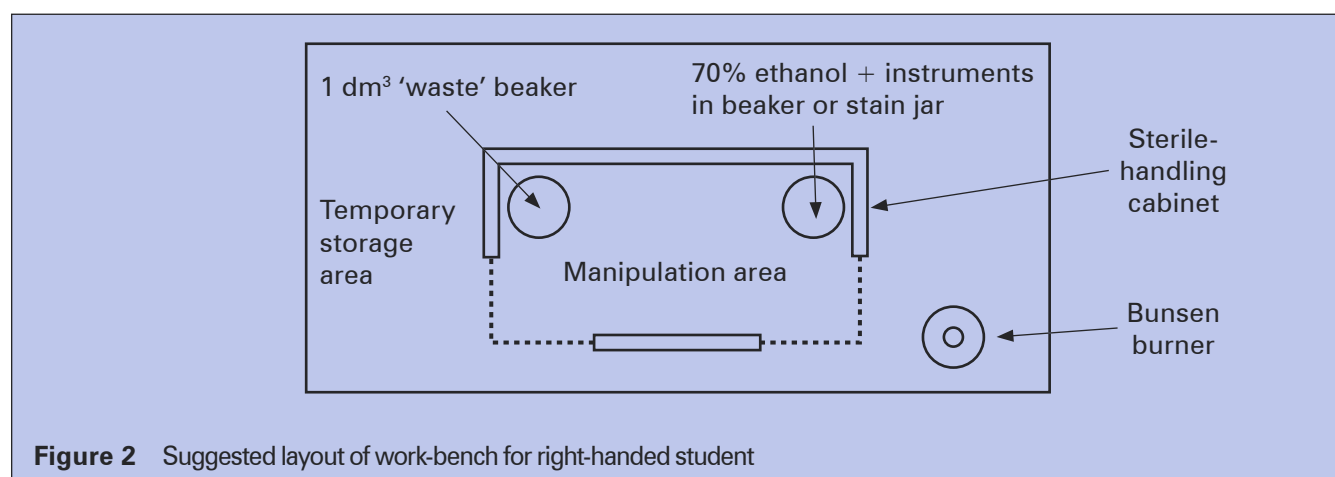
Each group will require:  
 Sterile-handling cabinet (if available)  
 Bunsen burner  
 Beaker (400 cm<sup>3</sup>) or stain jar + lid, containing approx. 200 cm<sup>3</sup> 70% ethanol solution (v/v)  
 Beaker (1000 cm<sup>3</sup>) or equivalent ('waste' beaker)  
 Sterile forceps  
 Sterile scalpel handle + blade  
 70% ethanol solution (v/v) (200 cm<sup>3</sup> approx.)  
 Marker pen  
 Parafilm or insulating tape

Contaminating micro-organisms will grow freely on the media used for plant tissue culture, so it is vital that all procedures are carried out using aseptic handling techniques, preferably in a sterile-handling cabinet.

All media and wash water used must be sterile and all instruments should be sterilized initially, and kept in 70% ethanol when not in use. Sterilization is achieved by steaming in an autoclave for 15 minutes at a pressure of 15 p.s.i.

Success requires careful, methodical organization of materials before work commences. Ensure that you have all the apparatus you require close at hand.

Figure 2 gives a suggested layout for a work-bench.



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1. Your work-bench/cabinet should be washed thoroughly with 70% ethanol. When the solution has evaporated, the Bunsen should be lit to create a permanent updraught. Windows and doors should be kept closed.

2. You are the most likely source of contamination so:

a) wash your hands with 70% ethanol;

b) wear a clean lab. coat;

c) do not lean over the bench or breathe directly onto sterile materials;

d) do not, at any time, pass your hands over sterile materials. (When transferring sterile materials from one container to another it is often best to arrange the containers in a line away from you rather than from left to right.)

3. Before use, all instruments should be flamed thoroughly by burning off the ethanol (take care) and then heating the instrument in the Bunsen flame for 15 seconds, ensuring that all parts that might touch the specimen are properly heated and allowed to cool in air or by immersion in a sterile liquid.

**Caution: Never return hot instruments to the ethanol beaker.**

4. When you open any glass container of, for example, sterile water, the neck should be passed briefly through the Bunsen flame so that an updraught is created from the mouth of the container.

5. Lids should never be exposed upside down nor placed on the work-surface.

## Experiment 1: Production of plantlets from floral organs of cauliflower

The upper surface of cauliflower curd consists of floral meristems. These would normally develop into flowers but, in the conditions employed in this experiment, they revert to the vegetative phase and develop into leafy shoots, without the formation of disorganized callus. Plantlets produced in this way show a high degree of genetic uniformity. This method is, therefore, ideal for the propagation and maintenance of varieties.

It is not economically viable to use these plants as a crop for consumption but the seeds produced by the crop are worth far more than the marketable cauliflowers. The crop for consumption is grown from this seed.

This method has been used for the improvement and propagation of early cauliflowers in Brittany and Cornwall.

### Apparatus

Each group will require:  
Basic set of apparatus (see p. 17, 'Students' notes')  
Screw-top jar, clean, preferably sterile (250 cm<sup>3</sup> approx.)  
Clean white tile or petri dish base  
Sodium chlorate(I) (hypochlorite) bleach solution (250 cm<sup>3</sup> approx.) + wetting agent  
Sterile water (500 cm<sup>3</sup> approx.)  
Cauliflower floret (clean, fresh, approx. 3 cm × 3 cm curd surface)  
Sterile petri dish containing growth medium (3)

### Apparatus

(after 3 weeks approx.)

Petri dish (up to 5, containing 20 cm<sup>3</sup> sterile growth medium without kinetin)  
Sterile petri dishes  
Basic set of apparatus

### Notes on apparatus

1. The chlorate(I) bleach solution + wetting agent can be either:  
a) 10% sodium chlorate(I) solution (v/v) (1.4% available chlorine) + 2 cm<sup>3</sup> Teepol; or  
b) 20% solution (v/v) Domestos (1.4% available chlorine).

2. The petri dishes should contain 20 cm<sup>3</sup> of an agar-based growth medium which includes 2.5 mg/dm<sup>3</sup> *kinetin* (a cytokinin), sucrose, vitamins and mineral salts.

### Procedure

Collect all the apparatus that you will need and prepare any solutions that are required.

Set up and sterilize your work-bench as suggested in the notes on aseptic handling techniques (p. 17).

1. Select a clean floret from a fresh cauliflower head. Place it on a tile and, holding it with forceps, carefully trim off 'mini-florets' from the curd (Figure 3a) to produce 20 'cuboids' of curd tissue, approximately 3 mm X 5 mm X 5 mm (Figure 3b). These will be your explants.



Figure 3a Section of curd.

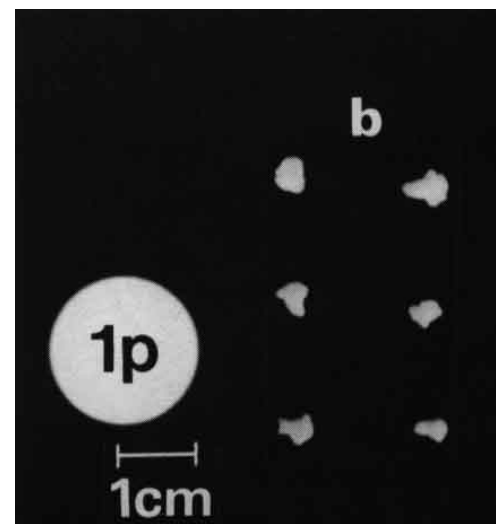


Figure 3b 'Mini-florets', the cuboid explants.

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*The next stage of the procedure is to sterilize the surface of the explants with a chlorate(I) solution.*

**Caution: You will be using a fairly strong bleach solution so take extreme care.** From now on you must use aseptic handling techniques (p. 17), so return your forceps to the ethanol beaker.

2. Quickly transfer your explants to a clean (preferably sterile) screw-top jar and add chlorate(I) solution to leave a small head space. Reseal the jar and shake the contents for five seconds.

3. Shake the jar for five seconds every minute for exactly 10 minutes.

4. After exactly 10 minutes, pour off the hypochlorite solution into the 'waste' beaker, using the jar lid to trap the sterilized explants.

5. Wash the explants four times as follows. Add approximately 100cm<sup>3</sup> sterile water to the jar, reseal it, shake for five seconds and pour off the liquid into the 'waste' beaker as in Step 4. The explants may be left in the last wash until they are required.

6. Using sterile forceps (cooled in the wash water) transfer six explants to each of the three petri dishes containing growth medium. The explants should be widely spaced and pressed gently onto the agar. Flame sterilize and cool your forceps when each dish is complete.

7. Seal each dish with Parafilm or insulating tape to reduce dehydration.

8. Label each dish clearly on its base and incubate them in the light at 20°C–28°C.

9. Examine each culture weekly, record and sketch any changes you observe. If some of the explants show signs of contamination, the remainder should be aseptically transferred to fresh medium (Steps 6–8).

When developing shoots have reached approximately 1–2 cm in length, the following procedure should be carried out using aseptic handling techniques (p. 17).

1. Carefully transfer the growth from one explant (Figure 3c) to a sterile petri dish and reseal both dishes.

2. Hold the material with sterile forceps and cut from the mass the larger shoots and connected material at their bases.

3. Transfer four shoots to a petri dish containing hormone-free medium. The shoots should be widely spaced and their basal ends pressed gently into the agar.

4. Seal each dish with tape or Parafilm and incubate at 20°C–28°C in the light.

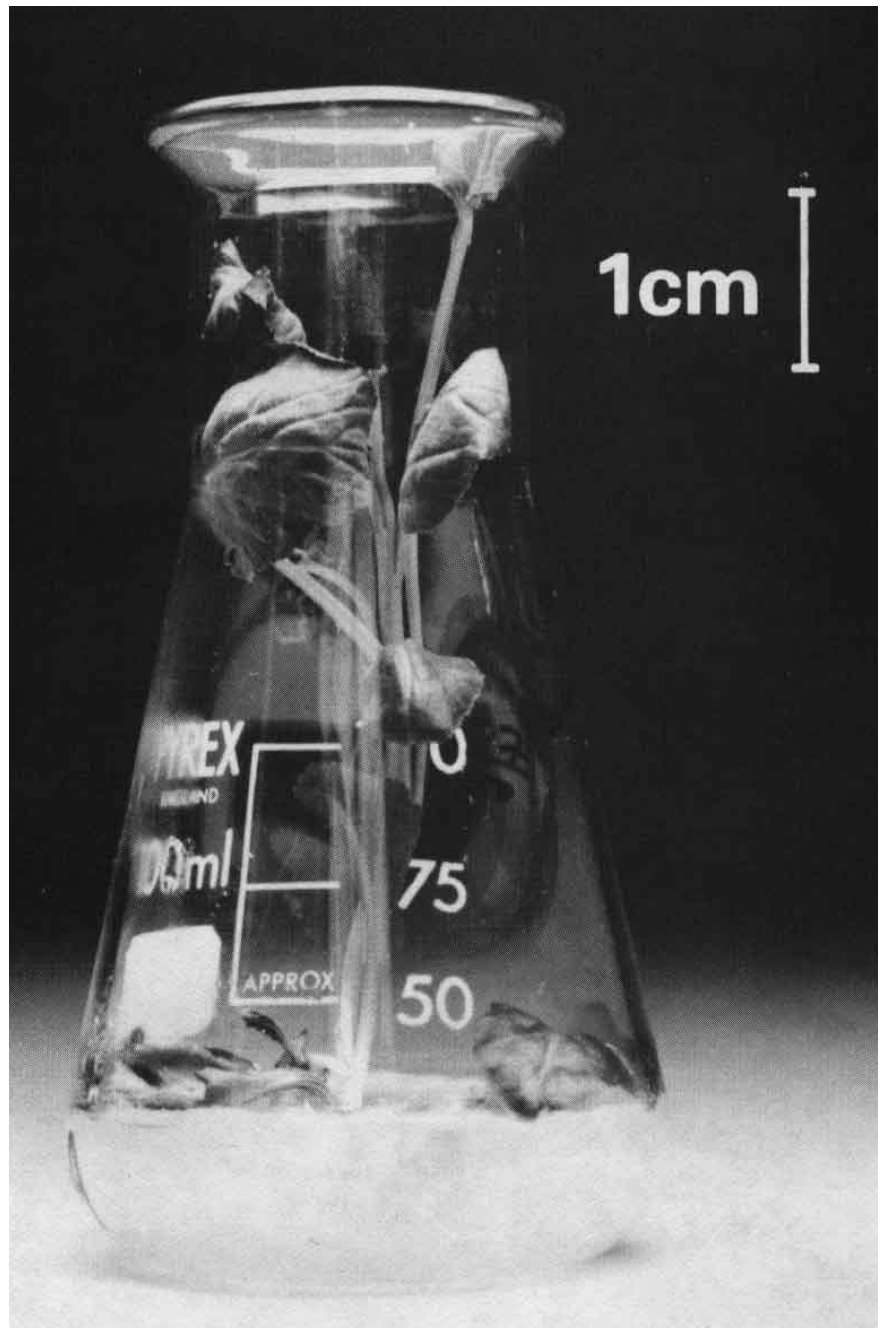
5. After a further 2–3 weeks the shoots should have developed roots (Figure 3d) and can then be transferred to individual petri dishes containing hormone-free medium or to small pots of



**Figure 3c** Growth from explant after three weeks.



**Figure 3d** Rooted plantlets.



**Figure 3e** Clone ready for potting.

sterile potting compost. In either case, the containers should be sealed to restrict dehydration, and incubated in the light at 20 °C–28 °C.

### Questions

1. From your results, estimate how many plants could be propagated from a single cauliflower head by using this method. State clearly any assumptions that are made and show your working.

2. In the past, vegetative propagation of cauliflowers has involved the use of cuttings from leafy shoots originating from the base of the stem or from the curd. What advantages, over this method, are offered by the use of tissue culture for micropropagation?

## Experiment 2: Studies on carrot callus cultures

Many crop plants offer no conventional method for vegetative propagation. One such species, the oil palm, is now being propagated on a commercial scale using a similar tissue culture technique to that employed in this investigation. Root explants are stimulated to produce undifferentiated callus by the provision of a suitable growth medium containing auxins. Eventually, embryoids spontaneously appear in the callus, and these are transferred to a medium which favours their proliferation. The shoots which develop are then transferred to a rooting medium and the whole plantlets, produced in this way, may then be hardened off and planted out.

In this investigation, explants are obtained from the tap root of a carrot and stimulated to produce callus by the synthetic auxin 2,4-D. The callus cultures are maintained and subcultured on this medium and their properties are investigated. Embryoids that arise are observed microscopically or transferred to a hormone-free medium to encourage their further development.

### a) Initiation and maintenance of carrot callus cultures

#### Notes on apparatus

1. The growth medium should be an agar medium containing mineral salts, sucrose, vitamins and  $0.1 \text{ mg/dm}^3$  2,4-D (2,4-dichlorophenoxyacetic acid).

2. The bleach solution + wetting agent can be either:

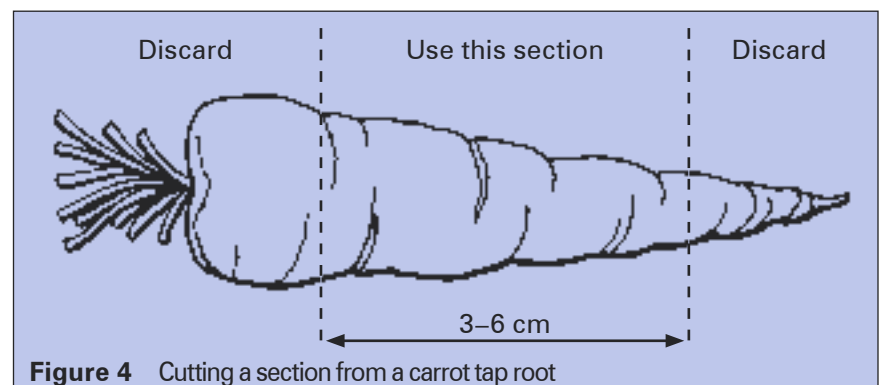
- 10% sodium chlorate(I) (hypochlorite) solution (v/v) (1.4% available chlorine) +  $2 \text{ cm}^3$  Teepol; or
- 20% solution (v/v) Domestos (1.4% available chlorine).

#### Procedure

You will be provided with a tap root of carrot (*Daucus carota*). If the tap root is undamaged and without internal air spaces, the interior of the root will be sterile. To eliminate tap roots with air spaces, only fresh, undamaged carrots which sink in water are used.

Collect all your apparatus, make up any solutions that you require and prepare your work-bench as suggested in the notes on aseptic handling (p. 17).

1. Cut a section of tap root 3–6 cm long, discarding both ends of the root (Figure 4). Remove the epidermis and any blemishes with a scalpel, ensuring that you know which end was the root pole.



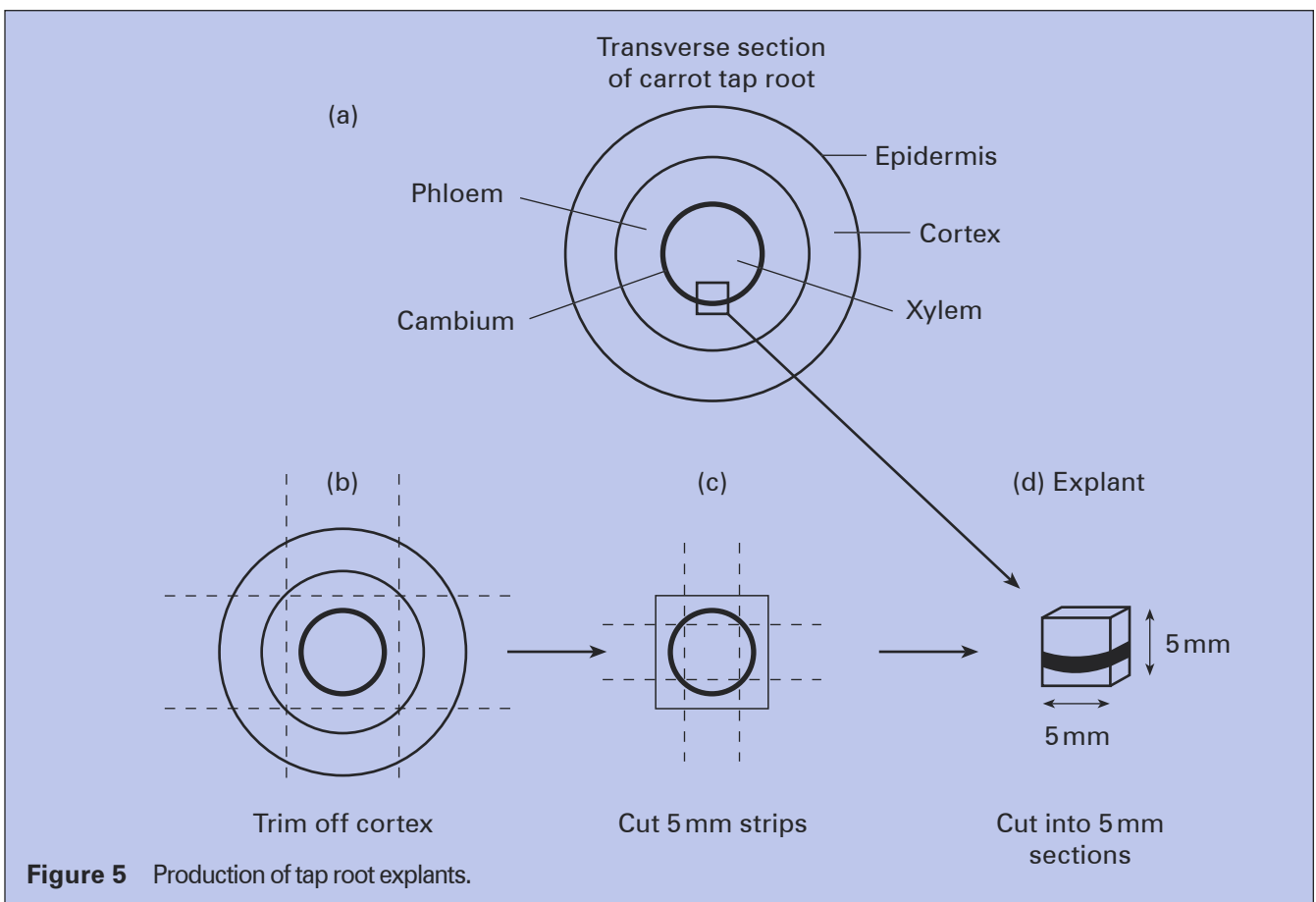
**Figure 4** Cutting a section from a carrot tap root

#### Apparatus

Each group will require:  
Basic set of apparatus (see p. 17, 'Students' notes')  
Tap root of carrot (*Daucus carota*)  
Scalpel (preferably sterilized)  
Sterile water ( $1 \text{ dm}^3$  approx.), preferably distilled  
Sterile petri dish (5)  
Sterile glass screw-top jar and lid  
Sterile petri dish containing growth medium (5)  
Bleach solution ( $250 \text{ cm}^3$  approx.) + wetting agent

The next part of the procedure is to sterilize the surface of the material by using a dilute solution of bleach. **The solution must be handled with care**, and the following procedures must be carried out aseptically (see p. 17).

2. Place the tap-root section in a sterile, lidded jar and cover it with a chlorate(I) bleach solution (approx. 1.4% available chlorine) containing a wetting agent (detergent). Reseal the jar and shake for five seconds every five minutes for 20 minutes.
3. Pour off the bleach solution into the 1 dm<sup>3</sup> 'waste' beaker.
4. The sterilized root is then washed four times by completely covering it with sterile water, resealing the jar, shaking for five seconds and pouring the water into the 'waste' beaker.
5. Transfer the root to the base of a sterile petri dish, cut 1 cm from each end and discard this material.
6. Insert a sterile scalpel blade into the central core of xylem at the shoot (broader) end of the section; this will hold the material steady.
7. Cut 3–5 transverse sections, 1–3 mm thick, across the tap root and transfer each, shoot-pole uppermost, to a fresh sterile petri dish, which should be resealed after each operation.
8. Cut smaller sections, explants approximately 5 mm square, from each of these transverse sections by cutting across the cambium (Figure 5a). The following method is recommended.



**Figure 5** Production of tap root explants.

i) Trim the cortex and some of the phloem from each transverse section (Figure 5b).

ii) Cut off 5mm-wide strips containing the cambium (Figure 5c).

iii) Each strip can then be subdivided to produce 5mm square explants, each containing parts of the phloem, xylem and cambium (Figure 5d).

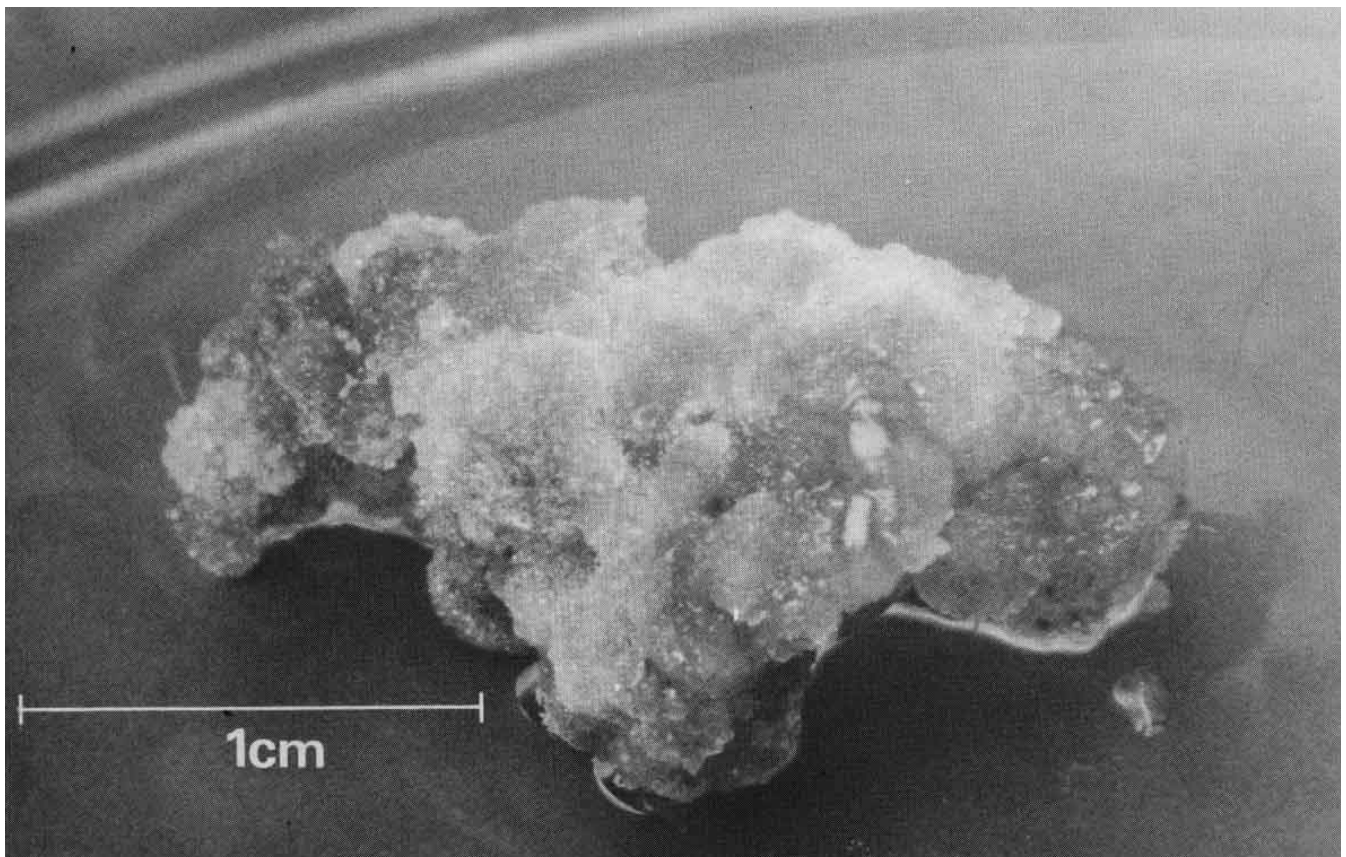
9. Transfer each explant, two per petri dish, root-pole downwards, to a complex sterile agar medium containing the hormone 2,4-D.

10. Seal each dish with Parafilm or insulating tape to reduce dehydration of the medium. Label each dish and incubate them upright, in the dark, at 25°C.

11. Examine your explants at weekly intervals. Record any changes that you observe. If your material is contaminated, you may transfer any undamaged explants to a fresh petri dish containing growth medium and incubate this as before (Steps 9 and 10).

#### **Maintaining growth of callus**

After 4–5 weeks, the callus produced will be well developed (Figure 6). To maintain its growth, it must be subcultured at regular 4-week intervals onto fresh medium. Unhealthy, dark necrotic tissue should be discarded.



**Figure 6** Well-developed carrot callus ready for subculture.

### **Apparatus**

Basic set of apparatus  
Sterile petri dish per callus to be subcultured  
Petri dish (per callus) containing growth medium + 2,4-D

### **Procedure**

Prepare your work-bench as before. Carry out the following subculture procedure using aseptic handling techniques (see p. 17).

1. Transfer a well-developed callus to a sterile petri dish with sterile forceps. Reseal both dishes.
2. Barely raising the lid of the dish, use a sterile scalpel carefully to cut off healthy (yellow or cream-coloured) pieces of callus, approximately 5mm cubes.
3. Transfer these callus cuttings to a petri dish containing fresh growth medium, ensuring that the cuttings are widely spaced.
4. Seal the petri dish with tape or Parafilm and label it with the date and nature of the culture. Incubate the dish upright, in the dark, at 25°C.

### **Questions**

1. Which tissues in the explant could have given rise to the callus produced?
2. Design and, if possible, carry out an experiment to eliminate one or more of these alternatives.
3. If the callus has not arisen from initially meristematic tissue, what does this suggest about the process of differentiation in plant tissues?

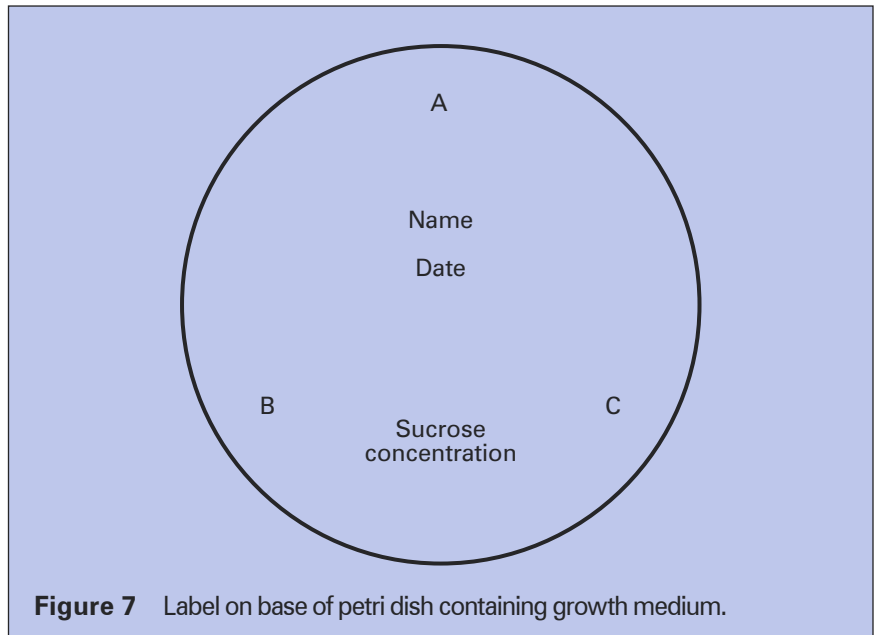
## **b) Effect of sucrose concentration on growth-rate of callus cultures**

### **Procedure**

1. Transfer the petri dish with fresh medium to an incubator at 25°C.
2. Label clearly each of the empty petri dishes 'A', 'B', 'C', so that you can identify them. Weigh each of them accurately and record their masses in the form of a table. Carry out all of the following steps using aseptic handling techniques. Prepare your work-bench accordingly (p. 17). When transferring callus, the lid of each dish should be barely lifted.
3. Using sterile forceps, carefully transfer one callus culture to each of the sterile petri dishes. Try not to carry over any of the growth medium from the original culture.
4. Reweigh each of the dishes and their contents. Record the new mass and determine the mass of each callus by difference.
5. Label the bottom of the petri dish containing growth medium, as shown in Figure 7.
6. Using sterile forceps, transfer the callus from Dish A to the growth medium, placing it in position on the agar above the label 'A'. Repeat this procedure with callus 'B' and callus 'C'.
7. Seal the dish with Parafilm or tape, and incubate it in the dark at 25°C.

### **Apparatus**

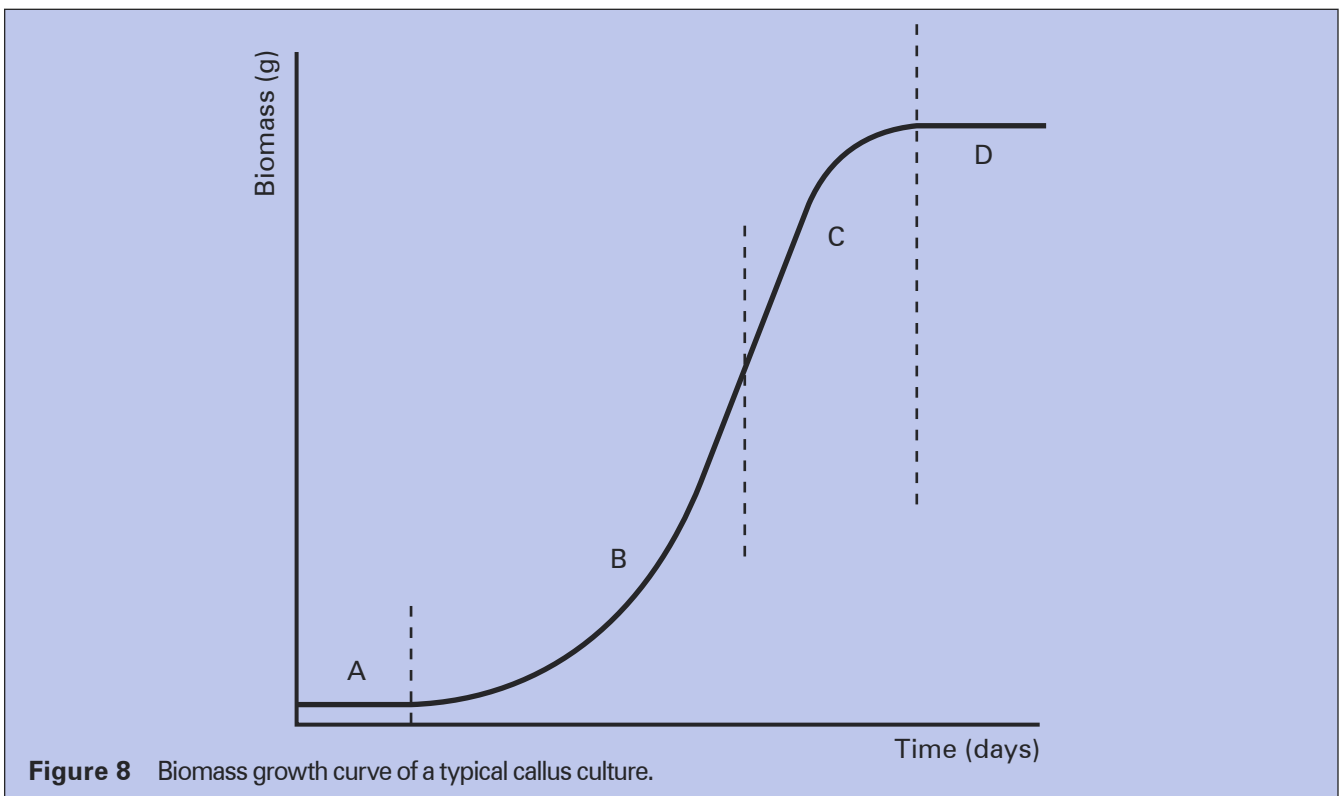
Each group will require:  
\*Basic set of apparatus  
Carrot callus cultures, 3/4 days after subculture (3)  
\*Balance, accurate to 0.005 g  
\*Sterile petri dish (3)  
Petri dish containing growth medium + 2,4-D + sucrose at a concentration in the range 0.75%–4% (w/v)  
\*Required weekly



8. Repeat Steps 1–7 at weekly intervals for six weeks, maintaining the code for each callus, 'A', 'B', 'C'.
9. Express your results in graphical form and summarize the data in a simple table.
10. Collect summarized data from other members of your class.

#### Questions

1. Does the evidence suggest that the growth of frequently subcultured callus is arithmetical, logarithmic or of some other kind? (If necessary, plot a further graph of  $\log_{10}$  biomass against time.)



2(a) Determine the mean time taken for the callus mass to double for each sucrose concentration and plot a graph of mean doubling time against sucrose concentration.

b) Describe and explain the effects of varying sucrose concentration upon the growth of the callus.

3(a) A callus culture that is maintained without transfer to fresh medium exhibits the growth curve shown in Figure 8. Which phase of the growth curve most nearly resembles the pattern of growth that you observed?

b) Explain the patterns of growth observed in Phases B, C and D in the light of your experimental results. What other explanations are there for the shape of this curve?

4. Why was it suggested that the fresh growth medium should be incubated before the callus was transplanted?

### c) Embryogenesis in carrot callus cultures

After eight weeks of growth on media containing 2,4-D, carrot callus cultures sometimes undergo developmental changes. 2,4-D stimulates disorganized growth but, under the low power of a microscope, organized structures may be observed at the edges of the callus. These putative embryoids may, if transplanted onto a hormone-free growth medium, give rise to shoots, roots and, ultimately, complete plants.

The following procedure can be employed, as circumstances permit, at approximately weekly intervals after the eighth week of culture.

#### **Apparatus**

Each group will require:  
Basic set of apparatus  
Sterile petri dish  
Petri dish containing sterile, hormone-free growth medium (2)  
Microscope, slides and coverslips  
Healthy carrot callus culture at least eight weeks old  
Glass rod

#### **Procedure**

Collect all your apparatus and prepare your work-bench as before. Carry out Steps 1–5 using aseptic handling techniques.

1. With sterile forceps, transfer a callus to a sterile petri dish. Reseal both dishes.

2. Barely raising the lid of the dish, use a sterile scalpel to cut off healthy (yellow or cream-coloured) pieces of callus, approximately 5 mm cubes, from the edge of the callus.

3. Transfer several cubes of callus to a petri dish containing hormone-free growth medium. Ensure that the cubes are widely spaced and pressed firmly onto the agar. Retain some cubes for Step 6.

4. Seal each dish with Parafilm or tape to reduce water loss. Label each dish with your name, date, details of the original callus and the medium's composition.

5. Incubate each dish upright, in the light, at 25°C. Examine your cultures at weekly intervals, recording any observations.

#### *Observation of embryoids*

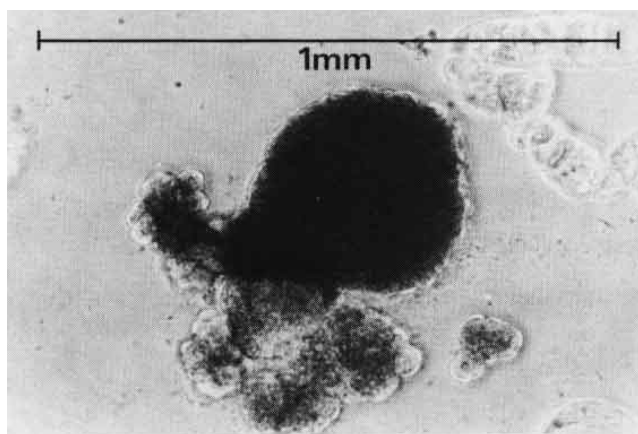
6. Remove and discard the dark, necrotic callus tissue.

7. Add 2cm<sup>3</sup> of water to the petri dish and, using a glass rod, gently break up as much as possible the remaining cubes of callus.

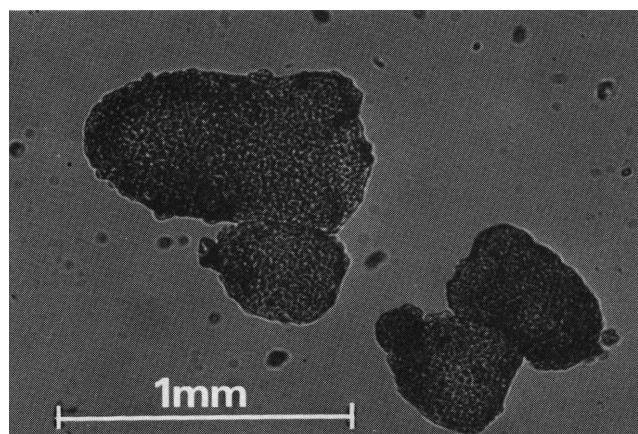
8. Transfer a drop of the resulting suspension to a clean microscope slide and cover with a coverslip.

9(a) Examine the suspension, initially under low power. Typical embryoid structures are shown in Figure 9a–9d.

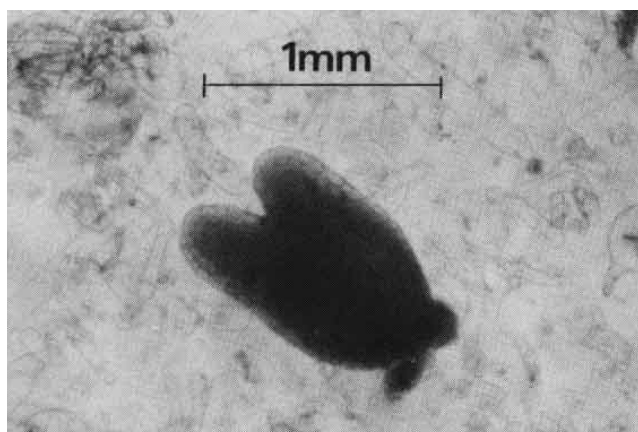
b) Draw any structures that you observe.



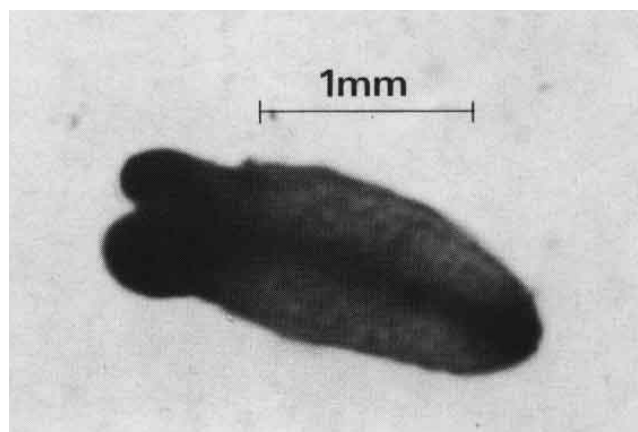
**Figure 9a** Globular stage in embryoid development.



**Figure 9b** Heart-shaped stage.



**Figure 9c** Torpedo stage.



**Figure 9d** Late torpedo, early cotyledonary stage.

### Question

1. Does your investigation support the statement that the nuclei of plant cells are totipotent?

### d) Cytodifferentiation in carrot callus cultures

Under the influence of the synthetic auxin 2,4-D, carrot explants grow to form a largely undifferentiated mass of cells, a callus. However, it is possible to observe differentiated xylem tracheary elements within the callus mass.

Increased production of xylem (xylogenesis) may be stimulated in cultures from explants of entirely parenchymatous tissue by providing a particular balance of auxins and cytokinins in the medium.

### **Apparatus**

Clean petri dish  
Mounted needle (2)  
Forceps  
Scalpel  
Phloroglucinol + concentrated hydrochloric acid  
Carrot callus (a subculture aged approx. three weeks)  
Microscope, slides and coverslips

### **Procedure**

1. With forceps, remove the callus to a clean petri dish.
2. Cut off a small piece of healthy tissue (a 2 mm cube approx.) from the edge of the callus and transfer it to a clean microscope slide.
3. Add a small drop of water and macerate the tissue thoroughly with the mounted needles.
4. Add two drops of phloroglucinol and leave for 10 minutes, then drain off the liquid.
5. Add one drop of concentrated hydrochloric acid.
6. Cover the material with a coverslip and observe, at first under the low power of a microscope. Lignin will be stained red/magenta.
7. Draw and label any lignified cells that you observe.



**Figure 10** Lignified structures from a carrot callus culture ( $\times 250$ ).

### **Questions**

1. When demonstrating cytodifferentiation in callus cultures, why is it important that the material used is a subculture rather than a primary callus grown from an explant?
2. Can you identify in your specimens any other differentiated cells?

### **Apparatus**

Each group will require:  
Actively growing callus culture of carrot (*Daucus carota*)  
Scalpel  
Boiling tube containing 5 cm<sup>3</sup> 1M hydrochloric acid  
Water bath at 60 °C or beaker (400 cm<sup>3</sup>) half full of water at 65°C  
Petri dish  
Forceps  
Mounted needle (2)  
Microscope slides and coverslips  
Aceto orcein stain (freshly prepared and filtered)  
Bunsen burner  
Microscope (low power objectives and magnification at least × 250)

## **e) Nuclear division in callus cultures**

### **Procedure**

1. Place the boiling tube of acid into the water bath to warm up while you are carrying out Step 2.
2. Transfer the callus to a petri dish lid and, using a scalpel, cut portions of active material from the pale, creamy-white outer parts of the callus.
3. Using forceps, transfer the callus fragments to the boiling tube of acid, and shake gently. Return the tube to the water bath and allow the material to hydrolyse for 10 minutes.
4. After 10 minutes, carefully decant most of the acid and transfer the hydrolysed callus fragments to a petri dish base.
5. Use a scalpel to cut sections approximately 1 mm thick from these fragments and place each section on a microscope slide.
6. Tease the fragment apart with the mounted needles.
7. Add two drops of aceto orcein stain and warm the slide gently until the stain steams, but does not boil. (*Note:* If the back of the slide is painful to the touch, then you are overheating it!)
8. Leave the material for 10 minutes to take up the stain. Carefully absorb excess stain with the edge of a piece of blotting paper.
9. Add one drop of fresh stain, apply a coverslip, and press down hard through several thicknesses of blotting paper. Warm again gently.
10. Observe the material under low power and then at a higher magnification. Nuclear material should be densely stained and, in actively dividing cells, chromosomes should be visible as heavily-stained rod-like structures within the cells.
11. Draw any patterns that you observe and identify the phases of mitosis that they represent.

### **Question**

1. Of the cells with nuclei, what proportion are undergoing mitosis?

## Experiment 3: Control of organogenesis in cultures of *Nicotiana tabacum*

In this experiment, you will investigate the effects of various combinations of growth substances on the nature and rate of organogenesis (organ, i.e. root and/or shoot, production) by *Nicotiana* explants. Such investigations are clearly a necessary step in the development of a reliable technique for the production of plantlets from the disorganized material used for genetic manipulation and for propagation. The possible role of plant hormones in development is also illustrated.

### Apparatus

Each group will require:  
Basic set of apparatus (see p. 17, 'Students' notes')  
Sterile water, 1 dm<sup>3</sup> approx.  
Sterile glass screw-top jar  
Sterile petri dishes  
Sterile petri dishes of agar medium containing mineral salts, vitamins, sucrose and hormones  
Sodium chlorate(I) (hypochlorite) bleach solution (250 cm<sup>3</sup> approx.) + wetting agent  
Seedlings of *Nicotiana tabacum*

### Notes on apparatus

1. The chlorate(I) bleach solution + wetting agent can be either:  
a) 2% sodium chlorate(I) solution (v/v) (approx. 0.25% available chlorine) + 2 cm<sup>3</sup> Teepol; or  
b) 4% Domestos solution (v/v) (approx. 0.25% available chlorine).

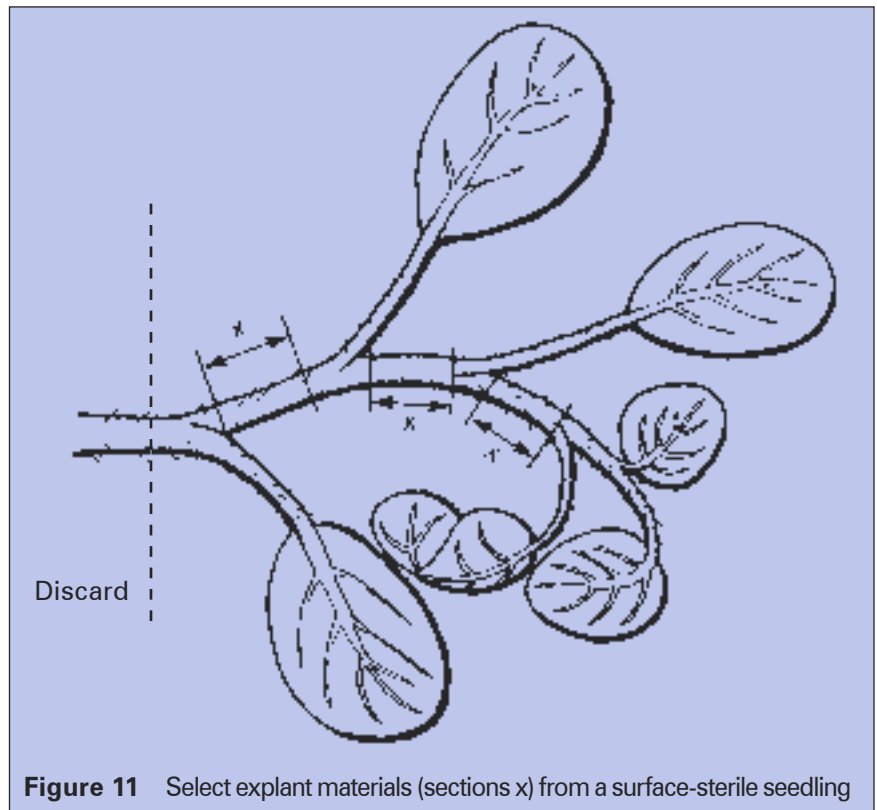
### Procedure

You will be given a jar containing one or more seedlings of *Nicotiana tabacum*, and a number of sterile petri dishes, each containing a complex agar medium. The media provided contain various concentrations and combinations of an auxin (either 1 Naphthyl-Acetic Acid [NAA] or Indol 3yl-Acetic Acid [IAA]) and a cytokinin (kinetin).

Collect all your apparatus, make up any solutions that you will require and prepare your work-bench as suggested in the notes on aseptic handling (p. 17).

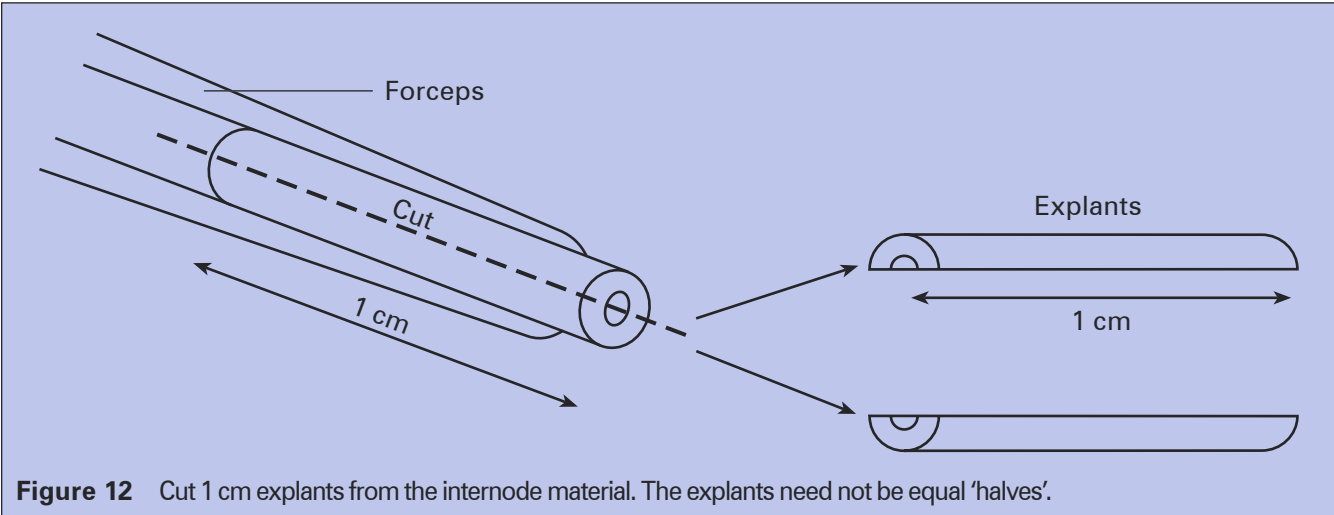
*The following procedure must be carried out using aseptic handling techniques.*

1. Using sterile forceps and a scalpel, remove the apical (upper) 7–10 cm of the stem of the seedling with its attached leaves and transfer the material to a sterile petri dish lid. If the seedling has been aseptically germinated and grown, surface sterilization (Steps 2, 3 and 4) may be omitted.
2. Transfer the plant material to a sterile, lidded glass jar and cover it with the bleach solution. Reseal the jar and shake it for five seconds. Shake for five seconds once every minute for exactly 10 minutes (or for the period suggested by your teacher).
3. After 10 minutes, pour off the bleach solution and wash the specimen four times by completely covering it with sterile water, resealing the jar, shaking for five seconds and discarding the water into the 'waste' beaker.
4. Transfer the specimen to a sterile petri dish and remove and discard the basal (lowest) 1 cm of the specimen.
5. Cut the specimen into sections, keeping only the internodes (see Figure 11) and transferring each to a separate sterile petri dish. Cut each internode into 1–2 cm lengths.



**Figure 11** Select explant materials (sections x) from a surface-sterile seedling

6. Hold each length with sterile forceps and split it lengthwise with a sterile scalpel (see Figure 12) to expose the pith and other internal stem tissue. These are your explants.



**Figure 12** Cut 1 cm explants from the internode material. The explants need not be equal 'halves'.

7. Transfer two explants, exposed pith downwards, to each of the different agar-based media. (Use a minimum of three dishes of each type.)

8. Seal each dish with Parafilm or tape to limit dehydration of the medium. Label each clearly and incubate them upright, in the light, at 26°C.

9(a) Examine your explants at weekly intervals and record any changes you observe. Uncontaminated, healthy explants from dishes showing contamination can be transferred to fresh growth medium and reincubated (Steps 7 and 8).

b) Count the numbers of roots or shoots occurring on the explants. (*Note:* A shoot appearing within one week of the initial incubation should be ignored.)

c) Tabulate your data and plot graphs of the number of roots and the number of shoots against time.

### Questions

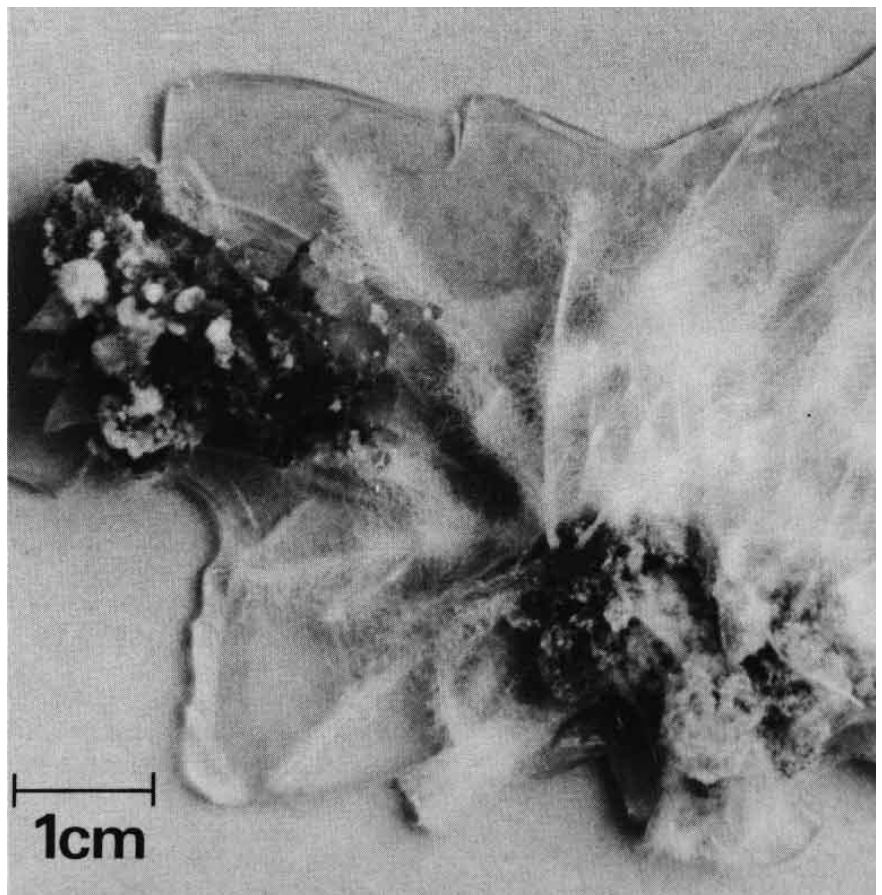
1. Determine the mean and standard deviation of the number of roots and of the number of shoots produced by a 1 cm length of explant on each of the different growth media after three weeks.

2. Which proportions of auxin and cytokinin stimulate the production of roots? Which stimulate shoot production?

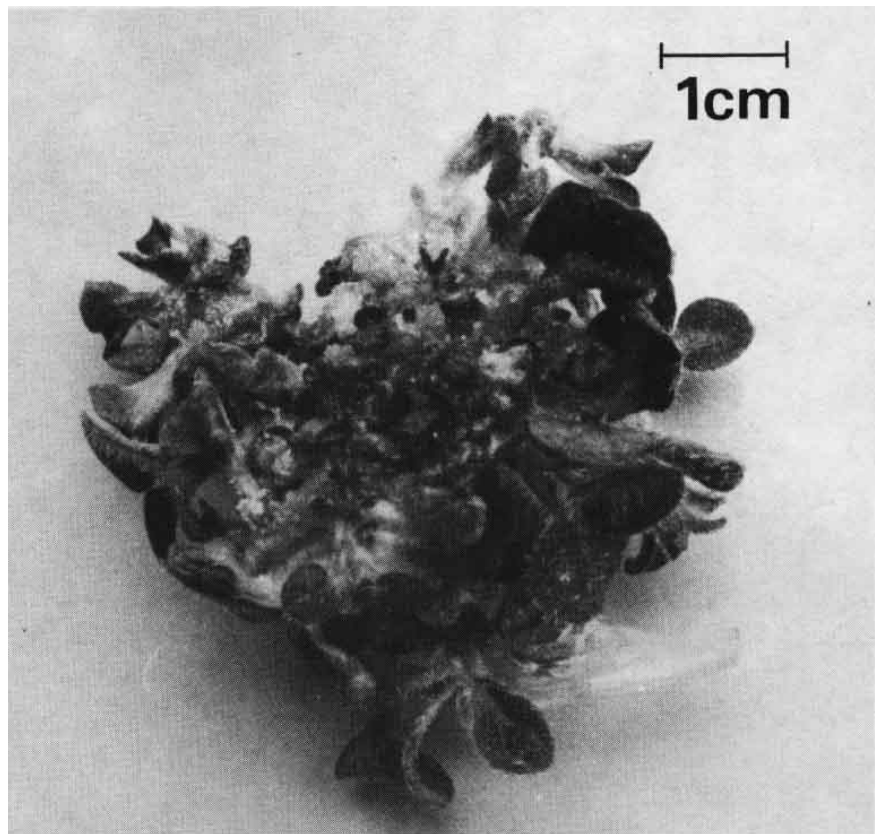
3(a) Which type of phytohormone would you expect to predominate in commercial 'rooting hormone' preparations sold for use with stem cuttings?

b) What properties, other than the ability to stimulate residual meristems to produce roots, should be possessed by such a commercial preparation?

4. Why should shoots developing within one week of incubation be ignored when collecting your data?



**Figure 13a** Organogenesis by *Nicotiana* stem explants (roots, root hairs clearly visible).



**Figure 13b** Organogenesis by *Nicotiana* stem explants (leafy shoots visible).

#### **Apparatus**

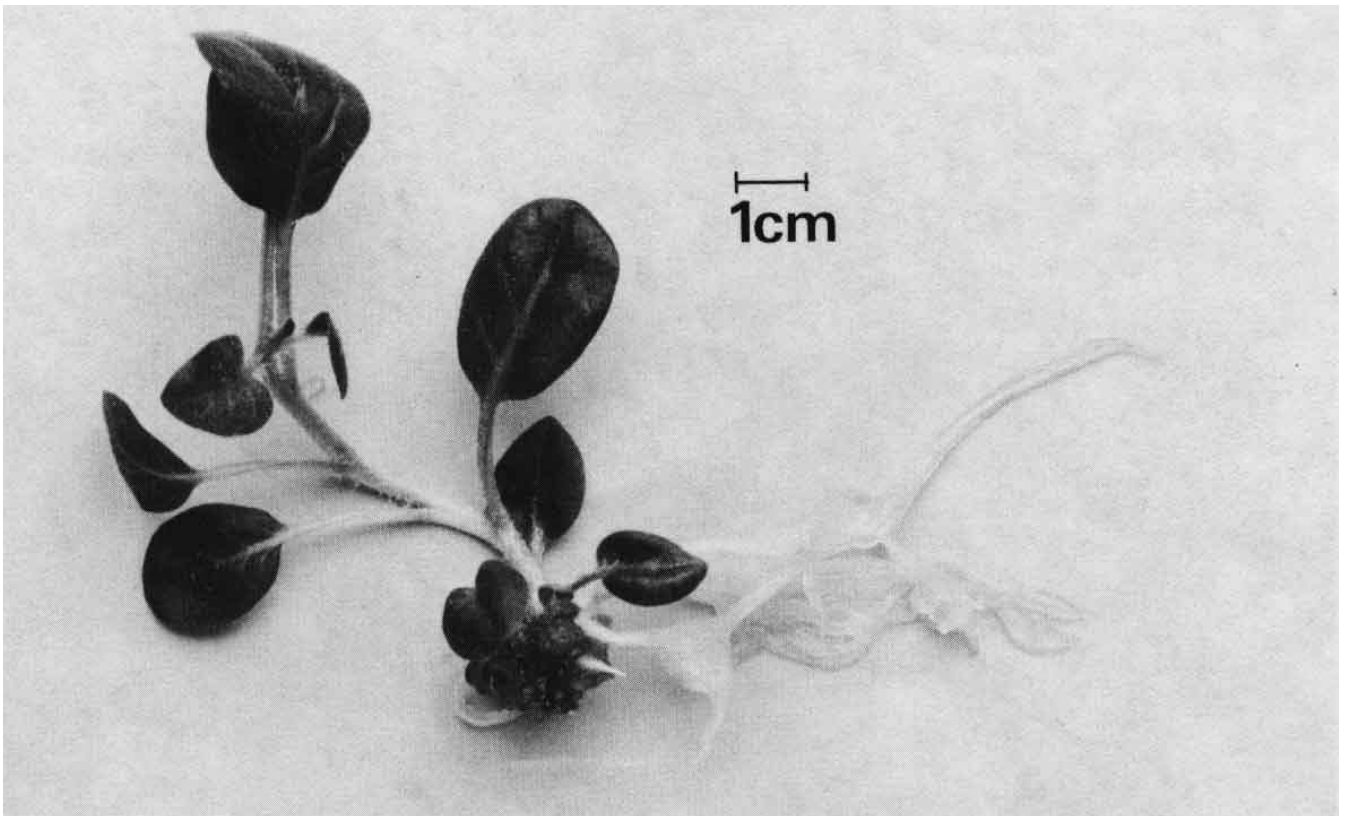
Each group will require:  
Basic set of apparatus  
Sterile petri dish  
Sterile petri dish containing  
hormone-free growth medium  
(2 or more)  
Pots of potting compost  
Plastic bags

#### **Extension work: production of plantlets**

After four or five weeks, shoots will be sufficiently developed to be transplanted to a hormone-free growth medium.

#### **Procedure**

1. Aseptically transfer a well-developed, shoot-producing specimen to a petri dish. Reseal both dishes.
2. Carefully separate four or more larger shoots from each other but retain some of the mass of tissue at the basal end of the shoot.
3. Aseptically transfer two shoots to each of the dishes of hormone-free medium, pressing the basal ends gently onto the surface.
4. Seal each dish with Parafilm or tape and incubate in the light at 20°C–28°C. Examine weekly.
5. When roots have developed, transfer the plants to small pots containing damp sterile potting compost. Cover each pot with a plastic bag to maintain high humidity and incubate the plantlets in the light at 20°C–28°C for two or more weeks.



**Figure 14** *Nicotiana tabacum* plantlet produced in seven weeks by tissue culture.

## Experiment 4: Control of organogenesis in cultures of petals of *Saintpaulia ionatha* (African violet)

The ability of *Saintpaulia* to regenerate from cuttings is renowned. This experiment investigates the effects of various combinations of the auxin NAA (1-naphthylacetic acid) and the cytokinins, kinetin and BAP (6-benzylaminopurine) upon organogenesis in cultures of African violet petals. Clearly, such investigations are a necessary part of the development of a reliable protocol for the propagation of plants by tissue culture. *Saintpaulia ionatha* is one of the many species of ornamental plant that are commercially propagated in this way.

### Apparatus

Each group will require:  
Basic set of apparatus (see p. 17, 'Students' notes')  
Sterile petri dish (2)  
Sterile screw-top jar (250 cm<sup>3</sup> approx.)  
Sterile water (1000 cm<sup>3</sup>)  
5% Teepol (detergent) solution in water (v/v) (100 cm<sup>3</sup> approx.)  
Sodium chlorate(I) (hypochlorite) bleach solution (200 cm<sup>3</sup> approx.) + wetting agent  
Petri dish containing growth medium (6 or more)  
Flowering African violet (*Saintpaulia ionatha*) or two mature flowers

### Notes on apparatus

1. The chlorate(I) bleach solution + wetting agent can be either:
  - a) 7% sodium chlorate(I) (1% available chlorine) + 1% Teepol in water (v/v); or
  - b) 14% Domestos in water (v/v) (1% available chlorine).
2. The petri dishes should contain 20cm<sup>3</sup> of a sterile, agar-based growth medium, including sucrose, vitamins, mineral salts and mixtures in differing proportions of an auxin (NAA) and a cytokinin.

### Procedure

Collect all the apparatus you need and make up any solutions. Prepare your work-bench as suggested in the notes on aseptic handling techniques (p. 17).

1. Using forceps, transfer two mature inflorescences from the *Saintpaulia* plant to a clean screw-top jar. Hold the inflorescences by their pedicels.

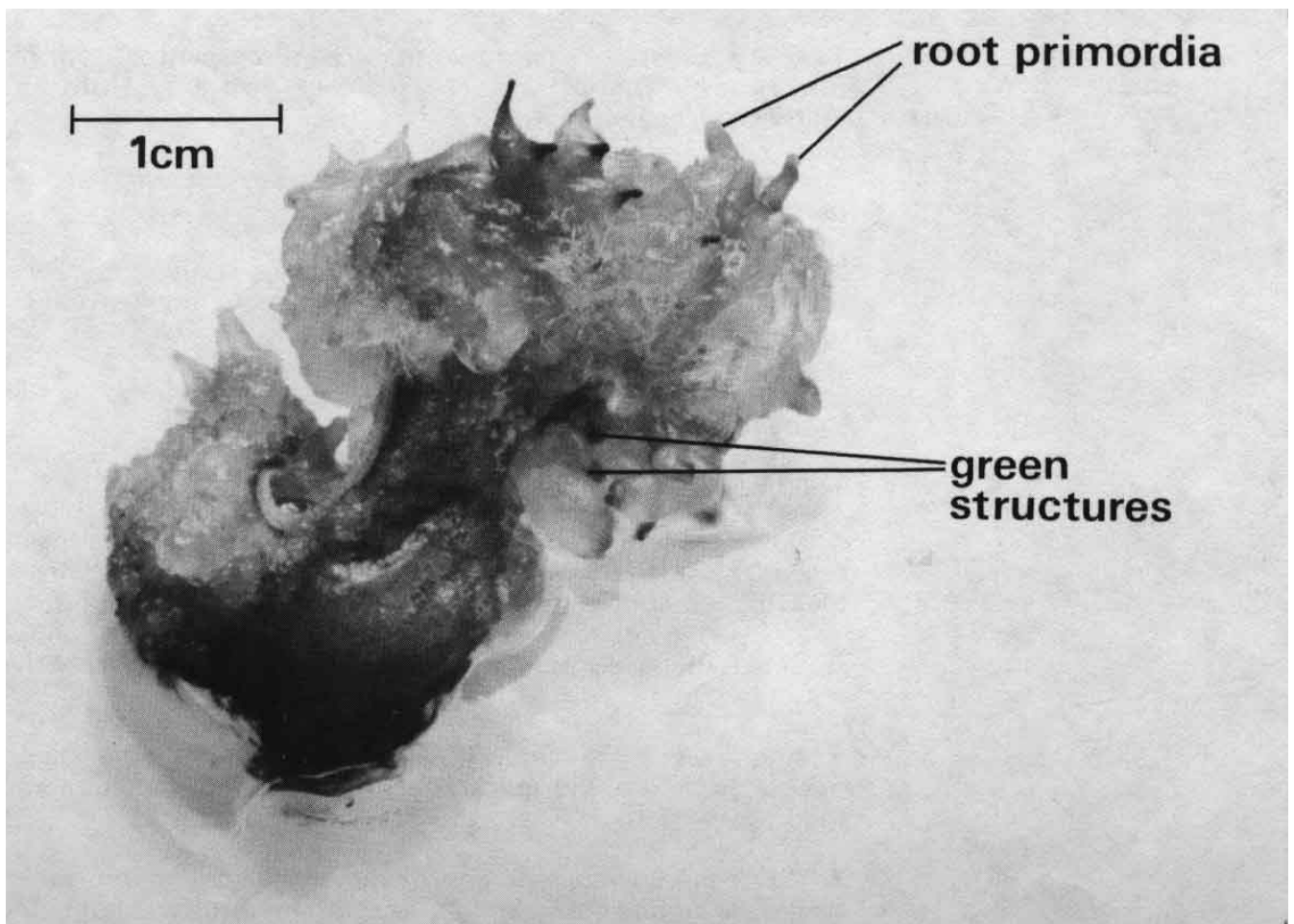
*The following procedures should be carried out using aseptic handling techniques (p.17).*

2. Cover the flowers with 100cm<sup>3</sup> detergent solution to dewax the material. Reseal the jar and, shaking frequently, leave it for one minute.
3. Pour off the liquid into the 'waste' beaker, using the jar's lid to prevent loss of the flowers.

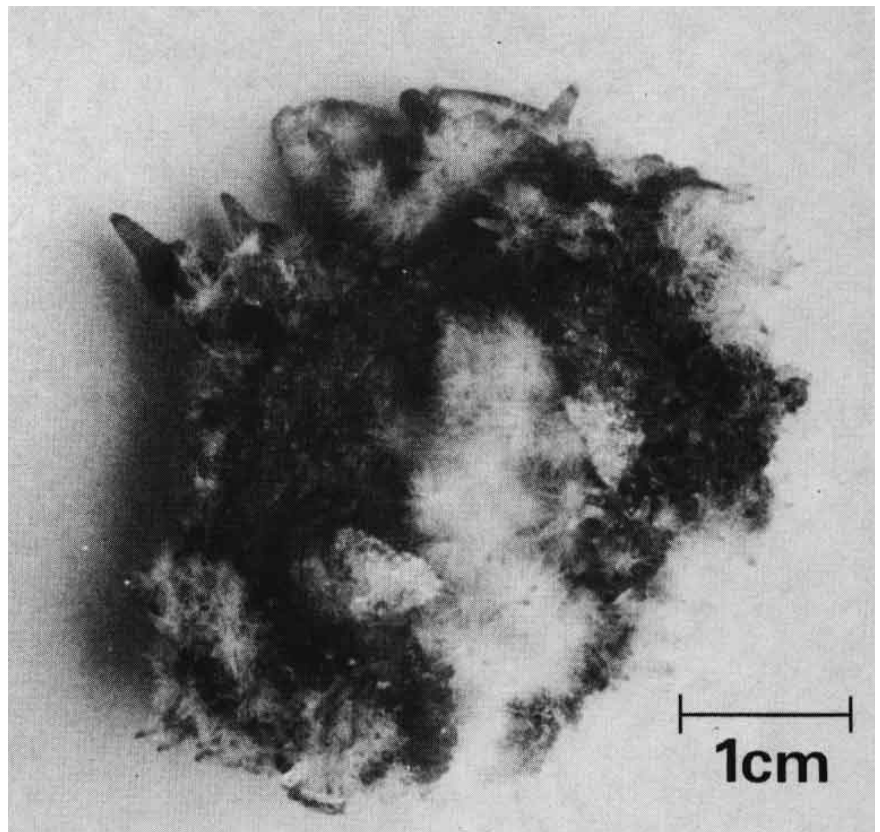
*You must now sterilize the surface of the petals.*

4. Cover the flowers with 200cm<sup>3</sup> of the chlorate(I) solution and reseal the jar. (**Caution: The chlorate(I) solution is a strong bleach, so it should be handled with care.**)
5. Shake the jar for five seconds every minute for exactly five minutes.
6. After exactly five minutes, pour off the liquid into the 'waste' beaker as before and quickly rinse the material four times as follows.
7. Cover the flowers with approximately 200cm<sup>3</sup> sterile water, reseal the jar and shake for five seconds. Pour the water into the 'waste' beaker. The material may be left in the final rinse water in the sealed jar until required.

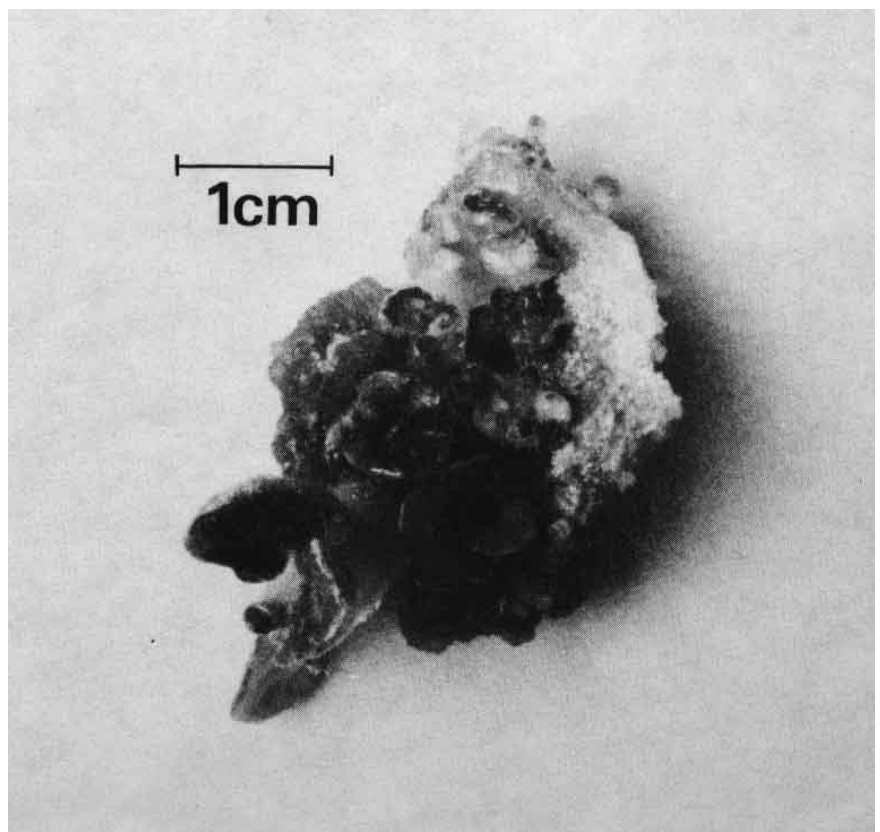
8. Transfer each flower to a separate sterile petri dish and replace the lids.
9. Prepare explants by using a sterile scalpel to cut approximately 1 cm × 1 cm squares of material from the petals of one of the flowers (Flower A).
10. Use sterile forceps to transfer two explants of petal material to a petri dish containing one of the growth media provided. The explants should be widely spaced and pressed gently onto the agar. Repeat this procedure for each of the different media, so that the same flower acts as the source of explants used for every medium.
11. Label each dish with your name, the date, the flower code, and the growth medium.
12. Repeat Stages 9, 10 and 11 using the other flower (B) as a source of explants.
13. Seal each petri dish with Parafilm or insulating tape to reduce water loss.
14. Incubate the dishes upright, in the light, at 26°C.
15. Examine the material at half-weekly intervals, recording and sketching any changes that occur.



**Figure 15a** Organogenesis by petal explants of *Saintpaulia ionantha*. Callus bearing root and shoot primordia.



**Figure 15b** Callus with more developed roots.



**Figure 15c** Callus with leafy shoots.

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### Questions

1. Which combinations and concentrations of NAA and cytokinins stimulate the production of (a) roots; (b) shoots; (c) both?
2. Kinetin and BAP are both synthetic cytokinins. What light does this experiment shed on the statement that high ratios of cytokinins to auxins stimulate the production of shoots?
3. The structures produced in this investigation have developed from differentiated tissue (epidermis). What does this tell us about the process of differentiation in plant cells? What support is given to the statement that the nuclei of differentiated cells are totipotent?
4. Predict the colour of the flowers produced by mature plants grown from your cultures.
5. Design and, if possible, carry out investigations into the effects of different light regimes on the development of *Saintpaulia* cultures. It is suggested that a medium containing NAA (2 mg/dm<sup>3</sup>) + BAP (0.2 mg/dm<sup>3</sup>) is employed.
  - a) Investigate the effect of varying daily light periods (0–24 hours).
  - b) Investigate the effect of varying light wavelengths.



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