# THE GROWTH OF TULIP TISSUES IN VITRO

by

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Thesis submitted to
The University of Nottingham
for the degree of
Doctor of Philosophy

May 1981

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# Abstract

# The Growth of Tulip Tissues *in vitro*

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Large numbers of adventitious shoots have been induced to form *in vitro* on floral stem sections of *Tulipa gesneriana* L. cultivar Merry Widow. In comparison with explants of scale and axillary bud, floral stem tissues showed the greatest potential for shoot production. A modified Murashige & Skoog medium containing inorganic salts and vitamins at full strength and supplemented with benzylamino-purine and naphthalene-acetic acid at 1 mg $l^{-1}$ induced shoots on 70%-90% of floral stem explants.

The stage of development of the bulb was found to be an important factor in determining the ability of explants to regenerate shoots. The shoot producing potential of floral stem tissues was greatest during the 'dormant' phase of the bulbs, but the ability to produce shoots was lost once rapid extension growth and greening of the floral stem had commenced.

Morphogenesis *in vitro* was found to be influenced by the origin of explants from within the floral stem. A study of endogenous plant growth regulators was made within the floral stem in order to elucidate their role in the organogenetic processes occurring *in vitro*.

A study of histological and morphological development *in vitro* showed that the shoot-like structures arise from the epidermal cell layer and have the potential to form whole plants.

To my Mother and Father

Contents

Acknowledgements

I would like to gratefully acknowledge the help and guidance throughout this work of my supervisor Dr. Peter Alderson.

I would like to thank Dr. John Hall of Leicester Polytechnic for the use of equipment and for his assistance in the analysis of plant growth regulators, Dr. Janet Hilton for her constructive criticism and proof reading, Miss Grace Fox for her efficient typing, Malcolm Millar of Kirton E.H.S., Boston, for supplying bulb material, and my mother for her constant support.

My sincere thanks to my parents for their financial support, which made this work possible.

## Abbreviations

| | |
|---|---|
| PGR | Plant Growth Regulator |
| ABA | Abscisic Acid |
| ABA-LS | ABA-Like Substance |
| Me-ABA | Methyl-ABA |
| BAP | Benzyl Amino Purine |
| C-LS | Cytokinin-Like Substance |
| GA | Gibberellic Acid |
| GA-LS | GA-Like Substances |
| IAA | Indole Acetic Acid |
| IAA-LS | IAA-Like Substance |
| IBA | Indole Butyric Acid |
| NAA | Naphthalene Acetic Acid |
| 2.4-D | 2.4-Dichlorophenoxyacetic Acid |
| GLC | Gas Liquid Chromatograph |
| ECD | Electron Capture Detector |
| TLC | Thin Layer Chromatograph |
| PVP | Poly-Vinyl Pyrrolidone |
| Rf | Ratio fraction |
| FAA | Formalin Acetic acid Alcohol |
| H | Heller (1953) |
| M & S | Murashige & Skoog (1962) |
| M [ x : x ] | defined in Section 2.5 |
| v/v | volume for volume |
| $^{o}C$ | degrees Centigrade |
| w/v | weight for volume |
| rpm | revolutions per minute |
| m.p. | melting point |
| vb, b, 1N, 2N, 3N, T | Floral stem explants - defined in Figure 4.6 page 85 |
| L.S.D. | Least Significant Differance |

# Chapter 1

## General Introduction

Until recently plant tissue culture was solely an experimental tool for a few biologists. Nowadays virtually all colleges, universities and many commercial organisations are involved in the *in vitro* culture of plant cells, tissues or organs.

There are four areas in which the application of plant tissue culture is feasible:

1. production of pharmaceuticals and other natural products (Barz *et al.*, 1977);
2. genetic improvement of crops (Broertjes & van Harten, 1978);
3. Recovery of disease-free clones (Ingram & Helgeson, 1980) and preservation of valuable germplasm (Withers, 1980);
4. rapid clonal multiplication of selected varieties (Murashige, 1974, 1977; Vasil & Vasil, 1980).

Tissue culture techniques for tulip could be of use in three of these areas. However, it is in the enhancement of clonal propagation that tissue culture is likely to have its greatest application. Procedures have been successfully established for the germination of isolated tulip embryos (Niimi, 1978) and similar techniques have been successfully used for the growth of immature tulip embryos of otherwise non-viable hybrids (Custers, pers. comm.). However, no reliable method has been developed for the clonal multiplication of tulip *in vitro*.

Vegetative propagation of tulip by traditional methods is very slow; only 2-7 daughter bulbs being produced by each flowering bulb in one year. For this reason, and also because of the very long juvenile or growth phase of the tulip bulb (2-3 years), the establishment of a new cultivar requiring the production of approximately 2,000 bulbs can require **10 years or longer.**

It is possible to reduce this time by the shortening of the annual cycle of growth. Sisa & Higuchi (1967, a & b) and Fortanier (1971; 1973), using appropriate environmental conditions have reduced the duration of this cycle from 12 months to 200 days, and under laboratory conditions the cycle was reduced to 6 months. However, using these techniques the introduction of a new variety could nevertheless require many years.

Methods used for increasing the propagation rates of other bulbs, e.g. planting scale leaves (Lilium), wounding by notching, scooping, or cross-cutting (Hyacinthus) and twin-scaling (Narcissus & Hippeastrum), have not been successfully used for tulip. However, the yield of daughter bulbs can be slightly increased by high temperature treatment of flowering-size bulbs - 'blindstoken' (Rees & Briggs, 1976). As this treatment kills the flower, it has been suggested that more food reserves are diverted to bulb formation following treatment. Nevertheless, the yield of daughter bulbs was increased by only 14-29%.

Thus a method for the rapid multiplication of tulip is required and plant tissue culture techniques which are being applied to an ever increasing number of plant genera, may prove to be useful for this purpose. Until recently it was thought that monocotyledonous species were particularly difficult to grow *in vitro*. For example, Partenan (1963), Carter *et al*. (1967) and Krikorian & Barquam (1969) have commented on the alleged refractory nature of monocotyledons regarding tissue culture. However, it has become clear that techniques applied to dicotyledons can be equally effective for use with monocotyledons *in vitro* with only minor modifications. This has been shown in a recent review (Vasil & Vasil, 1980) of clonal propagation by tissue culture in which over 100 monocotyledonous species were listed together with 205 dicotyledons which were capable of 'producing buds, shoots or embryoids *in vitro*'.

Multiplication *in vitro* can be carried out by utilizing one or a combination of several regenerative phenomena, viz.

1. *Enhancement of axillary bud production*. By placing existing meristems (axillary buds) in culture on media containing cytokinins, precocious development of axillary meristems can be induced, e.g. *Gladiolus* (Hussey, 1976c, 1977); *Freesia* (Hussey, 1980).

Methods of propagation *in vitro* based on the enhancement of axillary shoot formation have a considerable advantage

in that these meristems generally arise from several layers of cells and thus genetic changes are less likely to occur (Hussey, 1980), or can be avoided entirely (Murashige, 1974).

2. Adventitious meristem formation on organ explants. e.g. Freesia (Davies,1972; Bajaj & Pierik, 1974); Gladiolus (Ziv et al., 1970); Iris (Hussey, 1976a); Narcissus (Seabrook et al., 1976).

The number of axillary meristems formed in many monocotyledons (including tulip) is very small. Consequently, rapid multiplication of many monocotyledons is dependent on the production of adventitious buds (Hussey, 1975b). Adventitious shoot formation is a common means of vegetative propagation in vivo for some plants, e.g. Saintpaulia, Begonia and Streptocarpus. Many differentiated cells in mature organs are polyploid (D'Amato, 1975), and therefore explants used for obtaining adventitious shoots should be meristematic or as undifferentiated as possible (Hussey, 1980). The basal plate tissue of bulbs contains a large meristematic area with a high regenerative capacity, as shown by its potential to give rise to leaves, scales and stem. It is therefore an obvious choice of explant tissue, e.g. in Lilium as used by Simmonds & Cumming (1976).

Although basal plate tissue may be the most likely to be genetically stable, a number of workers have found that floral stem tissue can be a reactive tissue in vitro for

various bulbous plants. The first report of regeneration from a floral stem of a monocotyledon was by Sheridan (1968), who reported callus and plantlet formation on stem explants of Lilium using a Linsmaier & Skoog (1965) medium. Shoot production by floral stem of Lilium was also observed by Bigot (1974). Ziv et al. (1970) reported that inverted inflorescence stalk discs when placed on a modified M & S medium were the most reactive tissues of Gladiolus tested in vitro; Davies (1972) and Bajaj & Pierik (1974) have obtained regenerative callus from freesia stem immediately below un-opened flower buds. Using lower concentrations of PGR's shoot clusters were formed directly on the stem explants.

In his survey of several species of bulbs and corms Hussey (1975b)obtained both plantlets and callus capable of regenerating plantlets, from inflorescence stems of Hyacinthus, Muscari, Ornithogalum, Scilla and Ipheion. In 1976 Hussey reported the production of 100-150 bulbil initials on 1 mm flower stem sections of Iris hollandica. A high regenerative capacity has also been shown for floral stem segments of Nerine (Pierik, pers. comm.) and Hippeastrum (Fountain & O'Rourke, 1980; Blakesley, 1980; Alderson, pers. comm.).

Seabrook et al. (1976) reported the production of large numbers of shoots on stem sections of Narcissus after being placed with their morphological base uppermost. However, Hosoki & Asahira (1980) reported a method for the in vitro

propagation of Narcissus in which floral stem sections were placed base down onto the medium. In this report it was suggested by Hosoki & Asahira that the high potential for adventitious bud formation in young flower stems may be a result of the distribution of intercalary meristems in these tissues (Cutter, 1971). If this is correct and the adventitious shoots are formed from existing meristematic tissue genetic instability may not be a serious problem. However, as these meristems are not normally involved in vegetative regeneration, abnormalities may appear that would not normally be apparent, e.g. flower abnormalities in Freesia (Horsfield, pers. comm.).

3. Adventitious meristem production on callus tissue.

Induction of callus frequently occurs when high concentrations of PGR's are used in the culture medium and often shoots can be induced to form subsequently by transfer of the undifferentiated callus cells to a medium containing lower concentrations of PGR's e.g. Saintpaulia (Vazquez et al., 1977). Somatic embryogenesis may also be induced in many species by the transfer of callus to media without, or with only low concentrations of PGR's e.g. Asparagus & Iris (Reuther, 1977a & b).

Callus derived from many plant species shows a strong tendency to develop polyploid or other mutant cells which often dominate cultures that are serially maintained e.g. Saccharum (Heinz & Mee, 1969); Asparagus (Malnassy & Ellison, 1970); Ornithogalum (Hussey, 1976b). In some

species the increase in polyploid cells occurs very rapidly. Murashige et al. (1968) reported that the frequency of diploid cells in Citrus limon callus had decreased to 33% after only three serial subcultures (one per month). The production of callus in vitro can be a useful means of mutation breeding (Broertjes, 1976) thus any propagation system involving the production of callus has to be carefully monitored for genetic stability.

The first report of the culture of tulip tissue in vitro was that of van Bragt (1971b), who used tissue culture techniques to study the physiology of flower-stalk elongation. The first reported use of tissue culture with the aim of in vitro propagation was that of Bancillion (1974), who used a complex temperature pre-treatment to induce bud formation on various parts of the tulip bulb. Newly-formed buds were reported to have occurred in "fairly large numbers on fragments of floral stem, the base of chlorophyllous leaves and of flower" from bulbs that had been stored at 10°C for "a little more than 17 weeks after their harvest" prior to dissection. No bulb or root formation was reported on any of these "neo-formed" buds.

Nishiuchi & Myodo (1976) using eight cultivars (Tulipa gesneriana) and three botanical species of tulip, induced callus to form on scale explants of Tulipa praestans, and of the cultivars Apeldoorn, Red Emperor and Defiance. Callus formation was observed in six of the other varieties tested, but only after "prolonged culturing". Apel-

doorn was reported to require high concentrations of auxin (NAA) for optimum callus formation (10-15 mg $l^{-1}$), and kinetin inhibited multiplication of this callus, whereas no callus was formed in the presence of kinetin alone. Culture of scale pieces of Apeldoorn on a medium containing 5 mg $l^{-1}$ NAA & 1 mg $l^{-1}$ kinetin after nine weeks produced a number of shoot-like protuberances which were shown to have developed vessels and also to have accumulated starch grains in the surrounding tissues. The development of adventitious roots was achieved by prolonged culture (22 weeks), but only from callus on media containing NAA alone, i.e. not on cultures producing the shoot-like protuberances. A translation of the publication of Nishiuchi & Myodo (1976) was not available until 1979. Therefore no use could be made of their data in the experimental work carried out on scale tissue reported in Chapter 3.

Rivière & Muller (1976) reported the *in vitro* culture of axillary buds excised from bulbs of cultivar Paul Richter. Buds were successfully grown *in vitro*, rooted, and subsequently established *in vivo* within a period of six months. However, this represented only a slight increase in propagation rate above that of the natural rate of daughter bulb production. This was therefore not considered to be a useful method of propagation.

With the exception of van Bragt (1971b),all the reported studies on the growth of tulip tissues *in vitro* have been

carried out using media based on that of Murashige & Skoog (1962) (M & S). This medium has been used for the culture of many and varied tissues (Murashige, 1974) and has been the basis of media used for many bulbous genera (e.g. Ziv *et al.*, 1970; Hussey, 1975b, 1976 a & b; Seabrook *et al*, 1976).

In this thesis results of experiments designed to determine the conditions required to obtain morphogenic cultures of tulip, as the basis of a method of vegetative propagation of tulip *in vitro*, are reported. Details of experiments designed to study the role of the plant growth regulator (PGR) balance within the floral stem in the regeneration of shoots *in vitro*, and to study the histology and morphology of these morphogenic cultures are also reported.

# Chapter 2

## Materials and Methods - *in vitro*

## 2.1. Bulb material

**Tulip bulbs of the Triumph group, cv. Merry Widow and of the Darwin hybrid group cv. Apeldoorn (flowering size 12/13 cm** and 'seed' bulbs) were obtained from Kirton Experimental Horticulture Station, Boston. The bulbs were harvested during June or July 1977, 1978, 1979 and subsequently stored dry at 17°C in the dark.

## 2.2. Growth of bulb material

Bulbs were subjected to a double cooling treatment comprising a period of dry storage at 5°C (Merry Widow 6 weeks, Apeldoorn 9 weeks), followed by a further 3 weeks at 5°C after planting. The bulbs were grown in a growth room, subsequently at 9°C for one week, at 13°C for a further week, followed by growth at 17°C until senescence. Light was supplied from HLRG lamps for a ten hour photo-period with a photon fluence rate of $10 \pm 1 \times 10^{-5} Em^{-2} s^{-1}$ at the soil surface. The compost was composed of a 50%/50% (v/v) mixture of sphagnum peat and 2-3 mm grit, with a pH of approximately 6.5. The compost was brought to field water capacity, and the bulbs were planted by pushing them into the surface of the compost. The bulbs were not covered, thus reducing the level of contamination by micro-organisms.

## 2.3. Preparation of explants

a) Scale: The outer papery scale (tunic) and roots (when present) were removed, washed under running tap water, surface sterilized in 20% (v/v) 'Domestos' (a commercial bleach containing approximately 8% available chlorine Lever Bros., England) for 30 min and washed three times for 3 min each in sterile distilled water. The outer fleshy scale and the apical half of the bulb were removed. The remainder of the bulb was sterilized for a further 20 min in 20% (v/v) 'Domestos' and finally washed three times in sterile distilled water. Explants of scale tissue, approximately 5 mm in width and 10 mm in height, were excised to include some basal plate tissue.

b) Bud: Bulbs treated as for scale explant preparation were dissected and the buds between the scales were excised to include a small amount of basal plate tissue.

c) Floral stem: Floral stems were removed from non-sterile dry bulbs, (except where stated) surface sterilized for 10-15 min in 10% 'Domestos' and washed three times in sterile distilled water. Stems taken from bulbs after planting were washed under running tap water prior to surface sterilization.

d) 1 mm sections: Plastic syringes (10 ml) were modified by cutting off the needle end and inverting the

plunger which had been shortened to approximately one quarter of its original length. The modified syringes were sterilized by submersion in 100% ethanol for 10 min and dried before use in the air stream of a laminar flow clean air cabinet, where all surface sterilization and subsequent operations were performed.

A 3% agar solution, sterilized by autoclaving at $1.085 \times 10^{5} Nm^{-2}$ for 15 min and cooled to approximately $40^{o}C$ was poured onto surface sterilized floral stems placed centrally in the modified syringes.

Once the agar had solidified, each syringe was pushed into the spring clips of the sectioning apparatus (Figure 2.1, page 16) and thin sections of floral stem (and agar) were cut by drawing a razor blade (attached to a scalpel handle to help maintain sterility) across the top of the syringe. An inverted plastic petri dish, cut to fit the syringe, and sterilized in 100% ethanol was used to provide a working platform. Two full revolutions of the screw thread advanced the plunger 1 mm, thus giving a consistent section thickness. Floral stem sections were placed on a sterile glass plate and separated from the agar before being placed onto culture media.

## 2.4. Sterile technique

Surface sterilization and all subsequent operations were performed on sterile filter paper in sterile petri dishes

inside a laminar flow clean air cabinet (W.H.S. (Pathfinder) Ltd., model 64T horizontal flow), the surfaces of which had been previously swabbed using 100% (v/v) ethanol. Dissection instruments were sterilized in 100% (v/v) ethanol and subsequently flamed. The caps of culture vessels were flamed before both removal and replacement.

## 2.5. Media

The media described by Murashige and Skoog (1962) (M & S) and by Heller (1953) (H) were used as the basal media. Media constituents, concentrations of aqueous stock solutions and their storage period are described in the Appendix Section A1. The media were prepared by dissolving 3% (w/v) sucrose (except where stated) in glass distilled water followed by the addition of the required volumes of the stock solutions as described. Benzyl amino purine (BAP) and naphthyl acetic acid (NAA) were added (except where stated) before the pH of the medium was adjusted to 6.1 with 0.1M KOH. Glass distilled water was then added to make up the medium to its final volume. At this pH only 6.75 $gl^{-1}$ agar were required to provide a solidified but soft medium into which the explants could easily be inserted, and ensuring good contact between explant and medium. The media were autoclaved at $1.085 \times 10^{5} Nm^{-2}$ for 5 min to dissolve the agar (where present) and 10 ml aliquots were dispensed into 25 x 75 mm flat bottomed glass tubes covered by foil caps,

or 20 ml aliquots were dispensed into 40 x 75 mm screw-top glass jars. The culture vessels, containing the media were subsequently sterilized by autoclaving for 15 min at $1.085 \times 10^{5} Nm^{-2}$, and stored at $4^{o}C$ if not required within seven days. In the text the following abbreviations are used to represent media: Type of basal medium [NAA concentration in mg $l^{-1}$: BAP concentration in mg $l^{-1}$].
e.g. M & S medium [ 1 mg $l^{-1}$ NAA : 1 mg $l^{-1}$ BAP] = M [1:1]

## 2.6. Incubation

Cultures were incubated (unless stated otherwise) at $20^{o} \pm 1^{o}C$ for a 16 h photoperiod under Philips Warm White Colour 29 fluorescent tubes at a photon fluence rate of $3 \pm 1 \times 10^{-5} Em^{-2} s^{-1}$ at the culture level. Liquid cultures were aerated either by rotation at 2 rpm on a large vertical wheel, or by shaking at approximately 50 rpm on a horizontal shaker (L.H. Engineering Co., Stoke, model Mk.V).

## 2.7. Evaluation of results

The growth of cultures was recorded at regular intervals. However, in order to simplify the data, only the results for 16-20 weeks after inoculation have been presented, as shoot production had virtually ceased after this period *in vitro* (unless cultures were subcultured).

In the experiments reported in Chapter 3 morphological effects of treatments were recorded by a visual, subjective assessment. The proportion of cultures showing a specific response only was recorded, therefore subsequent statistical analysis of the data was not possible. In order to assign significance to the treatment differences in later experiments, quantitative assessments of shoot number and/or fresh weight gain were made. Photographic records were also made of representative cultures, as described in Chapter 6. **Each treatment was replicated ten times (except where stated).**

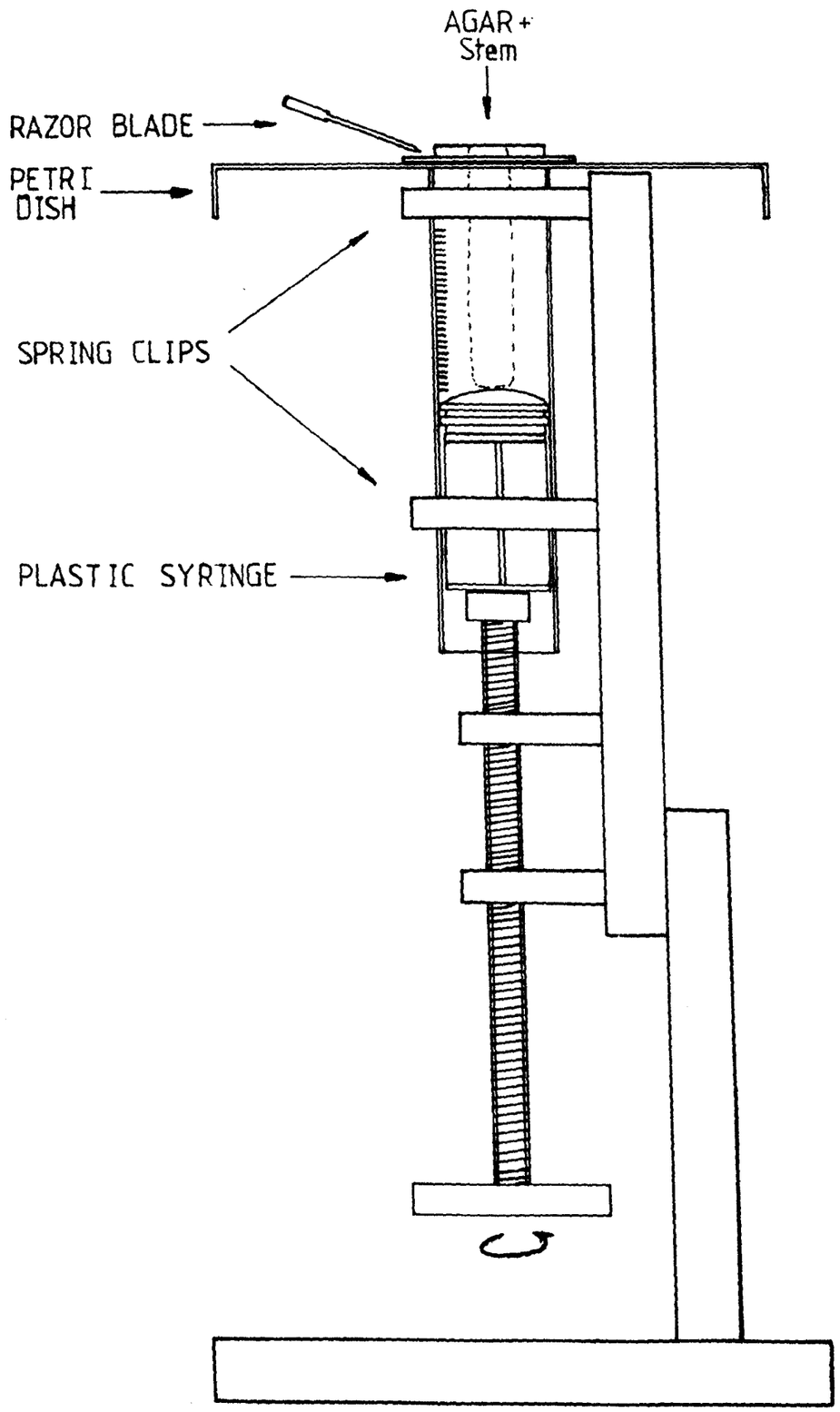
**Structures arising from the outer surface of an explant, that have or will develop into green, tubular, positively geotropic, leaf-like structures have been considered to be shoots (see Chapter 6 for histological description of shoots formed <u>in vitro</u>).**

When the proportion of cultures giving a specific response is presented as a percentage of a particular treatment, only cultures uncontaminated by micro-organisms have been considered. In the statistical analysis of mean shoot production and fresh weight gain, contaminated cultures were treated as missing values.

The development of floral stem explants is described as basal expansion as opposed to callus production. This is to distinguish between an open friable type of callus obtained from scale and bud explants, and the cell division in floral stem explants which is contained within an epidermal cell layer. This expansion is however caused by an increase in unorganised cells (see Chapter 6).

Figure 2.1.

Apparatus for cutting 1 mm sections of floral stem



Chapter 3

# Experiments *in vitro* with Bud, Scale and Floral Stem Explants

Examination of tulip bulb morphology (Figure 3.1, page 43) showed that there are a number of types of tissues that could be suitable starting material for culturing. The most commonly used explants in the establishment of morphogenic cultures from bulbous genera are scale and floral stem (e.g. Hussey, 1980).

A piece of tulip bulb scale including some basal plate tissue contains part of the meristematic region at the junction of the scale with the basal plate (Figure 3.1, page 43) from which daughter bulbs are initiated *in vivo* (Rees, 1972). If this initiation process could be increased on excised tissue by the manipulation of medium and/or environmental factors, this type of explant could be an ideal starting material for the vegetative propagation of tulip *in vitro*. It would simply be an enhancement of the natural multiplication process.

Hussey (1975b)showed that adventitious shoots were formed on floral stem explants of a range of Liliaceae genera, namely *Hyacinthus*, *Muscari*, *Ornithogalum* and *Scilla*, although he reported 'no reaction' for tulip. However, the possibility that tulip floral stem could be responsive *in vitro* was not ruled out.

Flowering size tulip bulbs, unlike many other bulbs, generally have one daughter bulb (axillary bud) in the axil of each scale. It is believed that daughter bulbs are in a juvenile state, as they are frequently vegetative during their first year of growth, they could therefore be a responsive tissue for *in vitro* multiplication by axillary bud proliferation.

## 3.1. Comparison of the growth of bud, scale and floral stem explants on media containing a range of concentrations of auxin and cytokinin

In this preliminary experiment three types of explant namely, bud, scale and floral stem were compared for their response *in vitro* on the M & S basal medium.

### Materials and methods

The basal medium (Appendix A1) was supplemented with auxin (NAA) at 0.0, 0.5 & 5.0 mg $l^{-1}$ and cytokinin (BAP) at 0.0, 1.0 & 10.0 mg $l^{-1}$, and all factorial combinations. Media were prepared as described in Section 2.5 and explants were prepared from bulbs of cultivar Merry Widow (Section 2.3), during November when the floral stems were approximately 20 mm in length (see Section 2.1 for storage conditions). Cultures were incubated as described in Section 2.6 with the exception that the light source consisted of Philips 400W HLRG lamps.

### Results and discussion

Growth of the original explants was observed in a large proportion of bud explant cultures on the majority of the media (Figure 3.2.a, page 44) but the assessment of 'growth' did not discriminate between increase in size of the explant and shoot development (proliferation). Consequently this large proportion of responsive cultures does not constitute a large number of shoots. In fact the number of cultures producing more than one shoot was very low and may not have involved proliferation of shoots, but simply the development of existing shoot initials present on the explants before excision. However it was possible to select the media

giving the most promising results for use in the following experiments. These were: M [ 0:0], M [ 0.5:0], M[0:1] and M [ 0.5:1]. Media M[5:0] and M [5:10] were not selected because the growth of explants on these media was twisted and distorted, possibly due to the high PGR levels (Hussey, pers. comm.).

The proportion of cultures producing callus on scale explants was dependent on the auxin concentration in the medium (Figure 3.2.b, page 44). Shoot production from these explants was very low and those growing may, as with the bud explants, have been initiated prior to explant excision. Shoots were only produced on scale explants on media containing 10 mg $l^{-1}$ BAP i.e. media M [ 0:10], M [ 0.5:10] and M [ 5:10]. These media plus medium M [ 0:0], as a control, were therefore selected for use in some of the following experiments.

Floral stem explants were the most responsive tissues <u>in vitro</u> (Figure 3.2.c, i & ii, page 44). A large proportion of cultures produced basal expansion (callus - as defined in Section 2.7), and many cultures gave rise to a number of shoots which were adventitious in origin because the floral stem explants did not contain nodal tissue. The most productive media for floral stem explants were M [ 0.5:1], M [5:1] and M [ 5:10], but as the highest shoot production was on media M [ 0.5:1] and M [ 5:1] which both contain 1 mg $l^{-1}$ BAP it was therefore decided to use an intermediate level of NAA, i.e. 1 mg $l^{-1}$. In order to use a full compliment of control media, zero levels of NAA and BAP were included in some of the following experiments, i.e. media M [ 0:0], M [ 0:1] and M [ 0.5:0].

## 3.2. Experiments with scale explants

From the results presented in the preceding Section (3.1) the comparison of bud, scale and floral stem explants showed that floral stem tissues were the most responsive *in vitro*. However, it would be advantageous to obtain shoot production from scale tissue because of the larger amounts of this type of tissue that are available from each tulip bulb, and for reasons of genetic stability (see Chapter 1). Consequently in an attempt to obtain shoot production from scale explants a series of experiments were conducted on explants of scale tissue.

### 3.2.a.i) Auxin test

In the previous experiment callus was obtained on scale explants when auxin was present in the medium, irrespective of the presence of BAP (Figure 3.2.b, page 44). Although callus may not be immediately useful for vegetative propagation, it may eventually be possible to regenerate shoots from callus cultures, so a study was conducted on the effects of different types of auxin on the growth of scale explants *in vitro*. The auxins tested were synthetic and naturally occurring and had a range of specific activities, as determined by Thimann & Schneider (1939) using the pea slit stem curvature assay, i.e. IAA 100%, IBA 190%, NAA 370%.

#### Materials and methods

Media containing auxin (NAA, IBA or IAA) at concentrations of 0.01, 0.1, 1.0 and 10.0 mg $l^{-1}$ were prepared as described in

Section 2.5. Explants were prepared and incubation conditions were used as for the previous experiment (Section 3.1).

Results and discussion

The amount of callus and number of roots produced on media containing the different auxins are shown in Table 3.1 (page 38) as a percentage of cultures responding. Both callus and root production appear to follow a similar pattern, namely an increase with auxin specific activity and also with auxin concentration.

Root formation was observed only on cultures where a large amount of callus had been formed. Production of roots in a large number of cultures, arising from within the callus, was similar to the observations of Nishiuchi & Myodo (1976). The production of 'protruberances resembling adventitious buds' after prolonged culturing reported by Nishiuchi & Myodo, was however, not observed.

3.2.a.ii) Subculture of callus

Materials and methods

Callus produced on scale tissue in the preceding experiment (Section 3.2.a.i) on media containing NAA at 1.0 & 10.0 mg $l^{-1}$ and IAA at 10 mg $l^{-1}$ was subdivided into approximately 2 mm cubes and placed on to M & S medium supplemented with 0.0, 1.0 & 10.0 mg $l^{-1}$ BAP, 0.0, 0.5 & 5.0 mg $l^{-1}$ NAA and all factorial combinations. Solid and liquid media were used.

Results and discussion

The callus was initially colourless or light brown in colour, and loose and friable in nature. But within approximately two weeks of being transferred it became dark brown or black and no further growth occurred on any of the media. The type of medium on which the callus was grown prior to being subcultured had no effect on its subsequent development after transfer. Callus growth only occurred in the presence of the initial explant tissue and lacked regenerative or even multiplication properties on the media tested.

As the production of callus appears to be a pre-requisite of root formation (Section 3.2.a.i), it may be that the callus formation is merely one stage in the initiation of adventitious roots. cf. The production of roots on cuttings *in vivo* (especially herbaceous plants). This may explain why callus removed from the original explant does not multiply or initiate shoot buds, a phenomenon also observed by Nishiuchi & Myodo (1976).

### 3.2.b. High cytokinin concentration test

In the preliminary experiment comparing various explants on a range of media (Section 3.1) the only media on which shoots arose from scale explants, contained a high level of cytokinin (10 mg $l^{-1}$), and shoot production was found to be independent of auxin concentration. It was therefore decided to determine the effect of high levels of cytokinin in the medium without auxin.

Materials and methods

Scale explants of the two cultivars Apeldoorn and Merry Widow, prepared as described in Section 2.3, were placed on M & S medium containing 0.0, 5.0, 10.0, 20.0, 40.0 and 80.0 mg $l^{-1}$ (prepared as described in Section 2.5).

Results and discussion

Explants of Apeldoorn produced very little callus, and no shoots on any of the concentrations of BAP tested. In contrast, substantial amounts of callus were produced by Merry Widow explants on low levels of cytokinin, although less callus was formed on high levels of cytokinin. Several cultures of Merry Widow scale produced shoots, but these occurred randomly and were not correlated with cytokinin concentration; thus supporting the hypothesis that buds could have been initiated, but were not macroscopically visible, before the cultures were set up.

### 3.2.c. Whole bulb scale

The tulip bulb at flowering size normally initiates one bud between each scale, occasionally more, but it was hoped to enhance this initiation process by placing scale explants, including basal plate tissue, on to culture media. In order to examine the number of buds initiated per scale, bulbs were dissected at an early stage of development (before the initiation of second generation daughter bulbs, Figure 3.3, page 45).

Materials and methods

The scales from bulbs of cultivar Merry Widow were dissected as described in Section 2.3 yielding 4-8 explants per scale depending on the location of the scale within the bulb. All the scale tissues from one bulb were dissected into explants and placed on to one type of medium, each explant in a separate vessel. Five bulbs were used per treatment and each bulb consisted of approximately five scales. There were therefore 25 scales per treatment. The media used were those selected in Section 3.1 and prepared as described in Section 2.5.

Results and discussion

With the exception of medium M [0.5:10] the number of shoots developing per scale (Table 3.2, page 39) was less than would normally occur *in vivo* (approximately one per scale). However, even medium M [0.5:10] only produced on average 1.9 shoots per scale, and it therefore appears that little enhancement of the initiation process was achieved.

### 3.2.d. Seed bulbs as explant source

Seed bulbs are small offsets (daughter bulbs) which are used by growers as 'seed' for growing on to flowering size. They are vegetative, producing only one large leaf, and may therefore be a suitable tissue to culture *in vitro* to obtain shoot proliferation.

## Materials and methods

Bulbs of cultivar Merry Widow were grown under the conditions described in Section 2.2 and scale explants were dissected (Section 2.3) at the time of flowering of large (12/13 cm) bulbs. 9 weeks after planting. This stage of bulb development was used as it was during the period of maximum expansion of the new scales (Rees, 1968). The media were those selected in Section 3.1, and control cultures contained explants of flowering size bulb scale.

## Results and discussion

Scale tissue produced a limited amount of callus but no shoots on any of the media. There was therefore no advantage in using explants from vegetative bulbs to obtain *in vitro* shoot production.

## 3.3 Experiments with bud explants

Shoot proliferation could not be induced on scale explants (Section 3.2). In this section data is presented from a similar series of experiments with bud explants (daughter bulbs). By removing the apical dominance of the bud it may be possible, under the correct conditions, to induce the proliferation of axillary meristems as has been reported for *Narcissus* and other bulbs (e.g. Hussey, 1976c).

### 3.3.a. High and low incubation temperature and culture medium salt concentration

Le Nard & Cohat (1968) showed that exposure of tulip bulbs to a period of low temperature stimulates the induction of bulbing, i.e. production of daughter bulbs. Tulip bulbs also require a period of exposure to low temperature for their normal growth and development, for the cultivar Merry Widow a period of nine weeks at 5°C is sufficient (ADAS, 1977). Exposure of bud explant cultures to low temperature for a similar period may induce growth and/or proliferation *in vitro* when transferred to a higher temperature.

The physiological changes that must occur in the bulb during cold treatment to manifest such effects on the growth of the bulbs may mean that the tissue will require different culture media for growth *in vitro* to those selected in Section 3.1. Consequently a wide range of PGR concentrations have been used on two types of basal media, one with high and one with low inorganic salt concentration.

Materials and methods

The PGR concentrations used in the M & S and Hellers media (detailed in Appendix A1 ), prepared as described in Section 2.5, were 0.0, 0.5, 5.0 mg $l^{-1}$ NAA and 0.0, 1.0, 10.0 mg $l^{-1}$ BAP and all factorial combinations. Explants were prepared as described in Section 2.3 and cultures were incubated in the dark at $5^{o} \pm 1^{o}C$ and transferred to the standard incubation conditions (Section 2.6) after 9 weeks. Control cultures were incubated in the standard conditions throughout the experimental period.

Results and discussion

No interaction was observed between PGR concentration and temperature or salt concentration and the data has therefore been presented as a combined result for the combination of all PGR concentrations (Table 3.3, page 40). The cold treatment had little effect, however there was a slight advantageous effect of the low salt medium in terms of callus and shoot production, but the number of shoots arising from one explant was very low (only 2-3). It therefore appears that axillary buds placed in culture are unable to respond to low temperature in the way that Le Nard & Cohat observed for mother bulbs.

### 3.3.b. Dissection of buds to remove apical dominance

The presence of expansion growth and the absence of shoot proliferation on bud cultures in the previous experiments suggests that the tulip bulb (bud) has a strong apical dom-

inance. In an attempt to remove this dominance bud explants were dissected in both a vertical (as shown by Hussey & Hilton, 1977 for Narcissus bulblets) and a horizontal plane.

## Materials and methods

The explants from bulbs of cultivar Merry Widow were initially prepared as described in Section 2.3, and the buds were cut into two equal parts horizontally, the bottom part being used as an explant, or they were cut into two equal parts vertically in which case both parts were used as explants. The explants were placed on media with the range of PGR concentrations used in Section 3.3.a. Control cultures were bud explants without further dissection.

## Results and discussion

Axillary bud proliferation did not occur (Table 3.4, page 41) and only a few buds produced more than one shoot. More of the buds cut horizontally than of those cut vertically produced new shoots, but a maximum of only 2-3 shoots were produced per culture (comparable with Section 3.3.a). Dissection did however induce the production of more callus which may have been related to the presence of more cut surfaces. It was indicated in a review by Yeoman (1970) on the role of the wound reaction in the early stages of callus formation, that the production of IAA at cut surfaces may be responsible for triggering the initial cell division. Consequently, the amount of callus produced on an explant could be dependent on the area of cut surfaces.

### 3.3.c. Seed bulbs as explant source

Vegetative material is known to be more responsive than floral tissue in traditional methods of plant propagation (Hartmann & Kester, 1975). Seed bulbs as described in Section 3.2.d, a vegetative material, may therefore be a responsive tissue *in vitro* as a source of bud explants.

#### Materials and methods

Bud explants were prepared as described in Section 2.3. from bulbs grown as described previously in Section 3.2.d. The explants were placed on media as used in Section 3.1 and buds from floral (12/13 cm) bulbs were used as control explants.

#### Results and discussion

Some of the explants produced expansion and extension growth, but there was no proliferation of shoots. The proportion of cultures producing more than one shoot was lower than the control cultures. It therefore appears that the regenerative potential of buds obtained from vegetative bulbs is even less than that of buds from floral bulbs.

### 3.3.d. Embryo cultures as explants source

For many plants it has been shown that the use of juvenile tissue will improve the rate of regeneration (Bleasdale, 1973). Tissue from embryo cultures has therefore been used as an explant source in a further attempt to obtain morphogenic cultures from vegetative material.

## Materials and methods

Seeds were collected from open pollinated cultivar Merry Widow in the summer of 1978 and stored at 20°C. Embryos were dissected from surface sterilized seeds and placed on Nitsch (1951) medium, as described by Niimi (1978). The embryos were grown under the standard incubation conditions described in Section 2.6 following a 10 week period at 5°C. Explants of bud (bulb) and also leaf (2 mm sections) were prepared and placed on M & S medium supplemented with the range of PGR concentrations previously described in Section 3.1.

## Results and discussion

The only development of explants that occurred was the expansion growth of a few of the buds, but no proliferation occurred on any of the media. It would appear therefore that vegetative, and juvenile tissue is not a good starting material for the initiation of morphogenic cultures.

## 3.4. Stage of bulb development in relation to the growth in vitro

It has been shown that for many plants, for example Nicotiana tabacum L. (Burk, et al., 1972), Freesia (Davies, 1972) & Hyacinthus (Hussey, 1975a), that the stage at which explants are removed from their donor is an important factor in determining their development in vitro. In order to establish the stage of development of tulip bulb material which was most responsive in terms of growth in vitro, samples of scale, bud and floral stem from bulbs at various stages during the growth cycle were placed in culture.

### Materials and methods

Bulbs of cultivars Merry Widow and Apeldoorn were stored and grown under the conditions described in Sections 2.1 and 2.2. The media used were those selected in Section 3.1 with the exception that for the later stages of development in which scale explants of daughter bulbs were cultured, the range of media was expanded to the full compliment of PGR concentrations used in Section 3.1. Media used are shown in Figures 3.4 (page 46) 3.5 (page 47) & 3.6 (page 48). Samples of explants were taken at the nine stages of development shown in Figure 3.3 (page 45).

### 3.4.a. Scale explants

Mother bulb scale was used as the source of explants up to stage 5 at which time food reserves were being transferred to the daughter bulb scales and the mother bulb was becoming

depleted (Gilford & Rees, 1974). After this stage explants were taken from daughter bulbs.

Results and discussion

The proportion of cultures forming callus decreased with age of mother bulb scale (Figure 3.4, page 46) but at no time was there a high proportion of callus producing cultures. Explants at the early stages of development did however, give rise to large amounts of callus in a few cultures. These high levels of callus production on explants taken early in the growth of the bulb, and the very small amounts of callus produced near to bulb harvest time are in agreement with the findings of Nishiuchi & Myodo (1976). It was suggested by Nishiuchi & Myodo that the reduced callus production at harvest time could have been caused by the accumulation of the growth inhibitor in the tulip bulbs which had been isolated by Hideo Ito (1960).

As found previously with cultures of scale explants the number of cultures giving rise to shoots was very low. At no time were there more shoots produced than could be explained by the presence of shoot initials before explants were placed in culture (cf. Section 3.1, 3.2). Explants of Merry Widow mother bulb scale produced more callus than explants of Apeldoorn (Figure 3.4.b, page 46) and medium M [5:10] was the most productive in terms of callus.

It is clear that the optimum media for callus production were not the same for daughter bulb scale (Figure 3.4.c, page 46) as for mother bulb scale (Figure 3.4.b, page 46).

For daughter bulb scale the optimum media contained lower concentrations of either or both NAA & BAP than those for mother bulb scale.

It has been shown that the stage of bulb development is important in determining the response of scale explants in terms of callus production, but it in no way reduces their recalcitrance in terms of shoot production.

### 3.4.b. Bud explants

Mother bulb axillary buds were used as explants up to stage 6 by which time initiation of buds within the daughter bulbs had occurred. After stage 6 these second generation axillary buds were used as explants.

#### Results and discussion

The proportion of cultures responding (i.e. showing a substantial increase in size) at the nine stages of development showed a similar trend for both cultivars (Figure 3.5.a, page 47). The proportion responding decreased down to a minimum just before the initiation of the second generation daughter bulbs, after which a slight recovery of response *in vitro* was evident. If the results for the nine stages of development are combined to compare the effects of media (Figure 3.5.b, page 47) M [0:1] was found to be the most productive. However there was no more than a maximum of three shoots formed on any of the media.

### 3.4.c. Floral stem explants

The explants were prepared as described in Section 2.3, except that during the later stages of development, to facilitate surface sterilization, a piece of stem of approximately 3 cm, containing the required explant, was cut from the stem prior to surface sterilization. After sterilization and washing (Section 2.3) an explant of approximately 5 mm in length was cut from immediately above the uppermost node of the stem, the centre of the 3 cm piece of stem. This size of explant was used throughout the experiment although the total stem length varied between 2.5 cm and 40 cm.

#### Results and discussion

Comparison of basal expansion ('callus' - see Section 3.1) for the two cultivars, Merry Widow and Apeldoorn showed that there was little difference between the two cultivars (Figure 3.6.a, page 48). A large proportion of explants of both cultivars formed callus up to stage 3, but after flower colour was visible (stage 4) callus production ceased. A comparison of the media for the responsive stages 1-3 (Figure 3.6.b, page 48) revealed that media M [1:1] and M [5:10] were the most productive in terms of basal callus on floral stem explants of Merry Widow and Apeldoorn.

A large number of shoots were produced from floral stem explants of Merry Widow, but at stage 1 only, i.e. during the 'dormant' phase of the growth cycle (Figure 3.3, page 45). After planting (stage 2 onwards no shoot production was observed on any of the media. Explants of Apeldoorn

did not produce as many shoots (10% of cultures) as those of Merry Widow (46% of cultures) when compared on media M [ 1:1] and M [ 5:10], the only media on which shoots were produced.

## 3.5. General discussion and explant comparison

The initial findings that scale tissue was recalcitrant *in vitro* (Section 3.1) were supported by the results reported in Section 3.2. Shoot production did not occur in sufficient quantity (as specified in Section 3.2.c) that it could not be explained by the possible presence of undetected shoot initials before the explants were placed in culture. It would appear that a radical change in approach will be necessary to cause the initiation of more than one shoot per scale on tulip bulb scale *in vitro*, if in fact these tissues are totipotent.

In the present study concentrations of cytokinin (BAP) as high as 10 mg $l^{-1}$ did not induce the proliferation of shoots on bud explants (Section 3.1), and there was also little success in reducing the strong apical dominance of these daughter bulbs (Section 3.3). It appears therefore that bud explants are not a suitable starting material for the establishment of morphogenic cultures, unless some means of reducing the apical dominance can be found. An understanding of the hormonal relations of the tulip bulb throughout its growth cycle (or at least during the initiation of buds), may prove necessary before the growth of tulip buds can be controlled satisfactorily *in vitro*. The present knowledge of endogenous PGR levels within the tulip is discussed in Chapter 5.

A comparison of the different types of explant on the most suitable media for each type, at the most productive stage

of development (data taken from Section 3.4a, b, & c) is shown in Table 3.5, (page 42) and representative photographs of the three explant types are shown in Plate 1 (page 49). It is evident that, although a large proportion of all types of explant produced callus, floral stem explants were the most productive in terms of shoot regeneration.

Table 3.1.

Callus and root production from scale explants on media containing a range of concentrations of the auxins IAA, IBA and NAA

a) Percentage of cultures producing callus

| Auxin | | | | |
|---|---|---|---|---|
| Concentration ($mg\ l^{-1}$) | 0.01 | 0.1 | 1.0 | 10.0 |
| IAA | 0 | 19 | 13 | 56 |
| IBA | 13 | 0 | 0 | 38 |
| NAA | 0 | 6 | 47 | 80 |

Control (zero auxin) = 13%

b) Percentage of cultures producing roots

| Auxin | | | | |
|---|---|---|---|---|
| Concentration ($mg\ l^{-1}$) | 0.01 | 0.1 | 1.0 | 10.0 |
| IAA | 0 | 0 | 0 | 13 |
| IBA | 0 | 0 | 6 | 31 |
| NAA | 7 | 6 | 27 | 20 |

Control (zero auxin) = 6%

Table 3.2.

Mean number of shoots produced from whole bulb scale tissue explants, cultivar Merry Widow

| Medium | Mean number of shoots produced per whole scale |
|---|---|
| M [ 0:0] | 0.1 |
| M [ 0:10] | 0.7 |
| M [ 0.5:10] | 1.9 |
| M [ 5:10] | 0.6 |

Table 3.3.

Callus and shoot production from bud explants cultured on Murashige & Skoog and Hellers basal media at 20°C and 5°C

| Percentage of cultures | Murashige & Skoog | | Hellers | |
|---|---|---|---|---|
| | 20°C | 5°C | 20°C | 5°C |
| Producing callus | 46 | 37 | 55 | 64 |
| Producing shoots | 15 | 11 | 17 | 15 |

Table 3.4.

The effect of bud explant dissection on subsequent callus and shoot production

| Percentage of cultures | Horizontally dissected | Vertically dissected | Not dissected |
|---|---|---|---|
| Producing callus | 75 | 95 | 46 |
| Producing shoots | 25 | 14 | 15 |

Table 3.5.

Comparison of explant types in terms of callus and shoot production for the most suitable media and stage of bulb development for each explant type

(Data taken from Section 3.4., a, b, & c)

| Explant type | Percentage of cultures responding | |
|---|---|---|
| | Callus | Shoots |
| Scale | 73 | 6 |
| Bud | 75 | 12 |
| Floral stem | 100 | 46 |

Figure 3.1.

Diagrammatic representation of the tulip bulb - showing explant origins

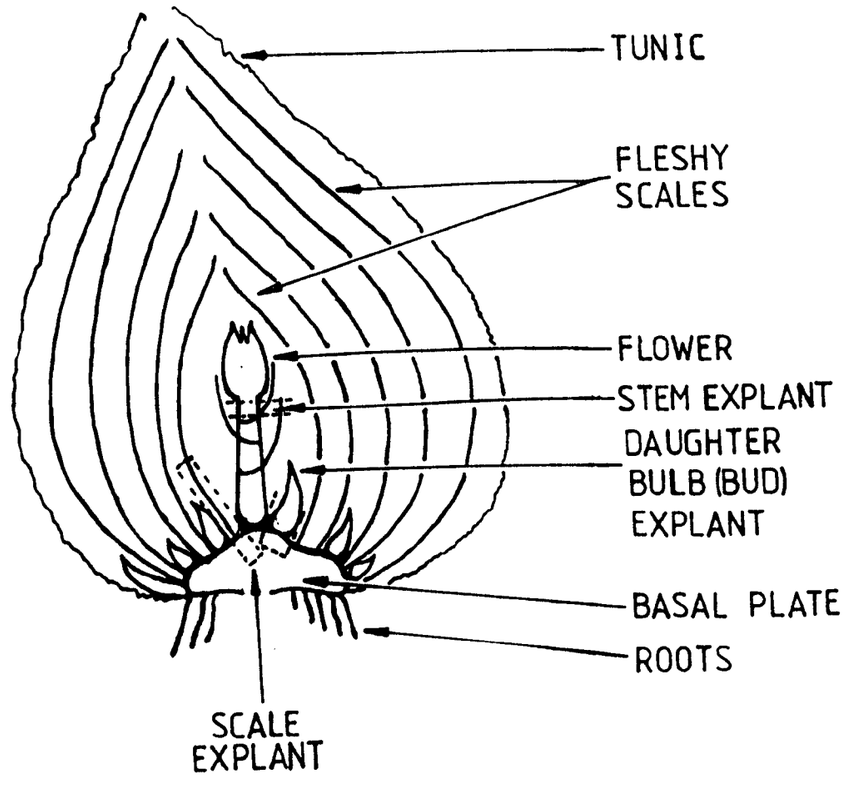


Figure 3.2. a, b, c.

Effect of media containing a range of auxin and cytokinin concentrations on the proportion of cultures exhibiting growth

a) Bud explants

b) Scale explants

c) Floral stem explants

(i) Basal expansion (callus)

(ii) Shoot production

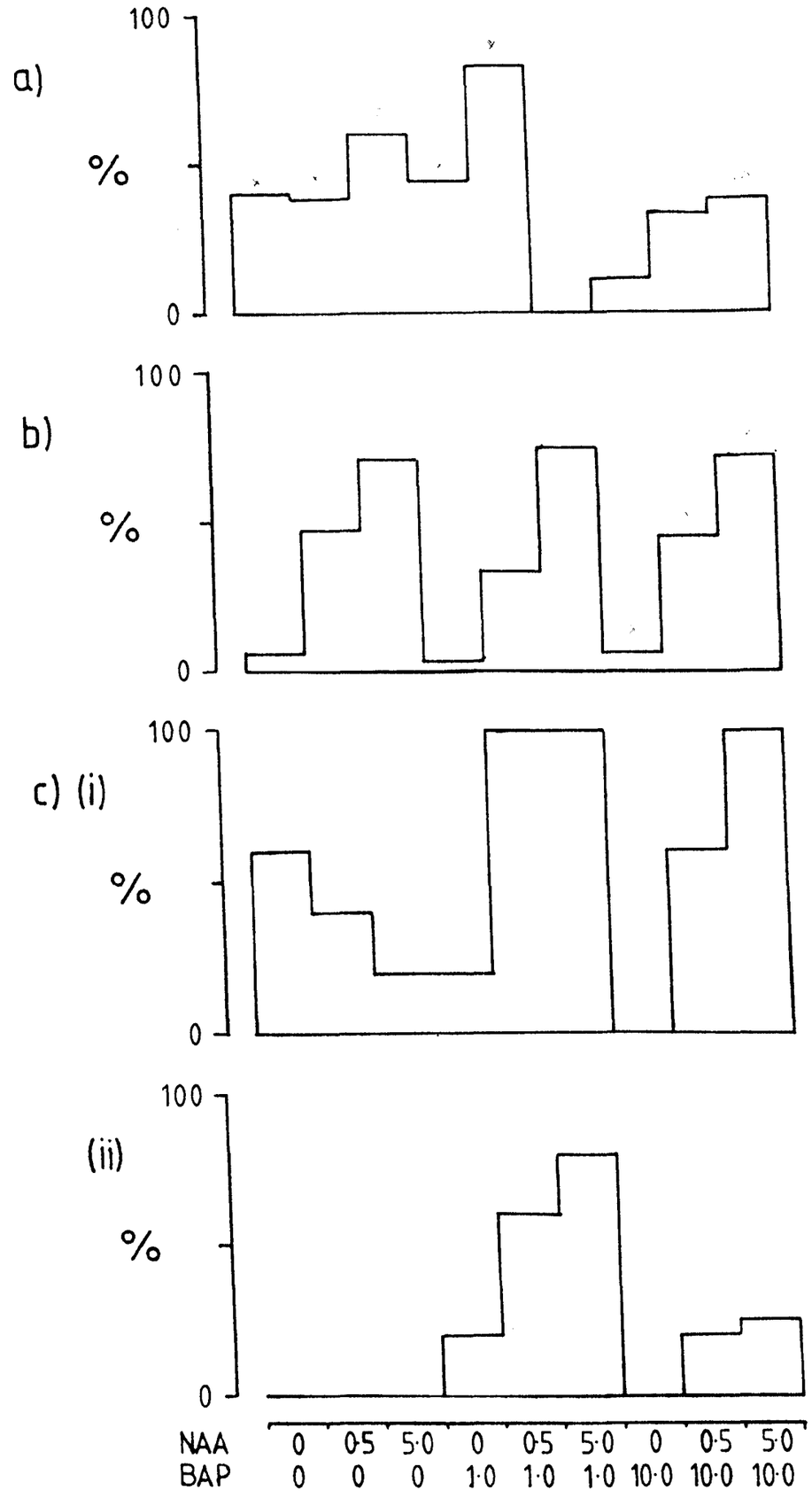


Figure 3.3

a) Annual cycle of the tulip bulb - field

b) Stages of bulb development - controlled environment

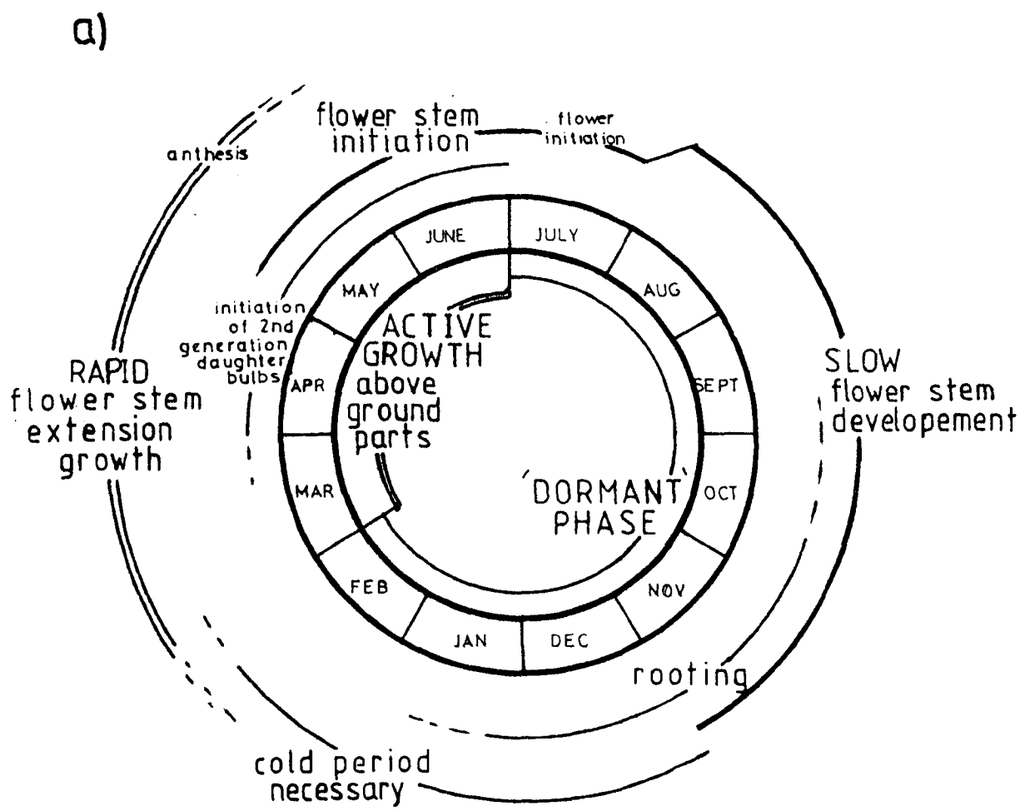


Figure 3.4. a, b, c.

Percentage of scale explants producing callus
in vitro

a) Effect of stage of bulb development

A - Apeldoorn

MW - Merry Widow

▼ Stage of explant origin change
- mother bulb daughter bulb

b) Effect of media (selected) on mother bulb scale explants

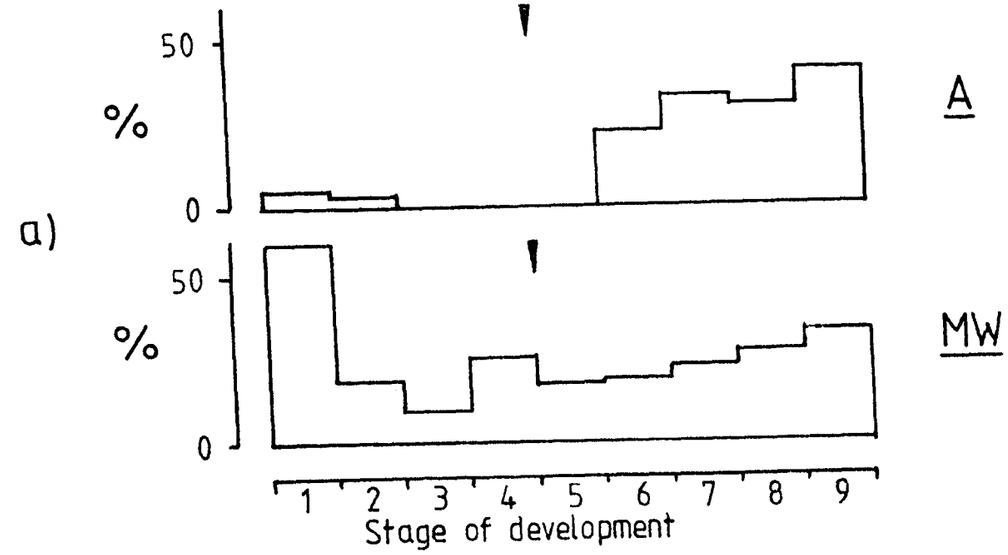
A - Apeldoorn

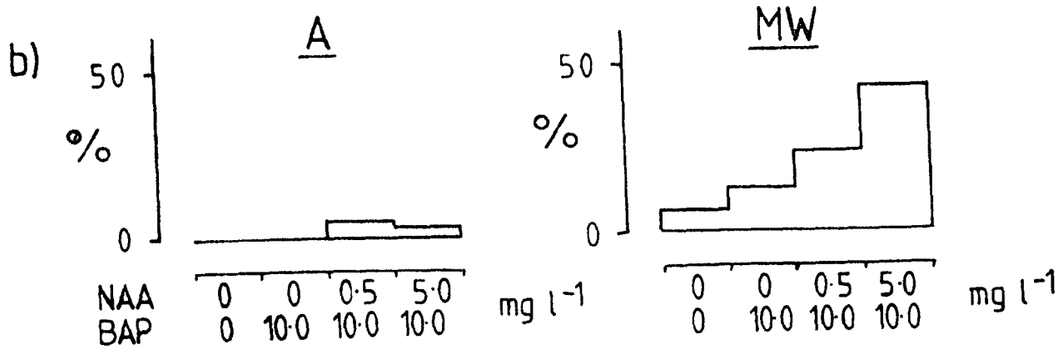
MW - Merry Widow

c) Effect of media (full range) on daughter bulb scale explants

A - Apeldoorn

MW - Merry Widow



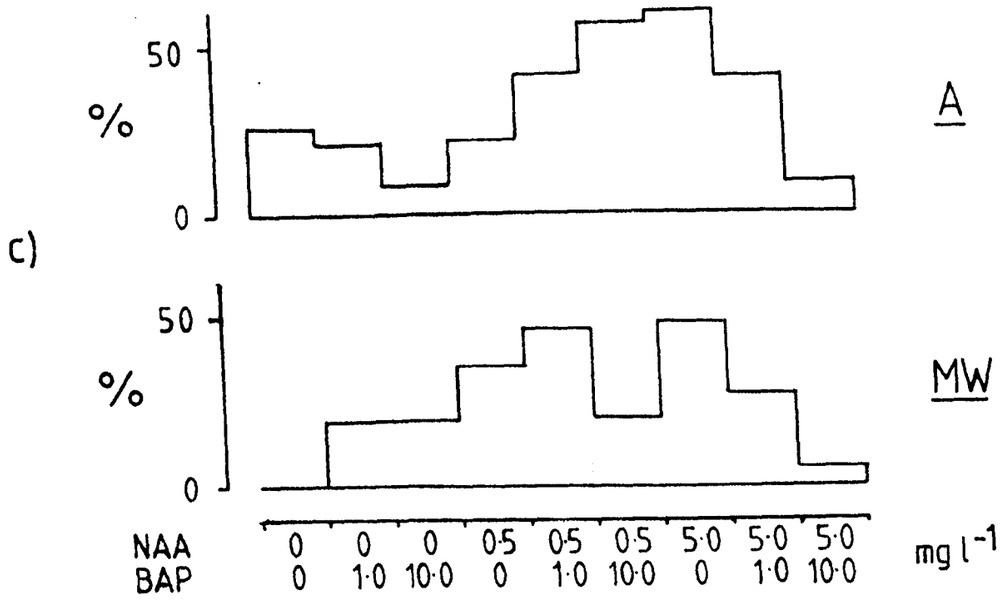



Figure 3.5. a, b.

Percentage of bud explants responding (as defined in Section 2.7) in vitro

a) Effect of stage of bulb development

A - Apeldoorn

MW - Merry Widow

▽ - Stage of explant origin change - mother bulb daughter bulb

b) Effect of medium PGR concentrations

A - Apeldoorn

MW - Merry Widow

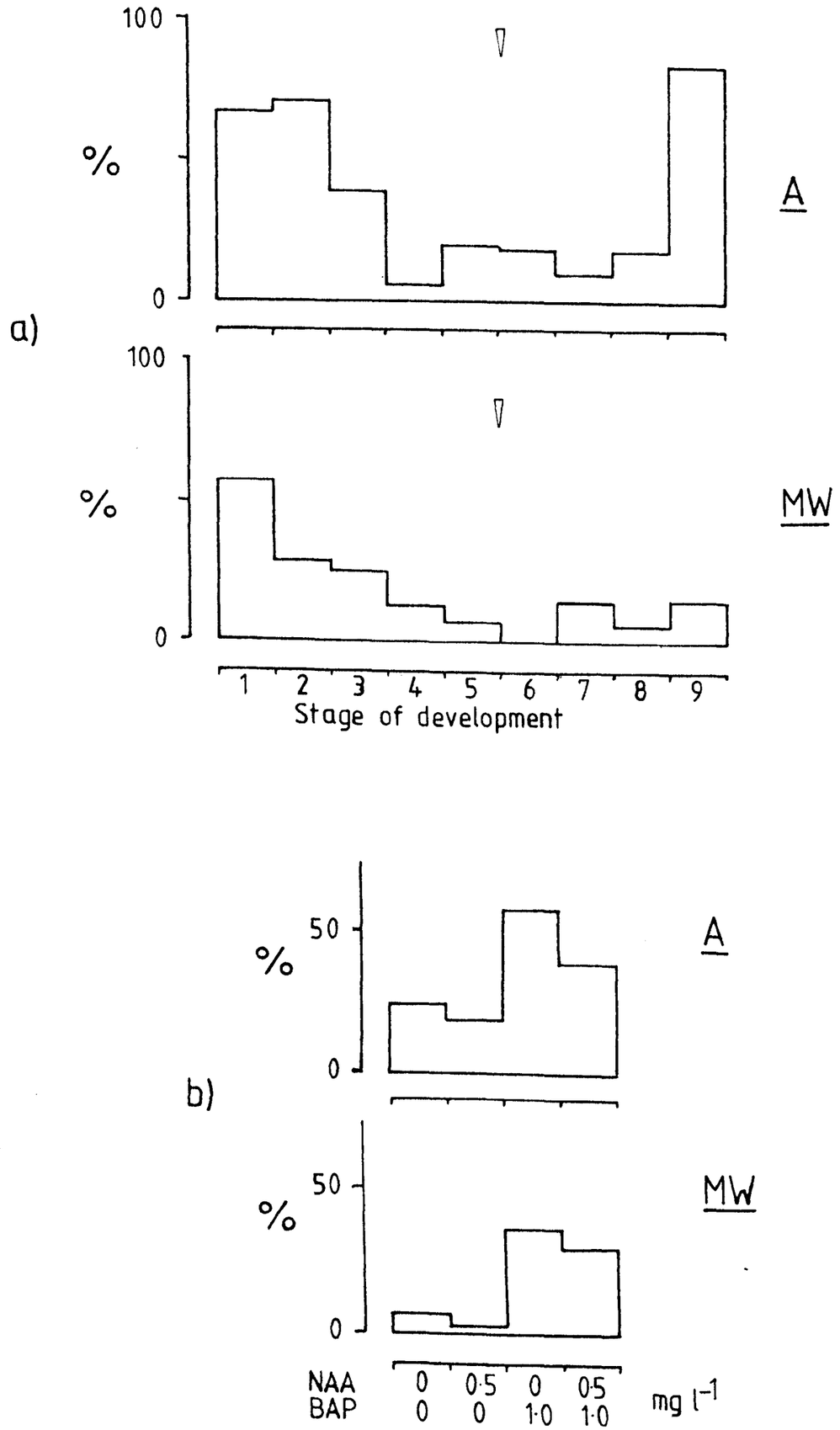


Figure 3.6. a, b.

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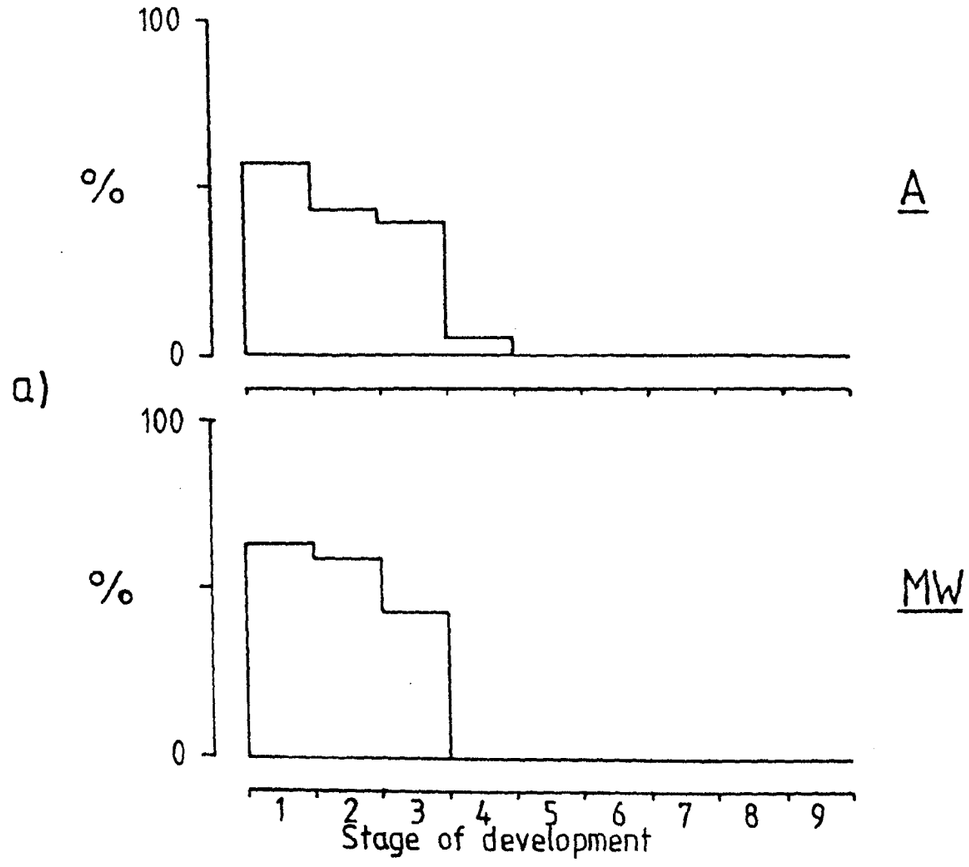
Percentage of floral stem explants producing basal expansion 'callus' (as defined in Section 2.7) in vitro

a) Effect of stage of bulb development

A - Apeldoorn

MW - Merry Widow




b) Effect of selected media on the percentage of floral stem explants producing callus. Mean of stages of development 1-3

A - Apeldoorn

MW - Merry Widow

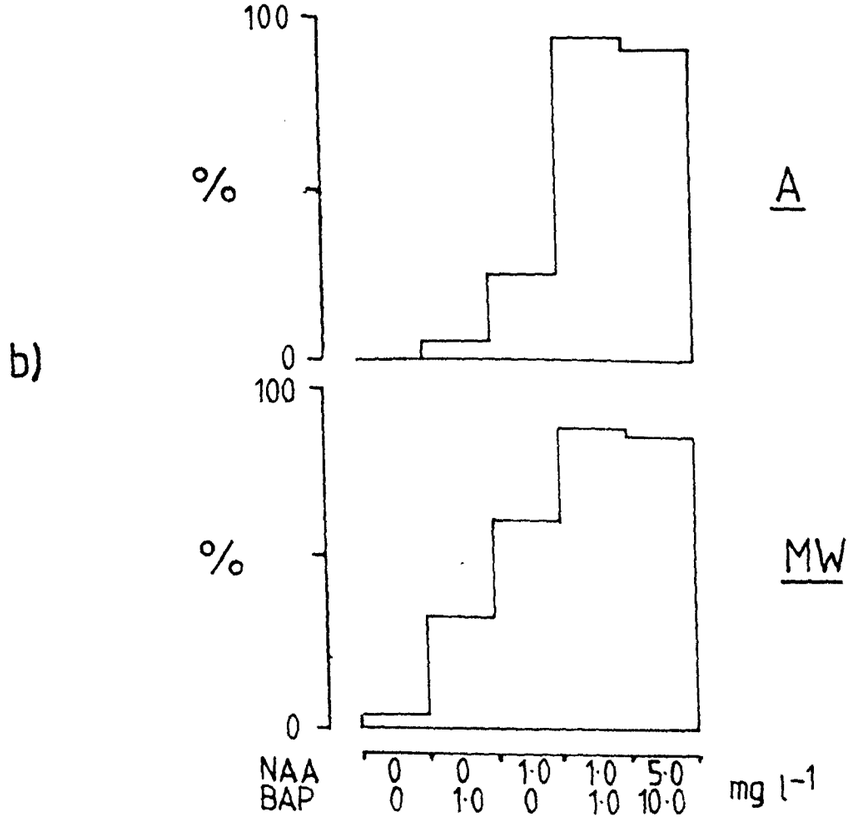


Plate 3.1

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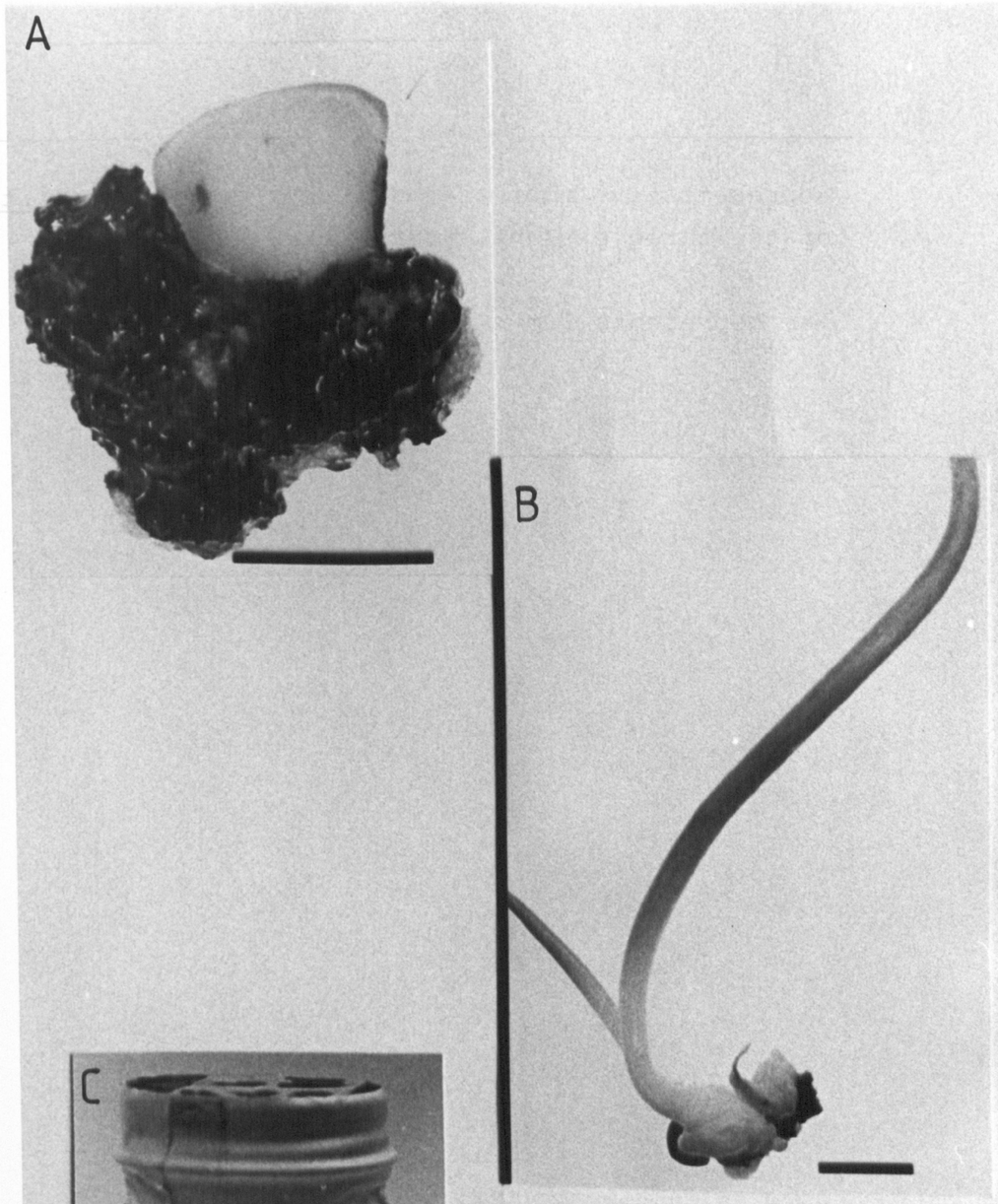
Representative photographs of the response <u>in vitro</u> of the three explant types

Bar represents 1cm

A - Scale

B - Bud

C - Floral Stem



Chapter 4

# Floral Stem Tissue *in vitro*

In view of this high regeneration of shoots on floral stem explants of cultivar Merry Widow in particular (Chapter 3, Section 3.4), a large proportion of the experimental work for this thesis has been concentrated on this type of tissue. Shoot production from bud and scale explants was very low in number as well as the proportion of shoot producing cultures, whereas explants of floral stem produced up to 20 shoots per culture (i.e. per 5 mm section of stem).

## 4.1. Medium composition

The composition of the medium was examined in an attempt to maximize shoot production on floral stem explants. Particular importance was attached to determining the optimum concentrations of PGR's and also their interaction with the pH of the medium. Experiments were also conducted to examine the source of cytokinin in relation to browning of the medium (Section 4.1.c) and the requirements of the floral stem explants for other media constituents.

### 4.1.a. Plant growth regulator (PGR) concentrations

The results presented in Section 3.3 suggest that the PGR concentrations in media M [ 1:1] and M [ 5:10] were the most productive of the concentrations tested. The following experiment was carried out to compare the shoot producing ability of a more detailed range of concentrations of NAA and BAP based on the 1:1 ratio of medium M [ 1:1].

#### Materials and methods

Media used consisted of M & S basal medium supplemented with a range of PGR concentrations (Figure 4.1, page 63) and prepared as described in Section 2.5. Floral stem tissues were excised from dormant bulbs (cultivar Merry Widow) during December 1978, which prior to use had been treated as described in Section 2.2. At the time of excision the floral stems were 20-25 mm in length, when measured from the basal plate to the base of the flower. The explant preparation and culture conditions are described in Sections 2.3 & 2.6, respectively.

Results and discussion

Shoot production occurred on a proportion of the explants on all of the media tested, and on some of the media a large number of shoots were produced (Figure 4.1, page 63). Maximum shoot production occurred on medium M [1:1], but this was not significantly different from several other media due possibly to the high variability in the number of shoots produced between replicates. These results suggest that an NAA:BAP ratio of approximately 1:1 was the most favourable and agree with the results presented in Sections 3.1 & 3.4, where medium M [1:1] gave a consistently large number of shoots on floral stem explants.

4.1.b. Medium pH

If tulip bulbs are grown in a substrate with a pH below 6.0 under natural conditions, physiological disorders can result. For this reason and because van Bragt (1971b) had cultured floral stems of tulip at pH 6.1, media with a pH of 6.1 were used in previously reported experiments. However, other workers have used media with a pH in the range 5.4-5.6 for tulip; Riviere & Muller (1976), Nishiuchi & Myodo (1976), and for other bulbous genera (e.g. Seabrook et al., 1976; Ziv et al.1970).An experiment has therefore been performed to determine the effect of pH of the medium on the growth of floral stem tissues in vitro.

The pH of an aqueous solution can affect the extractability of PGR's (Durley & Pharis, 1972) and also the response of plant material to PGR's in bioassay (e.g. Jones & Warner, 1967). It was therefore desirable to determine any interaction between pH and PGR concentration in the medium, and so a range of NAA & BAP concentrations were tested in the media.

Materials and methods

Media containing NAA & BAP at 0.25, 1.0 & 4.0 mg $l^{-1}$ in all factorial combinations were used (Figure 4.2, page 64). These concentrations were based on medium M [1:1] selected in Section 4.1 as a reliable medium for shoot production. Each medium was prepared at three pH's, namely 4.7, 5.4 & 6.1. Medium M [1:1] was also prepared at pH 6.8 in order to extend the range of pH above that of 6.1 previously selected for use. It was possible to test only medium M [1:1] at pH 6.8 because the supply of plant material was limited.

Explants were prepared from bulbs of cultivar Merry Widow as described in Section 2.3, during November 1979, when the floral stems were approximately 20 mm in length. Each non-nodal stem explant was excised 3-5 mm in length from below the lowest node, and these explants did not include any basal plate tissue. This type of explant was used in preference to those used in the previous section, because of the ease of their preparation from the base of the stem without removing any leaves. The

culture vessels were flat bottomed glass tubes containing 10 ml of medium (see Section 2.5). Each treatment was replicated ten times.

Results and discussion

A diagrammatic representation of the results is shown in Figure 4.2 (page 64). The area contained within each circle is proportional to the mean number of shoots produced by each treatment. The results are presented in this manner so that the effects of pH, PGR concentration and any interaction between them can easily be distinguished. The results are also presented in tabular form, with their statistical significance in the Appendix Section A2.

In general, there was little interaction between PGR concentration and medium pH. However, on medium M [0.25:0.25] there was an increase in the number of shoots produced corresponding to an increase in pH ($p = 0.05$ level of significance). This effect was not apparent on media containing higher concentrations of either BAP or NAA (M [0.25:1.0] and M [1.0:0.25] respectively.)

A comparison of the mean shoot production of each medium averaged over the three pH's (Figure 4.3.a, page 65) showed that medium M [1:1] gave rise to significantly more shoots than any other medium ($p = 0.05$). These results are consistent with those reported in Section 4.1.a. Figure 4.3.b (page 65) shows a comparison of the

range of pH for medium M [ 1:1]. The highest mean number of shoots was produced at pH 6.1, and shoot production at this pH was significantly greater than at either pH 4.7 or pH 6.8.

Low shoot production at low pH may reflect the preference of the tulip bulb for high pH (above 6.0) when grown in soil under natural conditions (ADAS, 1977). The results suggest, therefore, that the use of media with a pH of 6.1 in preliminary experiments was satisfactory. The apparent disadvantage of high media pH <u>in vitro</u> may have been caused by an effect of pH on the availability of PGR's. To study this subject in depth it would have been necessary to study membranes at the cellular level. An examination of this type was considered beyond the scope of this thesis.

The variability in the number of shoots produced per culture, within treatments, as described in the previous section (4.1.a) was not observed in this experiment. Also the mean number of shoots produced per culture in this experiment was consistently higher than that observed in the previous experiment. These differences between experiments could be explained on the basis of the varying origin of the explant from within the stem. The effects of this will be discussed further in Sections 4.2 & 4.3. Other differences between the experiments which may account for the reduced standard deviation include,

the type of culture vessel and the year in which the bulbs were harvested and subsequently cultured.

As the shoot production was very high on medium M [1:1] with a pH of 6.1, this medium was used in the majority of the following experiments.

### 4.1.c. Cytokinin test

In the preceding experiments a brown discoloration of the medium in some of the cultures was apparent particularly when both little growth and rapid death of the explant occurred. This response appeared to be dependent on the concentration of cytokinin in the medium. The discoloration may have been caused by the production of phenolic compounds by the plant material in response to the type of cytokinin in the medium (Hussey, pers. comm.). Also the response to a cytokinin can be specific to the plant material (Murashige, 1974). An experiment was therefore conducted to examine the effect of a range of compounds with cytokinin-like activity on the production of shoots by floral stem explants and on the discoloration of the medium.

#### Materials and methods

A list of the compounds tested is shown in Table 4.1, (page 61). They include both naturally-occurring and synthetic cytokinins and their ribosides, where available (Sigma, London). PBA was supplied by Shell Chemical Co. A yeast extract RNA having some cytokinin-like activity

(Skoog & Armstrong, 1970) was also included and because of it's low activity the concentrations used were ten times higher than those used for the other compounds. The specific activities of the other compounds varied considerably, as defined by Skoog et al. (1967), hence a range of concentrations, based on the 1 mg $l^{-1}$ shown to be the optimum concentration for both BAP & NAA (Section 4.1.b) was used. In the results presented in Section 4.1.a more browning and less shoot production was observed on explants from the top of the stem when compared to explants from the basal part of the stem used in Section 4.1.b. Non-nodal upper stem explants were therefore used in this experiment.

Media were prepared containing 1 mg $l^{-1}$ of NAA and 0.1, 1.0 & 10.0 mg $l^{-1}$ of all the cytokinin-like compounds except yeast extract RNA which was included at 1.0, 10.0 & 100.0 mg $l^{-1}$. The preparation of media was as described in Section 2.5, except that stock solutions of cytokinin-like compounds were adjusted to pH 6.1 and passed through a 0.22 µm Millipore filter, which had been previously autoclaved. The solutions were then added to the media after it had been autoclaved and cooled to approximately 40°C. Medium with BAP at 1 mg $l^{-1}$ (& NAA at 1 mg $l^{-1}$) which had been autoclaved was used as the control.

Explants 3 - 5 mm long were excised from between the uppermost node and the flower during January 1978 when the floral

stem was approximately 25 mm in length. Each treatment was replicated ten times.

Results and discussion

At their lowest concentration all of the compounds tested produced very few shoots (Figure 4.4, page 66) but at 1 mg $l^{-1}$ and 10 mg $l^{-1}$ some of the compounds produced as many shoots as the BAP control. Based on a visual assessment the media giving rise to good shoot production were discoloured as much as or more than the controls. It is possible therefore, that the browning of the medium is not due to cytokinin toxicity but is simply a consequence of growth *in vitro*, and may be explained by the release of waste or surplus phenolic compounds into the medium.

### 4.1.d. Gibberellic acid, organic compounds and salt concentrations

The addition of gibberellic acid ($GA_3$) to the medium has been found to be advantageous for the growth of tulip buds *in vitro* by Gibbon (Royal Bot. Gdns., Kew). The effects of a range of concentrations of $GA_3$ on the production of shoots by floral stem explants was therefore examined.

Hussey (1975b) showed that for a number of bulbous genera it is beneficial to use a medium containing a reduced number of organic constituents. In the present investigation effects of these minimum vitamins on tulip floral stem explants both with and without casein hydrolysate was examined.

Many workers have used low concentrations of inorganic salts in the medium (e.g. White, 1943 and Heller, 1953) including van Bragt (1971b) in experiments on the growth of tulip terminal buds *in vitro*. For this reason the low salt medium of Heller was included as a comparison with the M & S medium.

## Materials and methods

A list of the media used and their composition is shown in Table 4.2 (page 62) medium M [ 1:1] was used as a control. Preparation of the media was as described in Section 2.5. Explants were prepared as described in Section 4.1.c, except that they were excised during October, when the floral stem was approximately 10 mm in length. In consequence the explants were only 2-3 mm in length. A minimum of ten replicates per treatment were used.

## Results and discussion

All treatments, including the control M [ 1:1] produced a very low mean number of shoots per culture (Table 4.2, page 62). This may have been due to the dissection of the floral stems at an early stage of development (see Section 4.2 for the effects of stem size/age on shoot production).

The addition of $GA_3$ to the medium at any of the concentrations tested did not lead to any increase in the shoot production or shoot growth. There was a significantly

lower shoot production on media with low salt concentrations (H [ 1:1]), minimum vitamins (M [ 1:1]V), and minimum vitamins plus casein hydrolysate (M [ 1:1]VC), when compared to the control. It was therefore concluded that all or some of the organic constituents of the M & S medium were necessary for maximum shoot production on floral stem explants, and they were therefore used in all subsequent experiments. Although casein hydrolysate had no effect on shoot regeneration (M [ 1:1]-C cf. M [ 1:1]) it's absence resulted in fewer cultures producing basal expansion (a pre-requisite for shoot production, Chapter 6). The increased incidence of basal expansion in the presence of casein hydrolysate was not great, but there was no detrimental effect on shoot production, consequently it was included in the media in all subsequent experiments.

Table 4.1.

Compounds with cytokinin-like activity examined in the cytokinin test

| Common Name | Abbreviation | Chemical Name |
|---|---|---|
| Kinetin | K | 6- Furfurylaminopurine |
| Kinetin Riboside | KR | 6- Furfurylaminopurine Riboside |
| IPA | I | 6- (3-methyl-2-buten-1-ylamino)-purine |
| IPA Riboside | IR | " " -Riboside |
| BAP | B | 6- Benzylaminopurine |
| BAP Riboside | BR | 6- Benzylaminopurine Riboside |
| Zeatin | Z | 6- (trans-y-Hydroxymthyl-y-methyl-allylamino) -purine |
| Zeatin Riboside | ZR | " " - Riboside |
| PBA (SD 8339) | P | 6- (benzylamino)-9-tetra-hydro-propanyl)-9H -purine |
| RNA | R | Ribonucleic Acid. Sodium salt from Torula yeast (85%) |

Table 4.2.

The effect of GA, organic compounds and salt concentration on the shoot production and basal expansion (callus production)* of floral stem explants

| Abbreviation | Media | Composition | Mean number of shoots per culture | Percent cultures producing basal expansion (callus)* |
|---|---|---|---|---|
| M [1:1] | M & S | + 1 mg $l^{-1}$ NAA & BAP - Control | 2.3 | 100% |
| M [1:1:1] | M [1:1] | + 1 mg $l^{-1}$ GA | 1.0 | 25 |
| M [1:1:5] | " | + 5 mg $l^{-1}$ GA | 1.7 | 57 |
| M [1:1:10] | " | +10 mg $l^{-1}$ GA | 1.2 | 100 |
| M [1:1 V] | " | - M & S vitamins + minimum vitamins (Hussey 1975) | 1.8 | 63 |
| M [1:1]VC | M [1:1]V | + 500 mg $l^{-1}$ casein hydrolysate | 1.0 | 67 |
| M [1:1]-C | M [1:1] | - casein hydrolysate | 2.3 | 86 |
| H [1:1] | H [1:1] | - M & S inorganic salts + Heller (1953) inorganic salts | 2.0 | 100 |
| | | | L.S.D. = 1.0 (at p = 0.05 level) | |

* As defined in Section 2.7.

Figure 4.1.

Shoot production per culture on media containing a range of NAA and BAP concentrations

L.S.D. at the p = 0.05 level

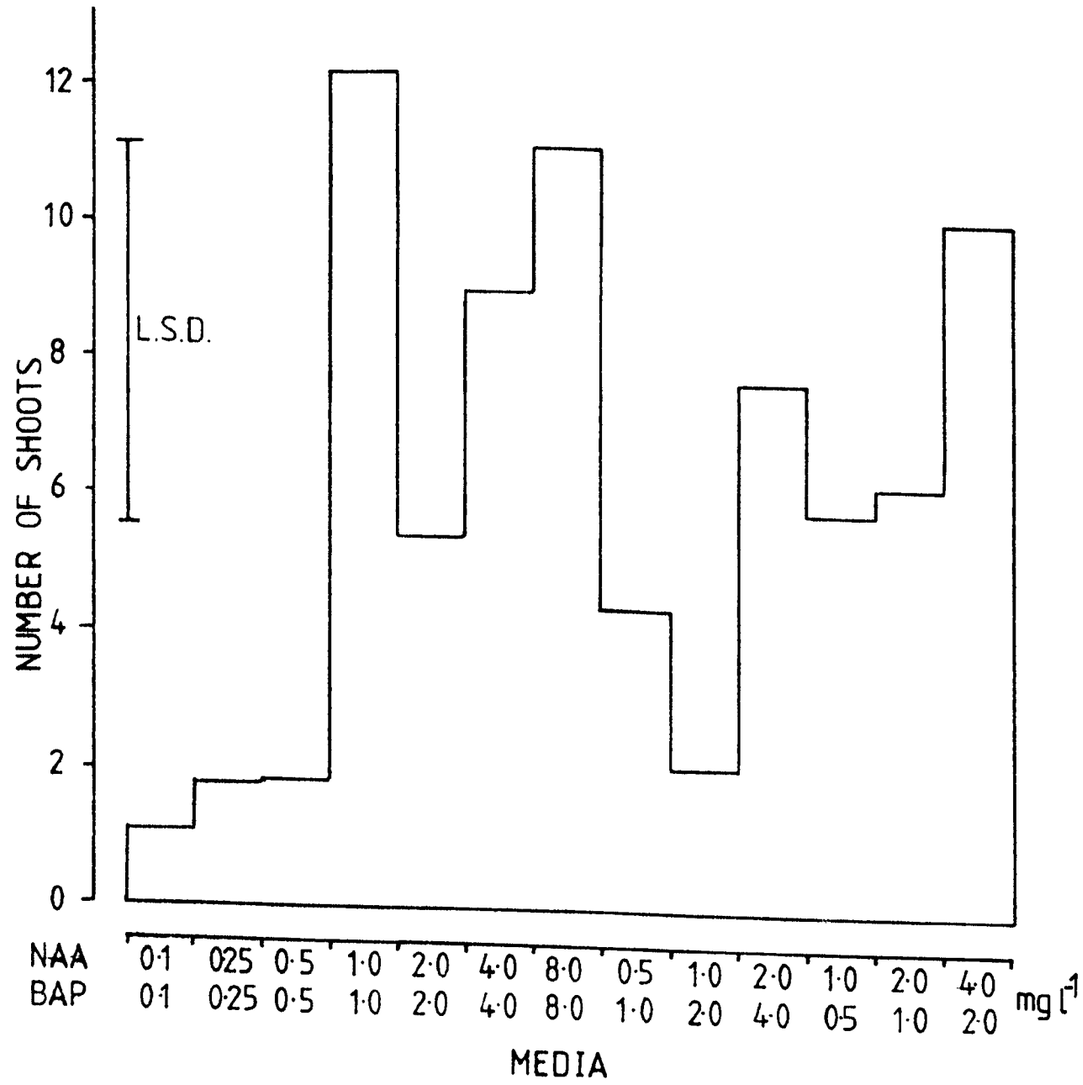


Figure 4.2.

Diagrammatic representation of the interaction between pH and PGR concentration in the medium, in terms of shoot production

The area contained within each circle is proportional to the mean shoot production of that treatment

= 10 shoots

N.B. These results are presented in tabular form, with their statistical significance in the Appendix Section A2

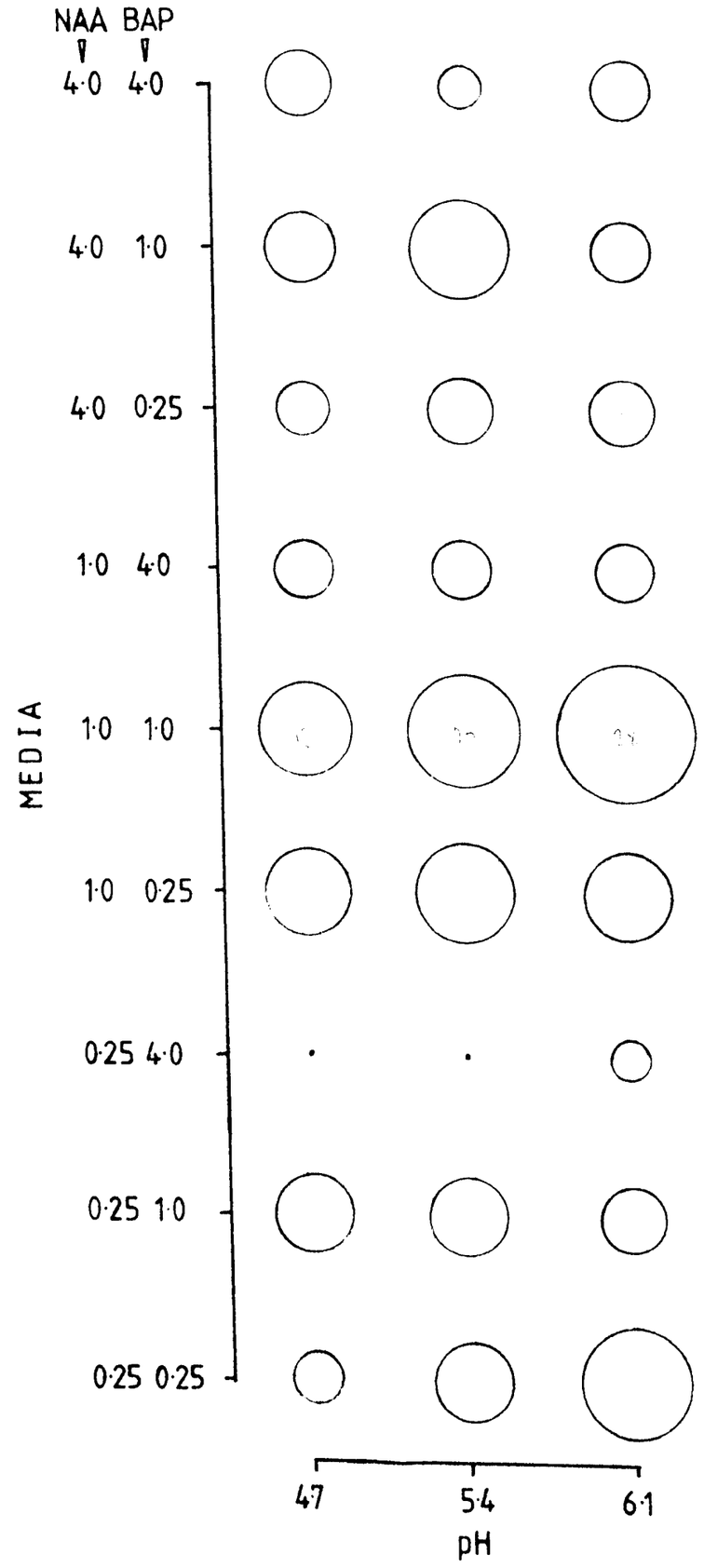


Figure 4.3. a, b.

a) Comparison of the mean shoot production, averaged over the three pH levels, of media containing a range of NAA and BAP concentrations

L.S.D. at the p = 0.05 level

b) Comparison of the mean shoot production on medium M[1:1] at varying pH levels

L.S.D. at the p = 0.05 level

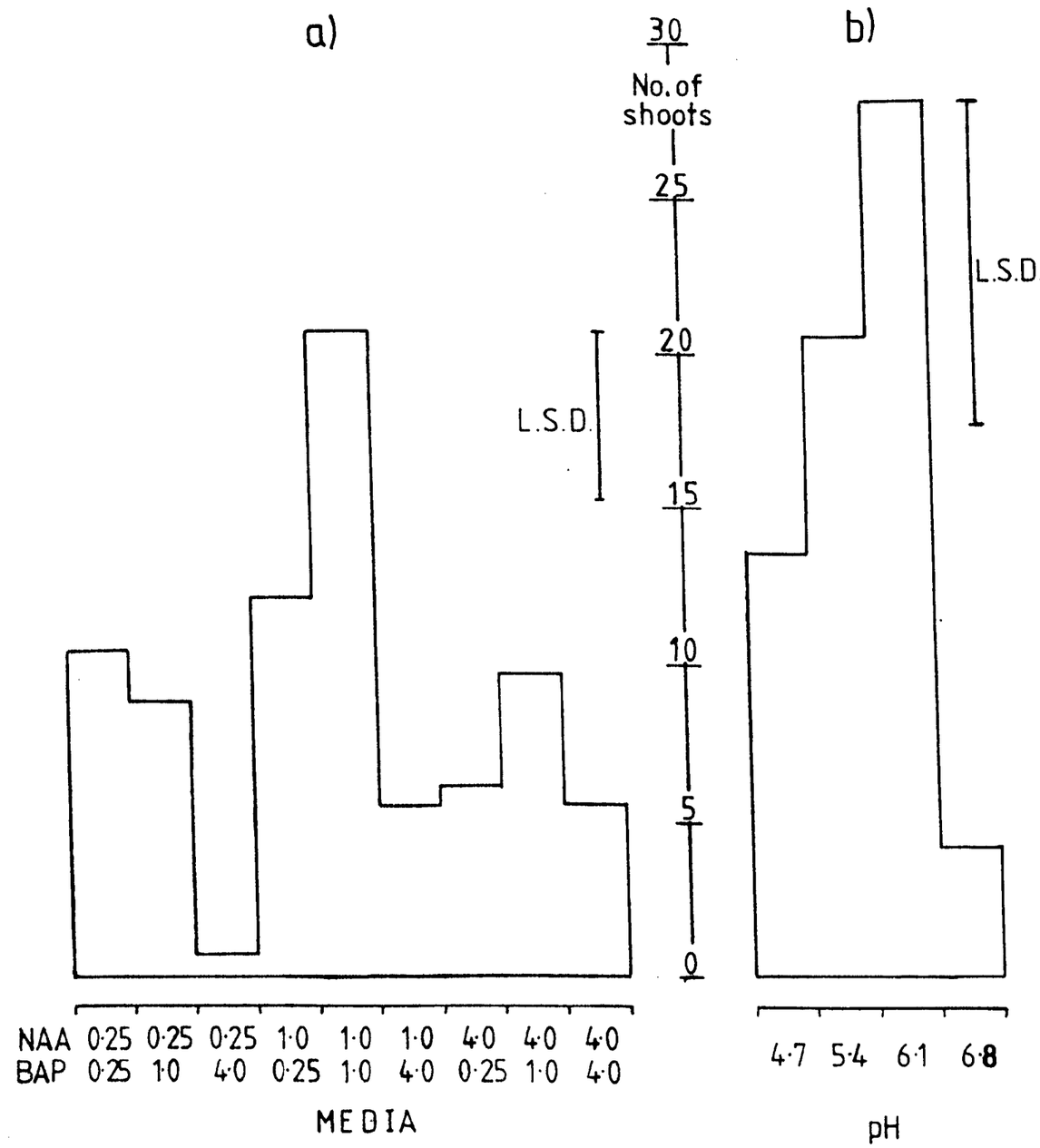


Figure 4.4. a, b, c.

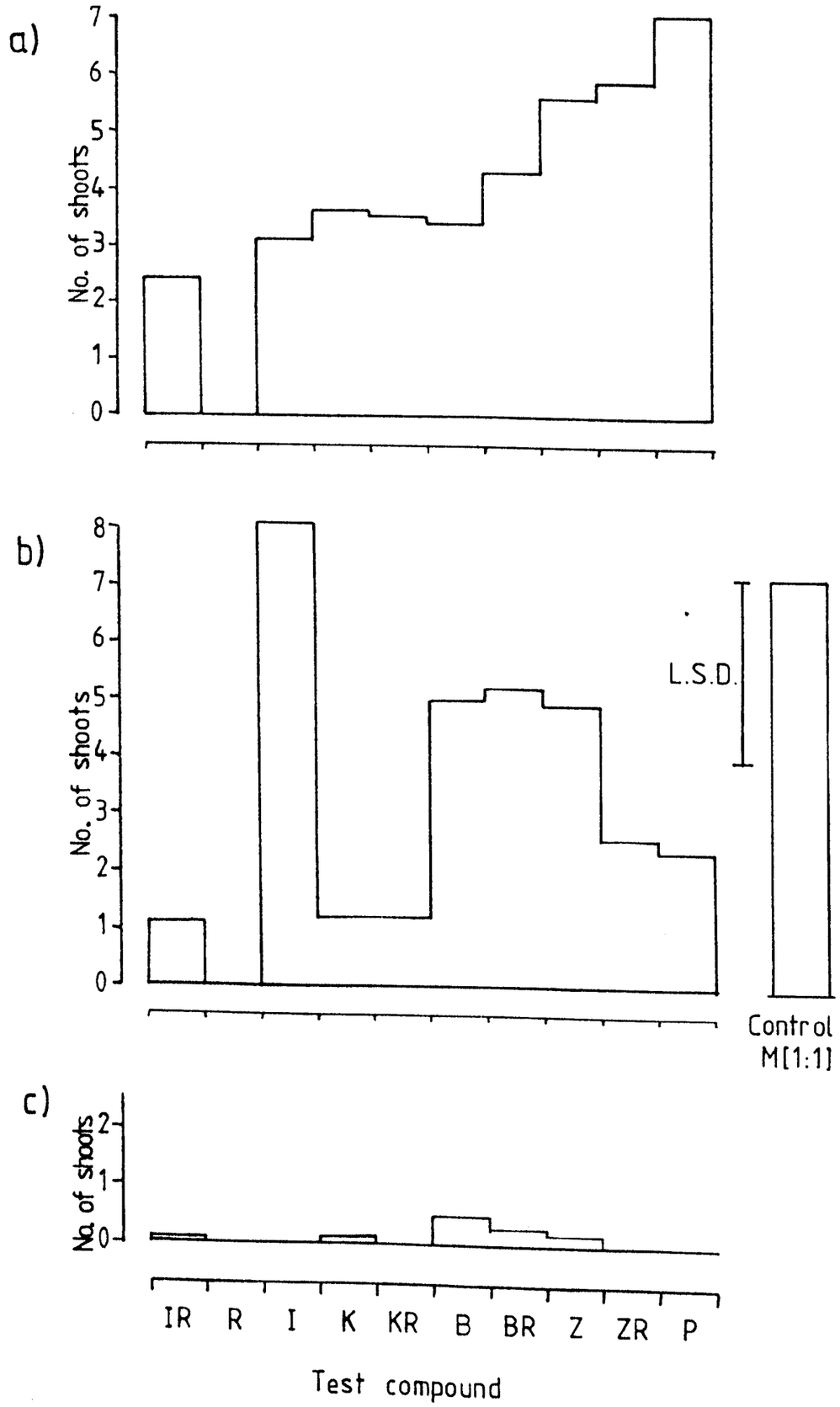
Comparison of mean shoot production by floral stem explants on media containing compounds with cytokinin-like activity. See Table 4.1, (page 62) for details of the chemicals

a) Test compounds at 10.0 mg $l^{-1}$

b) Test compounds at 1.0 mg $l^{-1}$

c) Test compounds at 0.1 mg $l^{-1}$

L.S.D. at the p = 0.05 level



## 4.2. Explant manipulation

Stage of development was found to be a critical factor in determining the shoot producing ability of floral stem explants (Section 3.4). It was shown that the most responsive floral stem explants were those taken during the dormant phase of the tulip bulb growth cycle (Figure 3.3, page 45). A further more detailed study has now been made during this 'dormant' phase to establish more accurately the most responsive stage of development for floral stem explants *in vitro*.

Indications from previous experiments (Section 4.1) of a different response *in vitro* from explants of different origin within the stem, has led to the study of the shoot producing ability of explants from all origins within the floral stem.

### 4.2.a. Effect of the stage of floral stem development on shoot production

The floral stem is initiated in the tulip bulb soon after lifting in June/July (Gilford & Rees, 1974). Flower initiation is complete by the end of July after which the stem develops slowly, increasing in length to approximately 25 mm by December/January when stored at 17°C. The following experiment was conducted to establish whether the stage at which the explants were cultured, during this slow development, had any effect on their ability to produce shoots *in vitro*.

Materials and methods

The explants were cut from non-nodal stem, between the top node and the base of the petals, and placed in culture at intervals from September to January. The culture medium used was M [ 1:1] (see Section 4.1.b). The size of the explant varied with the size of the floral stem. Table 4.3 (page 73) shows the measurements of explant and floral stem size, and the date on which explants were placed into culture.

Results and discussion

There was an increase in the mean shoot production per culture correlated with the increase in stem length or age (Table 4.3, page 73). However, there was no significant difference between stages 2, 3, 4 & 5. The large L.S.D. was due to a very variable response in the number of shoots produced per culture. Nevertheless these results indicate that a high level of shoot production can be obtained from explants cultured between October and January when the bulbs are stored at 17°C.

## 4.2.b. Effect of explant origin within the floral stem on shoot production

Previously the choice of the explant type has taken into account the ease of surface sterilization and of explant dissection. It is easier to obtain sterile explants from tissue wrapped in leaves when bulbs have been stored for long periods, but it is simpler to cut explants from

floral stem not wrapped in leaves. Choosing explants by this method does not take into account any variation in response *in vitro* due to the different origins of explants from within the stem, and because there has been considerable variation between experiments that could be due to these differences in explant origin, the following experiments have been conducted.

(i) Experiment 1

Materials and methods

Floral stems of 10-15 mm in length were dissected during November 1978 into five equal pieces, 2-3 mm in length (Section 2.3) and placed on medium M [1:1] (Section 4.1.b).

Results and discussion

There was considerable variation with treatment in the mean number of shoots produced per culture (Table 4.4, page 74) but the random error within treatments was such that the only significant variation was a decrease in shoot production correlated with increased distance of explant origin from the base of the stem. Part of the variability on number of shoots per culture was related to differences between individual stems resulting in variations in explant composition, i.e. the number of nodes per explant could vary between zero and two, and explants having two nodes did not produce any adventitious shoots, although the axillary buds in these nodes usually developed.

(ii) Experiment 2

In order to substantiate the results of explant position just reported a more detailed study was performed. In the previous experiment large explants were used and the position of the nodes within the stem varied slightly resulting in variability of explant composition (number of nodes). Division of the stem into a large number of explants would remove the possibility of there being more than one node per stem section.

Materials and methods

One problem which occurred in cutting the floral stem into a large number of sections was that, when in a responsive state *in vitro* (Section 3.4 & 4.2.a), it only attains a maximum length of approximately 25 mm. It was therefore necessary to devise a method of subdividing this quite small stem into many sections so a simple inexpensive apparatus was designed and constructed (Section 2.3.d) for the purpose, and using this apparatus sections of 1 mm in thickness could be cut consistently.

Under aseptic conditions floral stems were cut into 1 mm sections as described in Section 2.3. The stems used possessed three nodes and were approximately 24 mm in length, when measured from the basal plate to the base of the flower. The culture medium used was M[1:1] (Section 4.1.b) and 20 replicates were used per treatment.

Results and discussion

The shoot regeneration obtained on 1 mm sections of different origin within the stem varied significantly (Figure 4.5, page 75). The regeneration was very low from the basal region, reached a maximum in the region of the lower two nodes (central stem region), and declined again towards the apex. Few explants from the basal end of the stem showed any shoot production, callus growth, or even expansion growth, and many turned black within a short time of being placed in culture. It appears that the presence or close proximity of a node to an explant (before dissection) has an advantageous effect on the shoot production of that explant when placed in culture. The apparent lack of effect of the top node could be explained by the variability of its position within the stem, as it occurred between sections 16-20 inclusive. These differences between the regions of stem could be explained by a gradient of endogenous PGR's along the stem, giving an optimum interaction in the region of the nodes. This hypothesis is discussed further in Section 4.3 (and in Chapter 5).

The mean shoot production for all the explants was 7.1 shoots per culture, which is an average of 170 shoots per stem. This is a considerable increase in productivity compared with that of the stem divided into five explants only (Section 4.2.b.i), when a mean of 5.5 shoots per culture was produced, which yields an average of only 27.5 shoots per stem. The preparation of 1 mm sections was

considerably more complicated and time consuming but this could still be a useful technique if explant material was limited; for example in the bulking up of new cultivars or virus-free material.

Table 4.3.

Shoot production on explants from floral stems at various stages of development, during the 'dormant' phase

| Stage of development | Date | Total stem length (to base of flower) mm | Explant size mm | Mean number of shoots produced per culture |
|---|---|---|---|---|
| 1. | 25/9 | 5-7 | approx. 1 | 1.7 |
| 2. | 20/10 | 8-10 | " 2 | 3.3 |
| 3. | 16/11 | 10-15 | 3-4 | 4.2 |
| 4. | 14/12 | 15-20 | 4-5 | 4.1 |
| 5. | 21/1 | 20-25 | 5 | 6.0 |

L.S.D. = 3.1

Table 4.4.

Shoot production per culture from floral stems divided into five explants

| Explant origin | | Mean number of shoots produced per culture |
|---|---|---|
| Top of stem | 5 | 5.1 |
| | 4 | 7.7 |
| | 3 | 6.5 |
| | 2 | 5.0 |
| Bottom of stem | 1 | 3.3 |

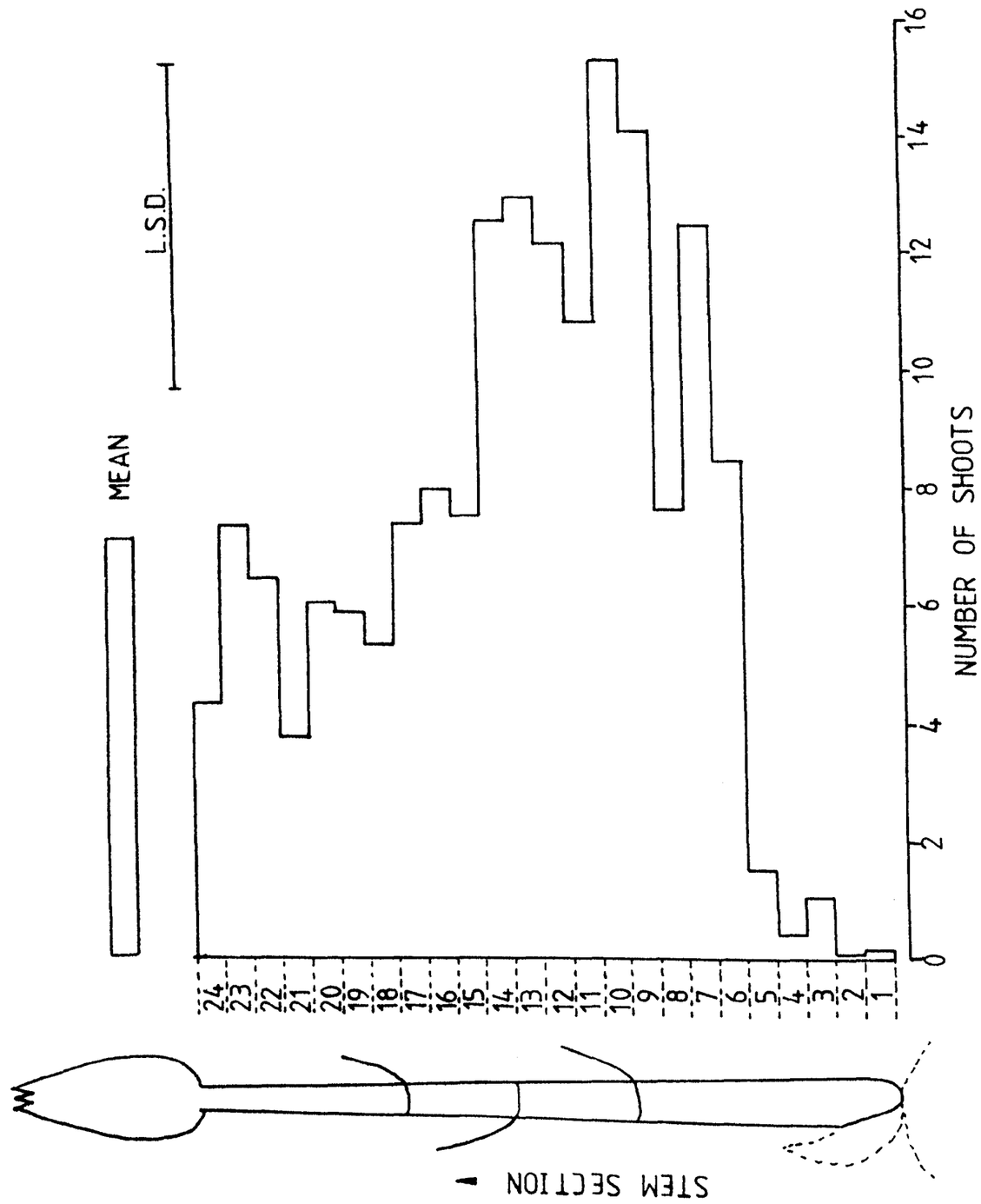
L.S.D. = 3.1 ($p = 0.05$)

Mean of all explants = 5.5

Figure 4.5.

Mean shoot production per culture from floral stems divided into 24 x 1 mm sections

L.S.D. at the p = 0.05 level



## 4.3. Explant x Media interactions

A gradient of endogenous PGR's within the stem may be responsible for the differences in the amount of shoot production from various regions of the floral stem (Section 4.2.b). To test this hypothesis it was necessary to extract and assay the PGR's from the different regions of the stem (Chapter 5).

The possibility of increasing the shoot production of the low regeneration regions of the stem by manipulating the levels of PGR's in the medium, and thereby compensating for any changes in endogenous PGR levels has been tested in the following experiments.

Growth responses to PGR's are normally logarithmic with concentration (e.g. bioassays, Hill, 1980), so based on medium M [1:1], media with PGR concentrations four-fold lower and four-fold higher were selected. The chosen concentrations should therefore manifest only a small change in response, but should be large enough to cover the range in which shoot production is likely to occur (as determined by previous experiments, Section 3.4, 4.1).

The explants were dissected on an anatomical basis, taking account of the position of the nodes as shown diagrammatically in Figure 4.6 (page 85), thereby avoiding the problems of variable explant composition experienced in Section 4.2.b.i), without having to divide the stem into a large number of sections as in Section 4.2.b.ii). With

only six sections the number of explants was reduced thus making it simpler to examine more treatments (cf. Section 4.2.b.ii).

### 4.3.a. Effect of explant and media on shoot production

The shoot production on floral stem explants varies with stage of development (Section 4.2.a), and therefore an interaction may exist between PGR levels in the medium and the stage of bulb development in terms of shoot production. Consequently examination of explant x media interactions was conducted at two stages of development.

#### Materials and methods

Floral stems at two stages of development were prepared as described in Section 2.3, divided into six explants of approximately equal thickness as described, and placed in culture. Stage 1 was cultured during October when the stems were 6-7 mm in length, and stage 2 at the end of December when they were approximately 22 mm in length. The media used were based on medium M [1:1] but with NAA & BAP at 0.25, 1.0 & 4.0 mg $l^{-1}$ and all factorial combinations.

#### Results and discussion

A diagrammatic representation of the effects and interactions between explant and media is shown in Figure 4.6 (page 85) (stage 1) and in Figure 4.7 (page 86) (stage 2). The area contained within each of the circles is

proportional to the mean number of shoots produced by that treatment, such that any interaction between treatments is shown clearly. Tables of these data are presented in the Appendix (Sections A3 & A4) together with their statistical significance.

At stage 1 of stem development the maximum number of shoots per culture for all the media was produced by the 1N explants, with the exception of media M [0.25:4] & M [4:4] on which the 2N explants appeared to produce the largest number of shoots. However, on these media all the explants showed a very low rate of regeneration and there was no significant difference between any of the explants. There was little interaction between explant and medium with the exception that some media (e.g. M [1:0.25]) gave rise to approximately the same number of shoots on most of the explants, whereas others gave a very large number on 1N explants and a very low number on the majority of the other explants (e.g. M [4:0.25]).

In order to investigate which media gave the highest overall shoot production the mean shoot production over all the explants was calculated (Figure 4.8.a, page 87). Most of the media produced a large number of shoots per culture, and only media M [0.25:4] and M [4:4] produced comparatively few. Medium M [4:1] gave rise to the largest number of shoots per culture but this was not significantly higher than M [1:0.25]. As both of these media have the same ratio of NAA:BAP, it is possible that this

ratio of PGR's is more important than their absolute concentrations in determining shoot production on floral stem explants.

The shoot production from each type of explant averaged for all the media (Figure 4.8.b, page 87) shows clearly that explants of the lowest node (1N) were the most productive and the non-nodal explants from the top and bottom of the stem (T & v⊬) were the least productive.

For stage 2 of the stem development (Figure 4.7, page 86) explant 1N produced a greater number of shoots per culture than the other explants for all the media with the exception of M [ 0.25:4]. This medium gave a very low rate of regeneration and no significant difference between any of the explants, as occurred in stage 1, but unlike stage 1, explants (especially 1N & 2N) on medium M [ 4:4] at stage 2 produced a large number of shoots.

Interactions between media and explant were much less for stage 2 than for stage 1, and the differences between media were also reduced, only becoming obvious when the mean of all the explants was calculated for each medium (Figure 4.9.a, page 88). As before, the majority of the media produced a large number of shoots per culture, but for stage 2 the highest shoot production was from media M [ 4:4], M [ 4:1] and M [ 1:4].

The mean number of shoots produced for stage 2 by 1N explants averaged over all the media was approximately the

same as for stage 1 (Figure 4.9.b, page 88 cf. Figure 4.8.b, page 87) but all the other explants were less productive for stage 2 than stage 1. Thus there was a slightly reduced grand mean (i.e. average over all explants & media) shoot production for stage 2.

There appears to be a preference for higher levels of PGR's in the optimum media for stage 2 compared with those for stage 1 (Figures 4.9, page 88 & 4.8, page 87). This is substantiated by a comparison between stages 1 & 2 of the average for all explant types of NAA & BAP concentrations (Figure 4.10, page 89). Optimum PGR concentrations at stage 1 were on average 1 mg $l^{-1}$ for NAA and 0.25 & 1.0 mg $l^{-1}$ for BAP, whereas at stage 2 they were 4.0 mg $l^{-1}$ for NAA and 1.0 & 4.0 mg $l^{-1}$ for BAP. It should be noted, however, that this representation of shoot production at different PGR levels is of little value for selecting the optimum concentrations of NAA & BAP in combination, i.e. the optimum medium, because each value is the mean of three levels of the other PGR. However it is useful for giving an overall view.

The media giving the maximum number of shoots per culture in this experiment were not the same as those found to be optimal in Section 4.1. This inconsistency could be caused by a number of factors that are different between the experiments. It has been shown that the stage of floral stem development and the explant type can alter the optimum PGR levels, and it is possible that bulbs

produced in different seasons may respond differently; great care should be exercised therefore in making overall generalizations. However, the optimum medium from the results in Section 4.1 (M[1:1]) has consistently given a large mean number of shoots at stages 1 & 2, and can be considered as a good medium for all floral stem explants.

### 4.3.b. Effect of explant and media on gain in fresh weight

In previous experiments in Chapter 4 all cultures have been assessed by counting the number of shoots produced per culture. However, this is quite difficult as it is necessary to perform this assessment without removing the explants from their culture vessels, in order to maintain their sterility. It is also a very time consuming task as some of the explants have the potential to produce over 30 shoots. An assessment based on weight of explants was therefore considered as an alternative, and in this section such an assessment is related to the shoot production recorded in Section 4.3.a.

#### Materials and methods

The initial fresh weight of the explant types varied considerably (Figure 4.11, page 90). It was therefore necessary to calculate the gain in fresh weight per culture in order to deal with a dimensionless quantity, i.e. a quantity that was proportional to explant growth.

Problems were encountered in the weighing of explants; the most serious of which was maintaining sterility. The use of a Sartorius top loading mg balance (model number 1205) made it possible to weigh explants in the laminar flow cabinet thereby avoiding contamination by micro-organisms. Nevertheless this sensitive balance could only be used to weigh the explants at the later stage of floral stem development (stage 2) as the initial fresh weight of stage 1 explants was at or below the sensitivity of the balance.

The initial fresh weight of the explants was measured immediately after their preparation before being placed in culture (for culture conditions, media etc. see Section 4.3.a). The fresh weight of explants was assessed again after 20 weeks in culture, at which time the number of shoots per culture was also recorded (Figures 4.7, page 86 & 4.9, page 88).

Results and discussion

Very large gains in fresh weight were made by all explants types on all media (Figure 4.11, page 90). (The data are presented in a similar format to that of Figure 4.7, (page 86); gain in fresh weight being proportional to the area contained within the circles.) A table of this data together with its statistical significance is presented in the Appendix (Section A5). Variation between treatments was much less for fresh weight than for shoot

production, thus any interactions between media and explant are masked on a fresh weight assessment. It appears therefore that assessment on a fresh weight basis does not totally reflect any treatment differences as shown by shoot production. This is confirmed by comparing Figures 4.12 (page 91) and Figure 4.9 (page 88) which show that the optimum media (a) and explant (b) for shoot production (Figure 4.9, page 88) do not agree with those found for fresh weight gain (Figure 4.12, page 91). Gain in fresh weight was almost totally independent of BAP concentration and proportional to NAA concentration (Figure 4.12.a, page 91) suggesting that the major contribution to fresh weight gain was in fact callus production, especially as the results presented in Chapter 3 suggested that callus production was dependent on auxin concentration alone. The large fresh weight increase related to callus production masked the effects of explant and media on shoot production expressed in terms of gain in fresh weight. Measurement of fresh weight gain is therefore considered to be of limited value as a simple method of culture (shoot production) assessment.

It was observed that the gain in fresh weight of explants 2N, 3N and T was greater than that of the lower stem explants (Figure 4.12.b, page 91). The final fresh weight was not significantly higher for the upper stem explants, but the initial fresh weight was substantially

lower (Figure 4.11, page 90). This low initial fresh weight of the upper stem explants could account, at least in part, for the low shoot production of these explants as observed in Sections 4.2 & 4.3. Unfortunately it was not possible to test this hypothesis by using explants of higher fresh weight because, as observed in Section 4.2.b.i) large pieces of stem do not respond in culture, they produce only axillary buds from the nodes and no adventitious shoots.

Figure 4.6.

Diagrammatic representation of the interaction between explant origin and medium, at stage 1 of floral stem development (i.e. mid October) in terms of the mean shoot production per culture

The area contained within each circle is proportional to the mean shoot production by that treatment

≡ 10 shoots

N.B. A table of this data plus statistical significance is presented in the Appendix Section A3

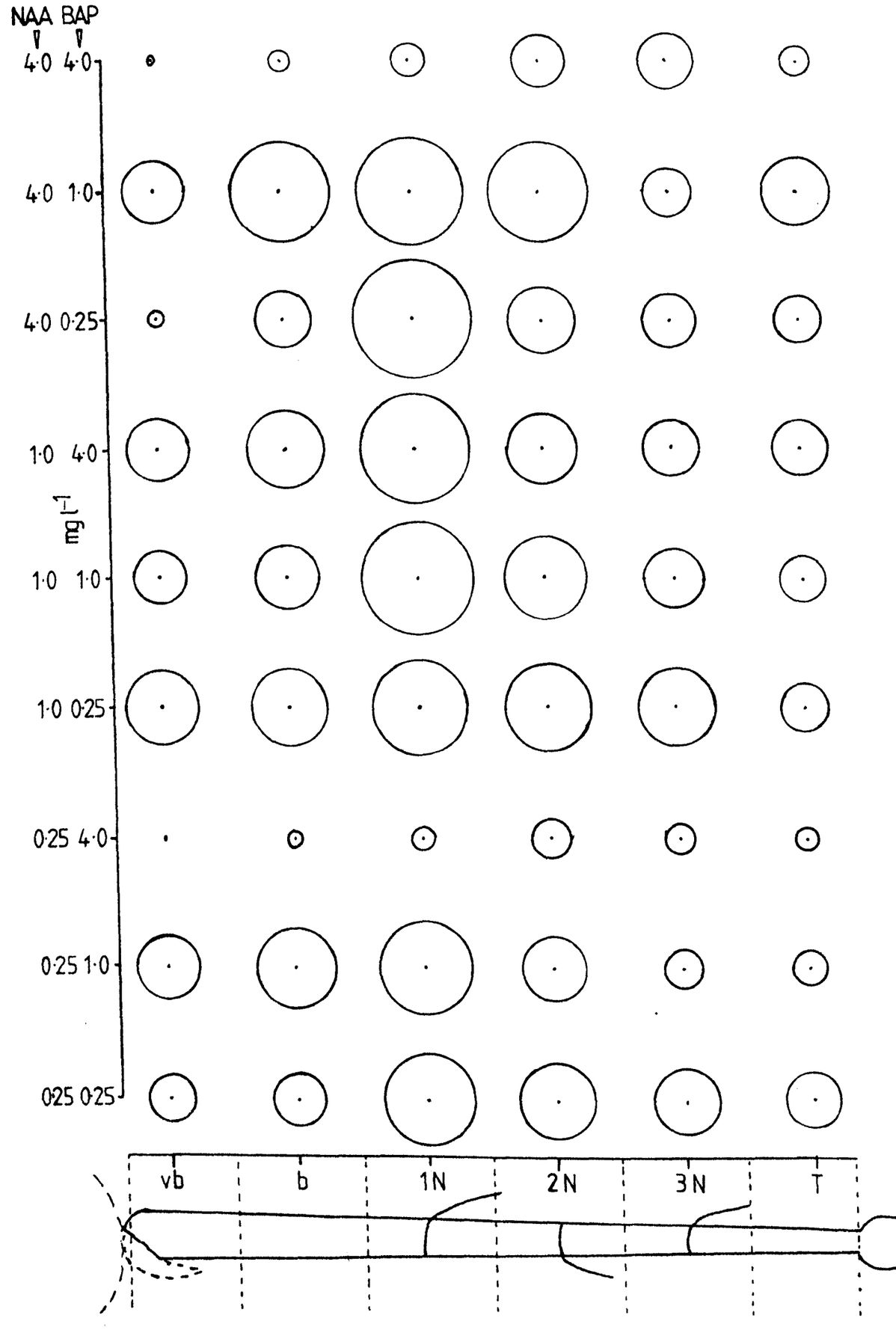


Figure 4.7.

Diagrammatic representation of the interaction between explant and medium, at stage 2 of floral stem development (i.e. end of December) in terms of the mean shoot production per culture

The area contained within the circle is proportional to the mean shoot production by that treatment

○ ≡ 10 shoots

N.B. A table of this data plus statistical significance is presented in the Appendix Section A4

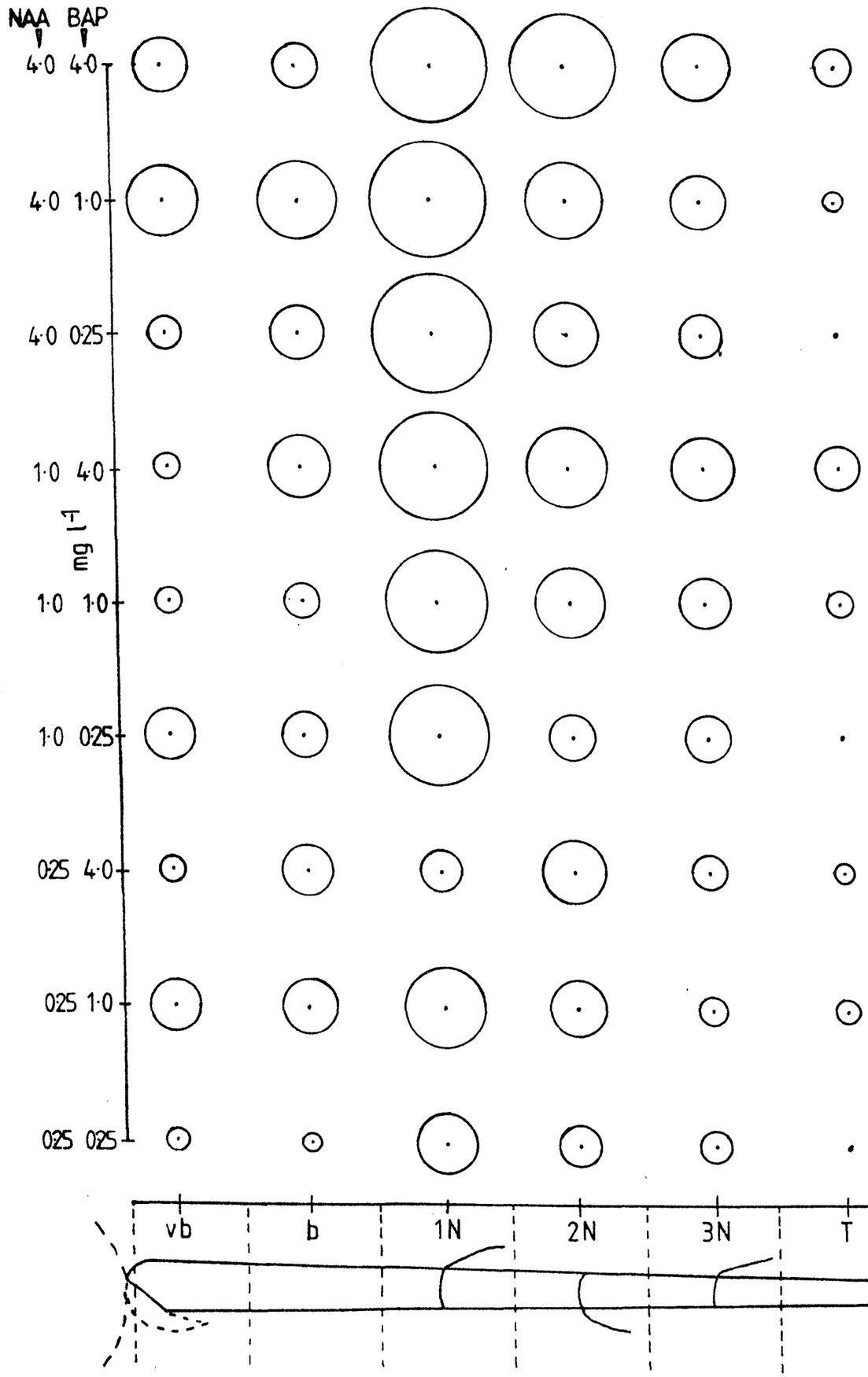


Figure 4.8. a, b.

Stage 1 of floral stem development:

a) Effect of medium PGR levels on the mean shoot production per culture, averaged over all explant types

b) Effect of explant type on the mean shoot production per culture, averaged over all media

L.S.D. at the p = 0.05 level

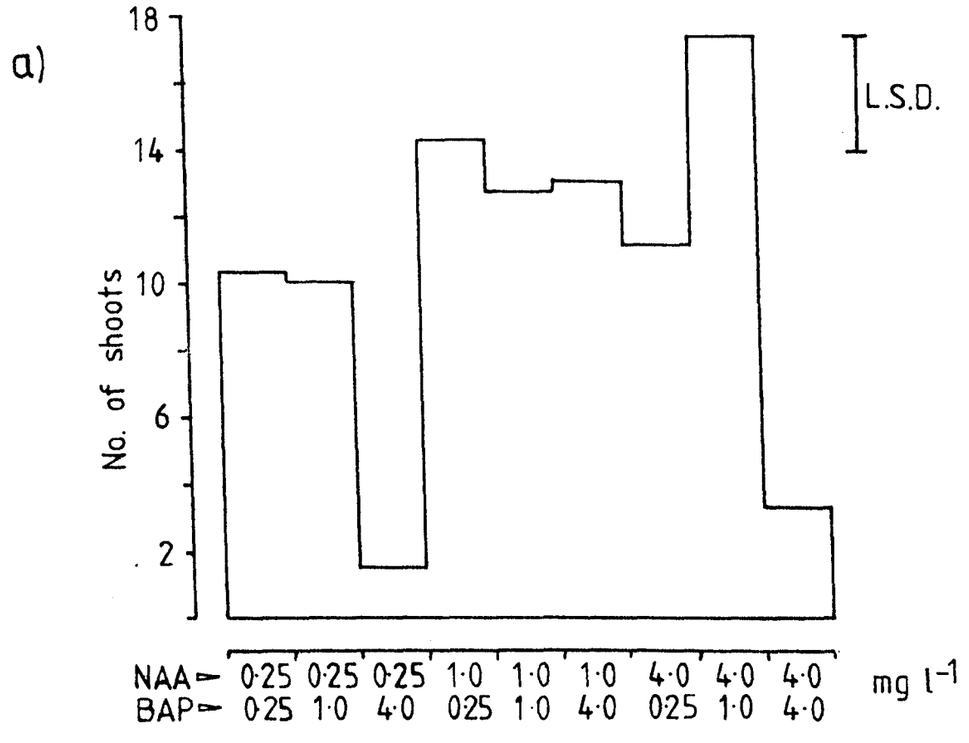


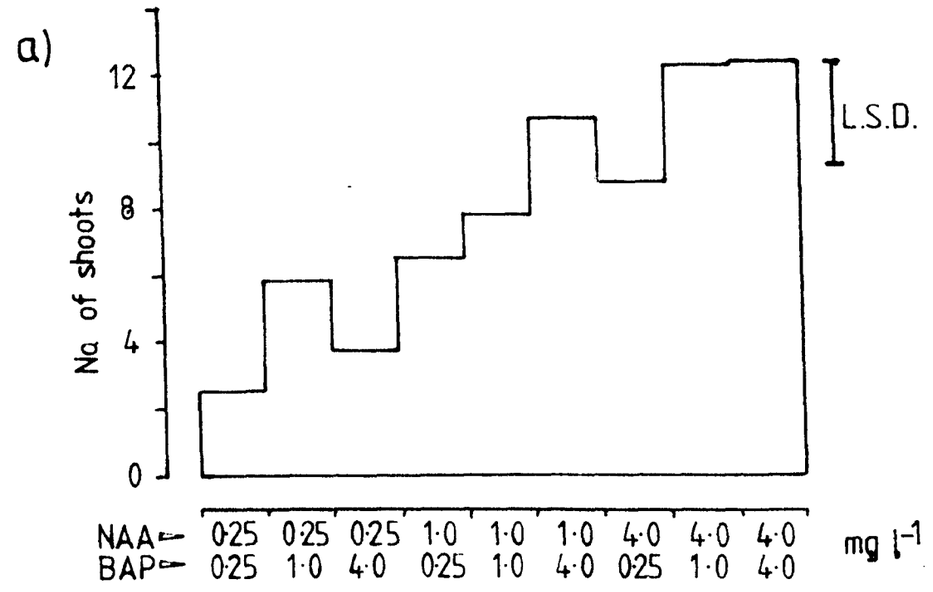
Figure 4.9. a, b.

Stage 2 of floral stem development:

a) Effect of medium PGR levels on the mean shoot production per culture, averaged over all explant types

b) Effect of explant type on the mean shoot production per culture, averaged over all media

L.S.D. at the p = 0.05 level



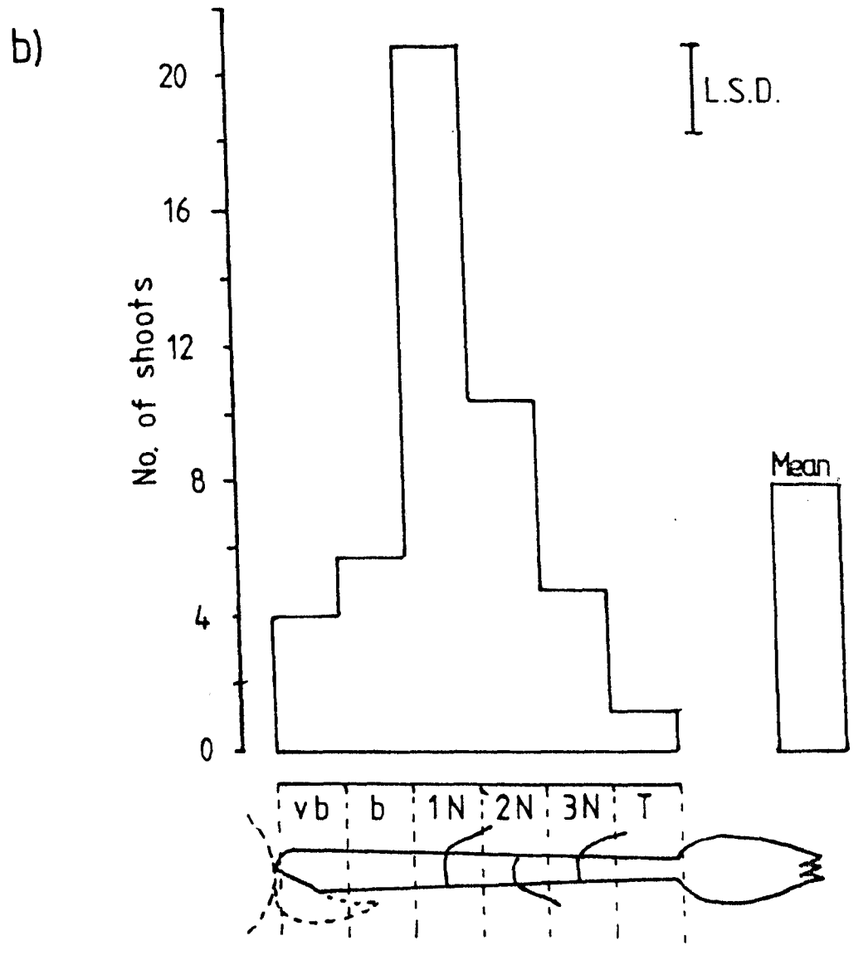



Figure 4.10.

Effect of PGR concentration on the mean shoot production per culture, averaged over all explants and all media containing each PGR concentration

a) Stage 1

(i) NAA

(ii) BAP

b) Stage 2

(i) NAA

(ii) BAP

L.S.D. at the p = 0.05 level

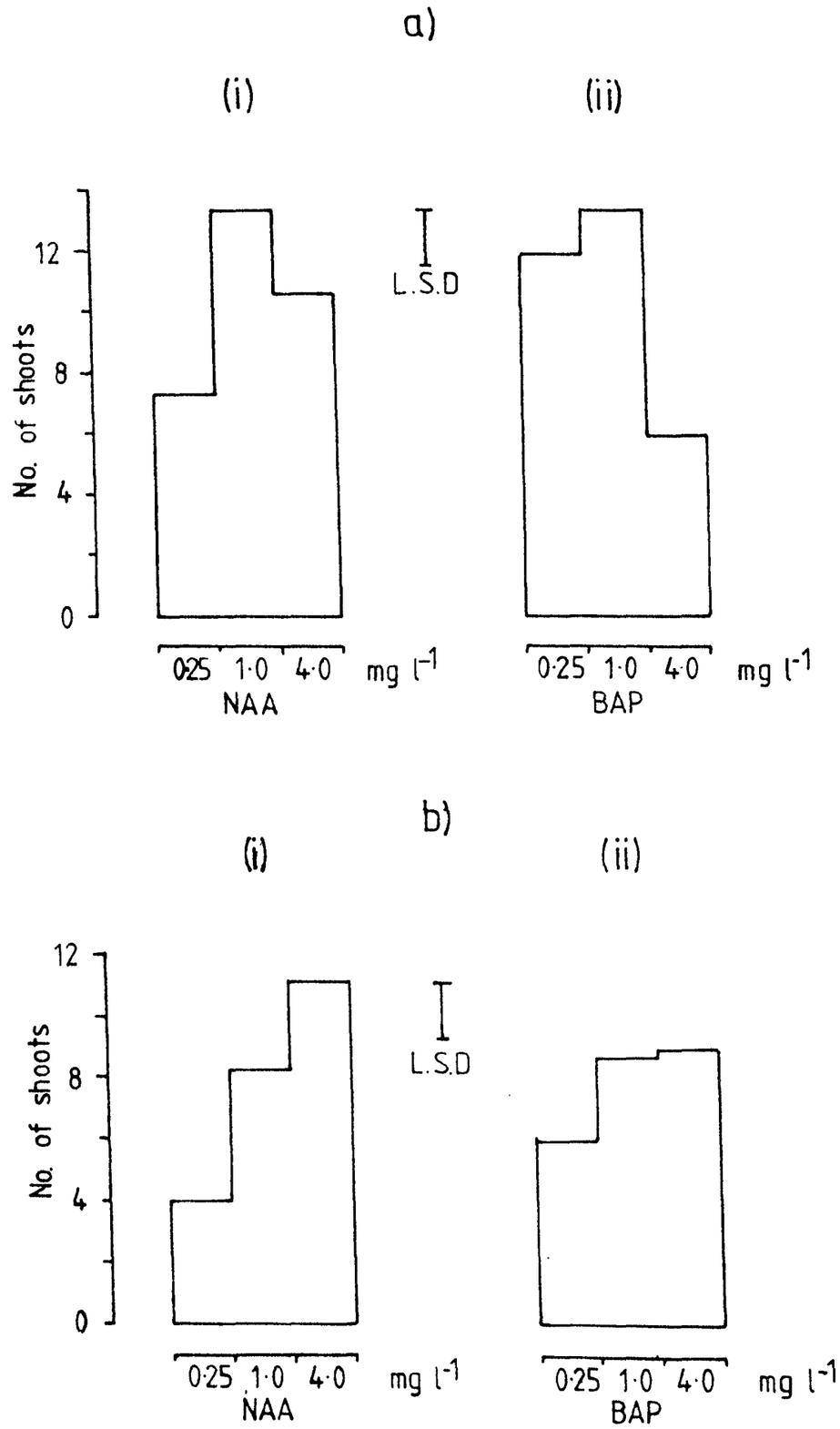


Figure 4.11.

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Diagrammatic representation of the interaction between explant and medium, at floral stem development stage 2 in terms of mean fresh weight gain per culture

The area contained within each circle is proportional to the gain in fresh weight by that treatment

○ ≡ 25 times initial fresh weight

The mean initial fresh weight of each explant type is shown

N.B. A table of this data plus statistical significance is presented in the Appendix Section A5

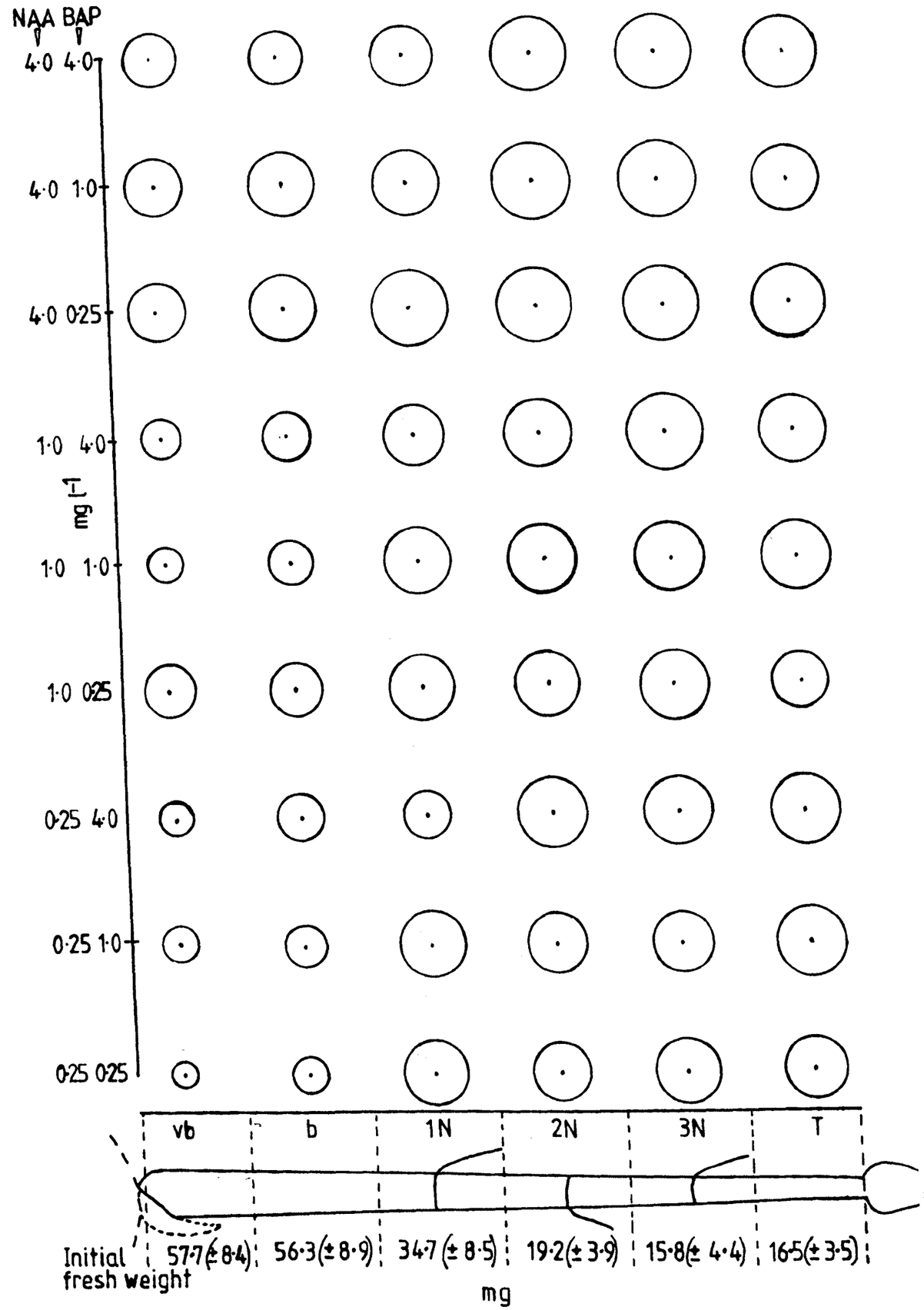


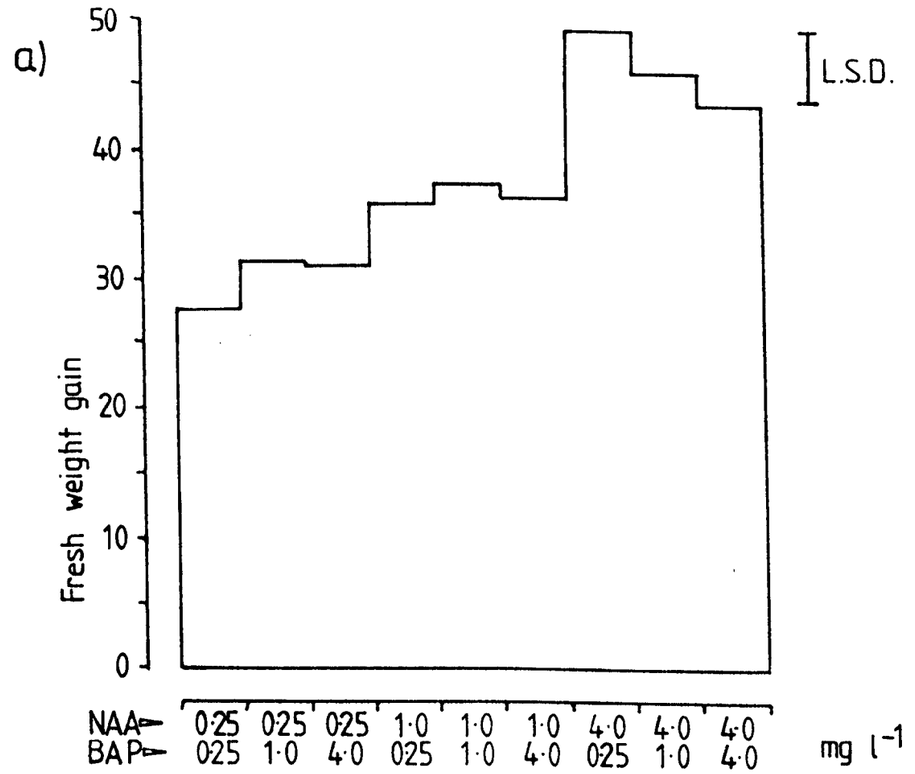
Figure 4.12. a, b.

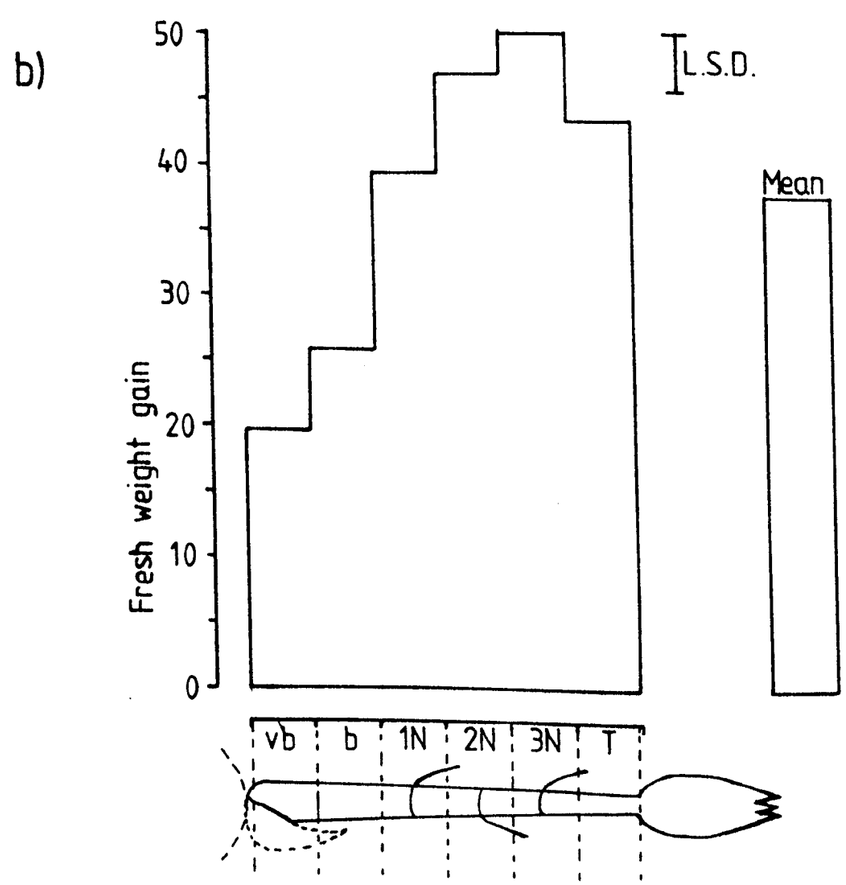
Mean fresh weight gain at floral stem development stage 2

a) Effect of medium PGR levels, averaged over all explant types

b) Effect of explant type, averaged over all media

L.S.D. at the p = 0.05 level



Chapter 5

# Endogenous plant growth regulator (PGR) studies

A gradient of one or more PGR's along the length of the tulip floral stem may account for the variation in shoot production from the different stem explants (see Chapter 4). Published reports on endogenous PGR levels in tulip (reviewed below) and other bulbous plants have not examined the floral stem in detail. For example, although Stewart & Stuart (1942) showed that the top of the lily stem contains 1000 times more auxin than the storage scales or bulb stem variation within the stem was not examined.

In studies of PGR's in tulip the major emphasis has been placed on correlating the levels of endogenous PGR's with their possible regulation of dormancy e.g. Rakhimbaev et al., 1978. With this objective the endogenous levels of gibberellic acid-like substances (GA-LS) have been studied in detail (Aung & De Hertogh, 1967,1968; Aung & Rees, 1974; Aung et al., 1971; van Bragt, 1971a; De Hertogh et al., 1971). In a study of 'bound' & 'free' GA-LS in tulip scale tissue van Bragt (1971a) found no correlation between endogenous levels of GA-LS and elongation of the floral stem. However the investigation was concerned with the levels of GA-LS in whole terminal bud (including floral stem) and not with variations in particular tissues. Similarly, the examination of endogenous cytokinin-like substances (C-LS) by Rakhimbaev & Solomina (1975) did not include any detail of variations in concentration within the stem, although they did report that the central part

of the bulb (presumably the floral stem) yielded much higher levels of C-LS than the storage organs. Differences between the 'central' part of the bulb and storage scales were also observed (Syrtanova et al., 1973), but again no detail within the floral stem was examined.

An indication of what may be occurring in the floral stem is available from studies of exogenously applied PGR's. Although un-cooled tulip bulbs will not produce normal extension growth of the floral stem, elongation of the lower internodes can be achieved by the exogenous application of GA (van Bragt & Zijlstra, 1971; van Bragt & van Ast, 1976; Rudnicki et al., 1976; Hanks, 1979; Rees & Hanks, 1979), whereas elongation of the upper internodes appear to be controlled by auxin (op den Kelder et al., 1971; Hanks & Rees, 1977; De Munk, 1979). These data strongly support the hypothesis proposed in Section 4.3, namely that there may be a gradient of PGR's along the length of the floral stem.

In order to test this hypothesis, a series of experiments were conducted to examine the levels of compounds exhibiting the properties of the following four important PGR groups; gibberellins (GA-LS), cytokinins (C-LS), auxin (IAA-LS) and abscisic acid (ABA-LS).

## 5.1. Materials and methods: Extraction, purification and assay of PGR'S

All the solvents used were re-distilled Analar grade. Hydrogen peroxide was removed from di-ethyl ether as follows: One litre of di-ethyl ether was shaken with 0.25 g of $FeSO_4$ and 50 ml of water. Following partitioning a further 50 ml of water was added and partitioned again. The whole procedure was repeated and the resultant di-ethyl ether was re-distilled over $FeSO_4$. Di-ethyl ether prepared in this way was used within one week of preparation.

### 5.1.a. IAA-like substances & ABA-like substances

Initially, procedures were established for the extraction and purification of IAA-LS and ABA-LS for their subsequent bioassay using the bean internode bioassay (Meudt & Bennett, 1978), and a modification of the barley endosperm bioassay (Jones & Warner, 1967) respectively. However, because of their low sensitivity and thus the large amounts of plant material necessary to detect biological activity in these bioassays, alternative methods of detection were investigated. Physico-chemical methods of detection, namely gas-liquid chromatography (GLC) and spectrofluorimetry, were used for IAA-LS & ABA-LS (by courtesy of Dr. J.F. Hall, Leics. Polytechnic.).

Procedures for the extraction and purification of IAA-LS were based on those described by Mousdale et al. (1978).

These procedures, although not producing very high yields for ABA-LS were found to give consistent and re-peatable results in trials, and were adopted for the extraction and purification of both IAA-LS and ABA-LS; the extract being divided immediately before the final partitioning.

Floral stems were dissected on ice and stored immediately at -20°C until required: the storage period did not exceed two months. Frozen samples were homogenized in 100% (v/v) methanol using a tissue:solvent ratio of 20:1, and allowed to extract at -20°C for 90 min. A methanolic solution of [$^{14}$C] IAA of approximately 5000 cpm was added at the beginning of the extraction for use in calculating the yield from the purification procedure. Following extraction the homogenates were centrifuged at 2000 g for 5 min in an MSE bench centrifuge, and the supernatant decanted and reduced to the aqueous phase <u>in vacuo</u> at 35°C. The aqueous residue was re-dissolved in 15 ml 0.5M phosphate buffer pH 8.5 and partitioned, once against an equal volume of petroleum ether, and twice against equal volumes of di-ethyl ether. After separation the aqueous phase was acidified with 1M HCl.

Following partitioning three times against equal volumes of di-ethyl ether at low pH, the organic phases were pooled and back-washed by shaking with half volume 0.5M phosphate buffer pH 8.5. The resultant aqueous phase was acidified to pH 3.0 with 1M HCl and partitioned three times against di-ethyl ether. The final pooled

ether fraction was evaporated to dryness in vacuo at 35°C and the dried residue re-dissolved in 0.1M phosphate buffer pH 8.5. This solution was applied to a pre-washed poly-vinyl-pyrrolidone column ( Biddington & Thomas, 1976) and the first 150 ml of solution eluting from the column were collected and acidified to pH 3.0 with 1M HCl. At this stage an aliquot was taken for ABA-LS extraction (see below). After acidification the eluate was partitioned twice against half volumes of di-ethyl ether and the pooled organic phases frozen to remove water. After filtering off the ice crystals the ether was evaporated in vacuo at 35°C, and the dried residue re-dissolved in 7 ml ethanol.

A 1 ml aliquot was taken for radioactive assay in order to calculated the recovery of purified substances following the purification procedures. The remainder of the extract was used in the fluorimetric assay.

The sensitivity of the GLC assay for ABA was such that only one tenth of the column eluate was required for further purification and assay of ABA-LS. In order to estimate the yield of ABA-LS an aqueous solution of [$^{14}$C] ABA of approximately 5000 cpm was added. The acidified aliquot was partitioned three times against equal volumes of ethyl acetate, the pooled organic phases frozen and filtered to remove water and finally the ethyl acetate was removed in vacuo at 35°C. The dried residue was re-dissolved in a small volume (1-2 ml) of methanol.

ABA was converted to its methyl ester (Me-ABA) by the addition of a solution of diazomethane in di-ethyl ether which had been generated on a small scale by the technique described by Schlenk & Gellerman (1960). After incubation for 10 min the excess diazomethane and solvent were removed under a stream of $N_2$. The dried residue was re-dissolved in 100-200 µl of methanol, applied to Merck silica gel GF254 TLC plates and developed in a chloroform:ethyl acetate:acetic acid (60:40:5, v/v/v) solvent. Spots of authentic Me-ABA marker were run in outer 'lanes' of the fluorescence plates. Subsequently, the band corresponding to these markers was scraped from the plates and eluted with methanol before the plates were allowed to dry. Rapid elution was necessary because allowing them to dry for just a few hours can reduce recoveries from about 90% to 10% or less (Saunders, 1978).

All procedures were performed under dim green safe light or at low light levels, because both IAA and ABA are deactivated in strong light (Mousdale et al., 1978; Saunders, 1978).

Assay procedure for IAA-LS

The ethanolic extract was divided into six equal aliquots (1 ml) and the following quantities of methanolic authentic IAA standard were added: 0 (blank), 0 (unknown), 20, 40, 60 and 80 ng. A few drops of toluene were added

to each tube and the solutions were evaporated to dryness in a stream of $N_2$ or by the use of a vacuum desiccator. The dry residue was incubated with a mixture of 0.1 ml acetic anhydride and 0.1 ml trifluoroacetic acid. After 15 min the reaction was stopped with 2 ml of methanol. Methanol was added to the control (blank) tube before addition of the reaction mixture, thereby allowing no reaction to occur in this tube.

Immediately after the reaction had been stopped the methanolic solutions were transferred to a silica fluorescence cuvette and the fluorescence intensity measured at 480 nm in a Perkin-Elmer Model MPF 43A Spectrophotofluorimeter using an excitation wavelength of 440 nm. A typical fluorescence emission spectrum of a floral stem extract is shown in Figure 5.1 (page 114). As there was a straight line relationship between the fluorescence intensity of 2-methyl-indole-$\alpha$-pyrone and the amount of IAA reacted, the amount of unknown IAA-LS per aliquot could be determined from a graph of fluorescence intensity plotted against IAA concentration. The IAA-LS per gram fresh weight of floral stem tissue was calculated from this figure by correcting for the number of aliquots, the recovery, and the initial fresh weight.

Assay procedure for ABA-LS

One $\mu$l samples of the methanolic extract were injected into a Gas Liquid Chromatograph (GLC) fitted with an electron capture detector (ECD). The column was OV17

10% on Gaschrome Q 100-200 mesh, injector 250°C, column 220°C for 1 min and 15° $min^{-1}$ to 280°C, detector 290°C, and a flow rate of approximately 60 ml $min^{-1}$.

Estimation of the yield of ABA-LS was obtained by comparing the peak area of the GLC trace with that of a known amount of authentic Me-ABA. All estimations were corrected for losses during purification based on the recoveries of the radioactively-labelled compounds.

5.1.b. GA-like substances

Frozen floral stem tissue samples (Section 5.1.a) were homogenized in ice-cold 80% (v/v) methanol and allowed to extract for 24-48 h at 0°C. The homogenates were centrifuged at 2000 g for 5 min and the supernatant decanted and reduced to the aqueous phase in vacuo at 35°C. Addition of 25 ml 0.5M phosphate buffer, pH 8.5 was followed by the addition of PVP at approximately 50 mg $ml^{-1}$. After the slurry had been vacuum filtered the aqueous phase was partitioned once against an equal volume of petroleum ether and subsequently three times against equal volumes of ethyl acetate. The pH of the aqueous phase was lowered to 2.5 with 1M HCl and partitioned three times against equal volumes of ethyl acetate. The pooled organic phases, after freezing and filtering to remove water, were reduced to dryness in vacuo at 35°C. The dried residue was re-dissolved in a small volume (100-200 µl) ethanol:ethyl acetate (1:1, v/v) and streaked on-

to a 0.25 mm silica gel TLC plate (Macherey-Nagel & Co., Duren, Germany). The developing solvent (chloroform: ethyl acetate:acetic acid, 60:40:5 (v/v/v)) was allowed to run 10 cm from the point of sample application. After being thoroughly air dried at room temperature 1 cm zones, scraped from the TLC plates were eluted three times with 0.5 ml ethanol:ethyl acetate (1:1 v/v). The pooled eluates, after centrifugation at 2000 g in an MSE bench centrifuge were reduced to dryness in a stream of $N_2$ and re-dissolved in 1 ml sterile water prior to bioassay.

The mobility of authentic GA's on TLC plates was also examined. Standard solutions containing $GA_3$, $GA_{4+7}$, and $GA_9$ were applied to TLC plates and developed as described above. The standard GA's were visualized on the plates following treatment with 5% (v/v) sulphuric acid in ethanol and drying in an oven at 100°C for 5 min.

Bioassay of GA-LS

GA-LS were estimated using a modification of the Jones & Warner (1967) barley half-seed bioassay, selected from the numerous GA-LS assays because of its sensitivity and reproducibility.

Half-seeds of Hordeum vulgare L. cultivar Himalaya, 1974 (Professor J.D. Maguire, Washington State University, USA) were used. Embryo-less halves of the barley seeds were surface sterilized for 20 min in 1% (v/v) sodium

hypochlorite. At the end of the sterilization period the half-seeds were washed at least three times in sterile distilled water and transferred to sterile filter paper in sterile petri dishes so that the cut ends of the half-seeds were not in contact with the paper, nor were they in contact with each other. The half-seeds were imbibed for 48 h at room temperature, ensuring that the filter paper was moist, but not flooded, with sterile water. At the end of the imbibition period ten sterile half-seeds were transferred to sterile conical flasks containing 0.5 ml of sterile 0.01M succinate buffer pH 4.8, 0.5 ml of sterile 0.1M calcium chloride and 1 ml of test solution. The conical flasks were incubated on an orbital shaker at 25°C for 24 h at approximately 50 Hz. After incubation the liquid was decanted, the half-seeds rinsed with 2 ml of distilled water and the combined solutions were centrifuged at 2000 g for 5 min in an MSE bench centrifuge. The supernatant was used as the enzyme solution and the $\alpha$-amylase activity induced was estimated using starch as the substrate, and iodine/HCl as the terminator of the reaction. The $\alpha$-amylase activity induced in the half-seeds by the test solutions was calculated from the absorbance of the resulting solution at 620 nm. Figure 5.2 (page 115) shows the results obtained in the above bioassay procedure when standard solutions of $GA_3$ were used as the test solutions.

5.1.c. Cytokinin-like substances

The procedure for extraction and purification of C-LS was based on that described by van Staden et al. (1972).

Frozen tissue samples (Section 5.1.a) were homogenized in ice-cold 80% (v/v) methanol and allowed to extract for 24-48 h at 0°C. The homogenates were centrifuged at 2000 g for 5 min (MSE bench centrifuge) and the supernatant decanted and reduced to the aqueous phase in vacuo at 35°C. Addition of 25 ml 0.5M phosphate buffer pH 8.5 was followed by the addition of approximately 50 mg $ml^{-1}$ PVP. When the slurry had been vacuum filtered the pH of the aqueous phase was adjusted to 2.5 with 1M HCl, and was partitioned once against an equal volume of petroleum ether, and three times against equal volumes of water-saturated n-butanol. The pH of the aqueous phase was raised to 8.5 with 1M KOH and partitioned against three equal volumes of water-saturated n-butanol. The pooled organic phases were reduced to dryness in vacuo at 35°C and the dried residue was re-dissolved in a small volume (100-200 µl) of ethanol prior to application to TLC plates (Section 5.1.b). The developing solvent (isopropanol: 0.88 ammonia:water, 10:1:1 (v/v/v)) was allowed to run 10 cm from the point of sample application. When dry, 1 cm zones scraped from the TLC plates were eluted three times with 0.5 ml ethanol. After centrifugation at 2000 g (MSE bench centrifuge) for 5 min, the pooled

eluates were reduced to dryness under a stream of $N_2$ and dissolved in 1 ml distilled water prior to bioassay.

Bioassay procedure for C-LS

The Amaranthus betacyanin bioassay (Biddington & Thomas, 1973) was used for the assay of C-LS because it was a very rapid assay requiring no special sterile precautions. Seeds of Amaranthus caudatus L. (Asmer Seeds Ltd., Leics.) sown in 16 x 10 cm plastic boxes on two layers of Whatmann 3MM filter paper moistened with distilled water, were allowed to germinate in darkness at 25°C for 72 h. The seed coats were removed and explants consisting of the uppermost portion of the hypocotyl plus the cotyledons were cut from the seedlings under dim green safe light. Sets of ten explants were transferred to 5 cm petri dishes containing three layers of Whatmann No. 1 filter paper, 1 ml 0.266M phosphate buffer pH 6.3 containing 1 mg $ml^{-1}$ tyrosine, and 1 ml of the test solution. The assay was incubated for 18 h in darkness at 25°C. At the end of the incubation period the explants were transferred to 3 ml distilled water. Betacyanin was extracted from the explants by freezing and thawing the samples twice and was estimated from the difference between the optical densities of the resultant solutions at 542nm & 620 nm (Bigot, 1968) measured on a Pye SP500 spectrophotometer. Figure 5.3 (page 116) shows the results obtained in the above bioassay procedure when standard solutions of BAP were used as test solutions.

5.1.d. Plant material

In an attempt to study the PGR's within the stem and to relate the results to those obtained in the culture of floral stem explants, sample stems were dissected into six explants, as described in Section 4.3.

The floral stem when in it's most responsive state *in vitro* (Section 4.1) has a maximum length of 25 mm, measured from the basal plate to the base of the flower, consequently when it is divided into six explants the amount of material per explant is very small in terms of fresh weight. Figure 4.11 (page 90) shows the mean fresh weight of the six floral stem explants. Extracts of the three explants 2N, 3N & T were pooled in order to keep the number of bulbs and the time required for dissection within practical limits. For similar reasons the total amount of material available from dissected floral stem, was used instead of using a standard fresh weight for all samples. It was therefore necessary to correct the PGR yields for fresh weight variations. To do this it was necessary to convert bioassay data for GA-LS and C-LS into ng equivalents of $GA_3$ and BAP respectively.

## 5.2. Results and discussion

### 5.2.a. GA-like substances

Figure 5.4.a. (page 117) shows the levels of biologically active 'free' acidic-ethyl-acetate-soluble GA-LS extractable from floral stem explants at their most responsive stage *in vitro*. There are differences in the Rf zones showing GA-LS activity between explant material, and although these differences show no marked trend along the stem, the results of a second experiment shown in Figure 5.4.b (page 117) are in broad agreement. In order to compare the concentrations of GA-LS per gram fresh weight it was necessary to convert the $\alpha$-amylase activity (Figure 5.4.a & b, page 117) into $ngGA_3$ equivalents (see Section 5.1.b) using the standard dose/response curve (Figure 5.2, page 115).

The yield of $ngGA_3$ equivalents per sample (Table 5.1, page 110) was very low and near to the limit of sensitivity of the barley half-grain bioassay. This was also noted in preliminary experiments, but could not be avoided due to severe limitations on the quantities of plant material available for PGR extraction. Nevertheless, there was found to be a consistent and significant increase in the $ngGA_3$ per gram fresh weight along the length of the stem (presented graphically in Figure 5.7.a, page 120). These results should be treated only as an indication that a gradient of GA-LS may exist within the floral stem of tulip because of the variation between

the two experiments. In order to verify these findings it will be necessary to obtain large quantities of material for extraction and/or to establish more sensitive detection methods.

5.2.b. Cytokinin-like substances

The activity of 'free' n-butanol soluble C-LS extractable from floral stem explants as assayed by the production of betacyanin in Amaranthus explants is shown in Figure 5.5.a & b (page 118). The concentration of C-LS was calculated in terms of ngBAP equivalents from the standard dose/response curve shown in Figure 5.3 (page 116). BAP was used as the test compound as it was the source of cytokinin used for the in vitro studies.

Although there were variations between replicates in the Rf zones showing C-LS activity (Figure 5.5. - a. cf b., page 118) the yields of ngBAP equivalents closely agree both in terms of absolute concentrations and in treatment effects (Table 5.5, page 111). The mean of the two experiments is shown in Figure 5.7.b (page 120). The erratic changes in C-LS concentration along the stem may reflect areas of cell division within the stem, as it is generally believed that cell division is stimulated by application of exogenous cytokinin (Miller, 1965).

### 5.2.c. IAA-like substances

The results of the fluorimetric assay of 2-methylindole-$\alpha$-pyrone in terms of the original IAA content of the extract are shown in Table 5.3 (page 112), together with their respective recoveries and fresh weights per sample. The resultant yields of ngIAA-LS per gram fresh weight averaged for the two replicates (Figure 5.7.c, page 120) show no significant variation between the stem pieces, therefore it appears that there is no gradient of IAA-LS along the floral stem. The results presented do not, however, preclude the possibility of changes in sensitivity of the tissue to IAA by varying stem explants. It has been suggested by De Munk (1979) that the tulip stem may have an endogenous sequence rhythm of development independent of applied auxin and possibly also of endogenous auxin. Such a response mechanism has been described by Söding (1936) as 'readiness for growth of the cells'. A change in auxin sensitivity may explain the effects observed with stem tissue *in vitro*. The auxin sensitivity may be controlled by an auxin co-factor or by another PGR.

### 5.2.d. ABA-like substances

The yield of substances with similar retention times to those of authentic Me-ABA and 2-trans-Me-ABA are shown in Table 5.4 (page 113). As the ECD on the GLC responded equally to both Me-ABA and its isomer the mean

total ngABA per gram fresh weight was calculated and is presented graphically in Figure 5.7.d (page 120), also shown are the separate means for the two isomers.

When exposed to ultra violet light Me-ABA is isomerised to its geometrical isomer 2-trans-Me-ABA and an equilibrium mixture containing approximately equal proportions of the two isomers is established. The 2-trans isomer of ABA is thought to occur naturally in only very small amounts (Saunders, 1978) however, in tulip floral stem extracts there appears to be larger amounts of this isomer than Me-ABA. Even if the purification of the extract had been conducted in the light, the amount of 2-trans isomers would not have exceeded that of the cis-trans isomer, because an equilibrium mixture would have been established. In contrast scale tissue extract contained only a small amount of the 2-trans isomer (Figure 5.6, page 119). This anomaly may be explained by the presence of an interfering substance co-chromatographing with the 2-trans-ABA, thus increasing the peak area. However, this explanation was not supported by the results obtained when the samples were exposed to daylight. The two peaks disappeared proportionately, and there was no apparent conversion of the peak corresponding to the Me-ABA to that of 2-trans-Me-ABA.

## 5.2.e. Discussion

The data for GA-LS and C-LS has been converted into ng equivalents of $GA_3$ and BAP respectively, for the reasons given in Section 5.1.d, but in view of the criticisms of Hill & Kimble (1969) and Reeve & Crozier (1974) on the use of ng $GA_3$ equivalents, great care should be exercised in drawing conclusions from such data. However, without positively identifying each component of the GA-LS & C-LS extract, and obtaining their dose/response curve for the $\alpha$-amylase & betacyanin assay respectively it is not possible to avoid the problems of non-parallism between dose/response curves of the unknown GA-LS or C-LS and that of $GA_3$ or BAP, and the problems of interacting compounds co-chromatographing with GA-LS's or C-LS.

Hanks & Rees (1980) have recently reported an in depth study of the GA-LS in field-grown tulips from planting until flowering. The tulip bulb/plant was divided into ten components, but the floral stem was examined as a whole, and consequently the results cannot be related to individual stem explants. However, it was reported that very much higher concentrations of GA-LS were present in the flower than the stem in unplanted bulbs. Therefore, this source of GA-LS could be responsible for the gradient of GA-LS in the stem that was observed in the present study.

The results presented in this chapter and their significance *in vitro* will be discussed further in Chapter 7.

Table 5.1.

Yield of ng $GA_3$ equivalents per sample and per gram fresh weight from floral stem tissues

Extract No. 1

| Stem section: | vb | b | N | T+ |
|---|---|---|---|---|
| ng $GA_3$ equivalents per sample | 0.66 | 1.93 | 1.09 | 1.60 |
| Fresh weight (g) | 3.0 | 3.0 | 1.6 | 2.0 |
| ng $GA_3$ equivalents per gram fresh weight | 0.22 | 0.64 | 0.68 | 0.80 |

Extract No. 2

| Stem section: | vb | b | N | T+ |
|---|---|---|---|---|
| ng $GA_3$ equivalents per sample | 1.08 | 2.27 | 1.76 | 3.31 |
| Fresh weight (g) | 3.0 | 3.0 | 2.0 | 2.6 |
| ng $GA_3$ equivalents per gram fresh weight | 0.36 | 0.76 | 0.88 | 1.27 |
| Mean ng $GA_3$ per gram fresh weight (extract 1 & 2) | 0.29 | 0.70 | 0.78 | 1.04 |
| | | L.S.D. = 0.13 | | |

Table 5.2.

Yield of ng BAP equivalents per sample and per gram fresh weight from floral stem tissues

Extract No. 1

| Stem section: | vb | b | N | T+ |
|---|---|---|---|---|
| ng BAP equivalents per sample | 68.3 | 8.06 | 57.0 | 2.64 |
| Fresh weight (g) | 3.0 | 3.0 | 1.6 | 2.0 |
| ng BAP equivalents per gram fresh weight | 22.8 | 2.69 | 35.6 | 1.32 |

Extract No. 2

| Stem section: | vb | b | N | T+ |
|---|---|---|---|---|
| ng BAP equivalents per sample | 29.8 | 12.5 | 27.4 | 2.61 |
| Fresh weight (g) | 3.0 | 3.0 | 2.0 | 2.6 |
| ng BAP equivalents per gram fresh weight | 9.94 | 4.16 | 13.7 | 1.00 |
| Mean ng $GA_3$ per gram fresh weight (extract 1 & 2) | 16.4 | 3.4 | 24.7 | 1.16 |

L.S.D. = 6.3

Table 5.3.

The yield of IAA-LS extractable from floral stem tissues

Extract No. 1

| Stem section: | vb | b | N | T+ |
|---|---|---|---|---|
| ng IAA per sample | 9.3 | 9.5 | 7.1 | 8.8 |
| % recovery | 56.4 | 59.5 | 56.9 | 62.0 |
| Corrected ng IAA per sample | 16.5 | 16.0 | 12.5 | 14.1 |
| Fresh weight (g) | 2.95 | 3.24 | 1.79 | 2.47 |
| ng IAA per gram fresh weight | 5.6 | 4.9 | 7.0 | 5.7 |

Extract No. 2

| Stem section: | vb | b | N | T+ |
|---|---|---|---|---|
| ng IAA per sample | 10.9 | 11.8 | 9.8 | 11.2 |
| % recovery | 42.8 | 49.3 | 44.6 | 47.7 |
| Corrected ng IAA per sample | 25.5 | 23.9 | 22.0 | 23.5 |
| Fresh weight (g) | 2.57 | 2.78 | 1.88 | 2.39 |
| ng IAA per gram fresh weight | 9.9 | 8.6 | 11.2 | 9.8 |
| Mean ng IAA per gram fresh weight (extract 1 & 2) | 7.8 | 6.8 | 9.4 | 7.8 |

L.S.D. = 1.9

Table 5.4.

The yield of ABA-LS extractable from floral stem tissues

Extract No. 1

| Stem section: | | vb | b | N | T+ |
|---|---|---|---|---|---|
| ng per sample | cis-trans | 47.8 | 37.0 | 36.8 | 53.2 |
| | 2-trans | 112.1 | 75.5 | 52.6 | 72.0 |
| Fresh weight (g) | | 2.95 | 3.24 | 1.79 | 2.47 |
| ng per gram fresh weight | cis-trans | 16.2 | 11.4 | 20.6 | 21.5 |
| | 2 trans | 38.0 | 23.3 | 29.4 | 29.1 |
| | Total | 54.2 | 34.7 | 49.9 | 50.6 |

Extract No. 2

| Stem section: | | vb | b | N | T+ |
|---|---|---|---|---|---|
| ng per sample | cis-trans | 36.8 | 49.9 | - | 45.8 |
| | 2-trans | 71.3 | 101.9 | - | 61.9 |
| Fresh weight (g) | | 2.57 | 2.78 | 1.88 | 2.39 |
| ng per gram fresh weight | cis-trans | 14.3 | 17.9 | - | 19.2 |
| | 2-trans | 27.7 | 36.7 | - | 25.9 |
| | Total | 42.0 | 54.6 | - | 45.1 |
| Mean ng per gram fresh weight (extract 1 & 2) | cis-trans | 15.3 | 19.6 | (20.6) | 20.3 |
| | 2-trans | 32.8 | 30.0 | (29.4) | 37.5 |
| | Total | 48.1 | 44.7 | (49.9) | 47.9 |

L.S.D. = 6.9

Figure 5.1.

The fluorescence emission spectrum of a typical floral stem extract as shown on a Perkin-Elmer Spectrophotofluorimeter model MPF 43A.

Excitation wavelength of 440 nm

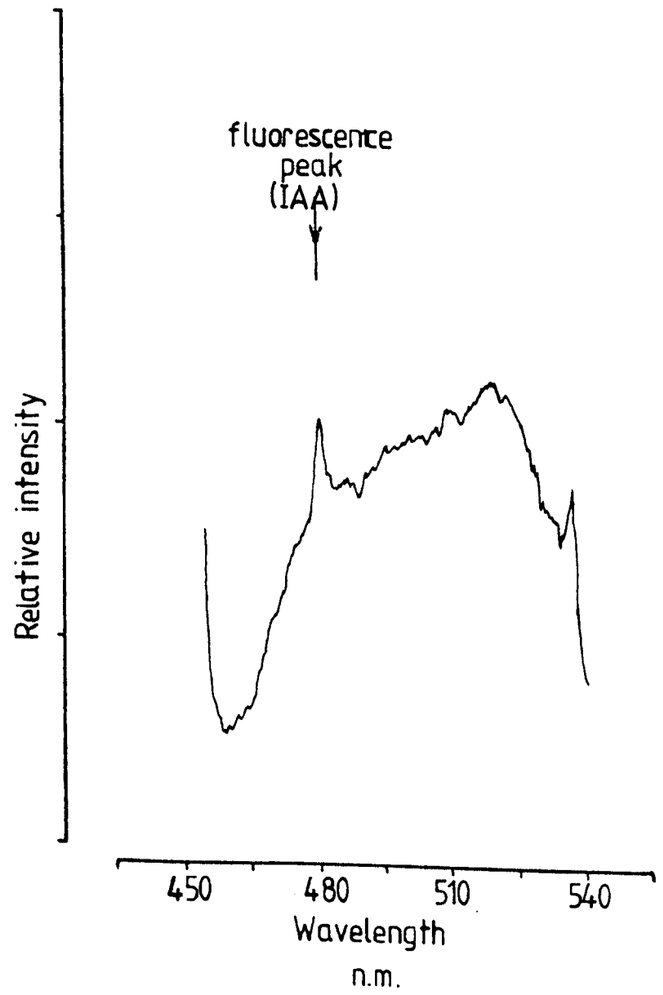


Figure 5.2.

Dose/response curve for the barley half-seed bioassay (Jones & Warner, 1967) $GA_3$ as test solution

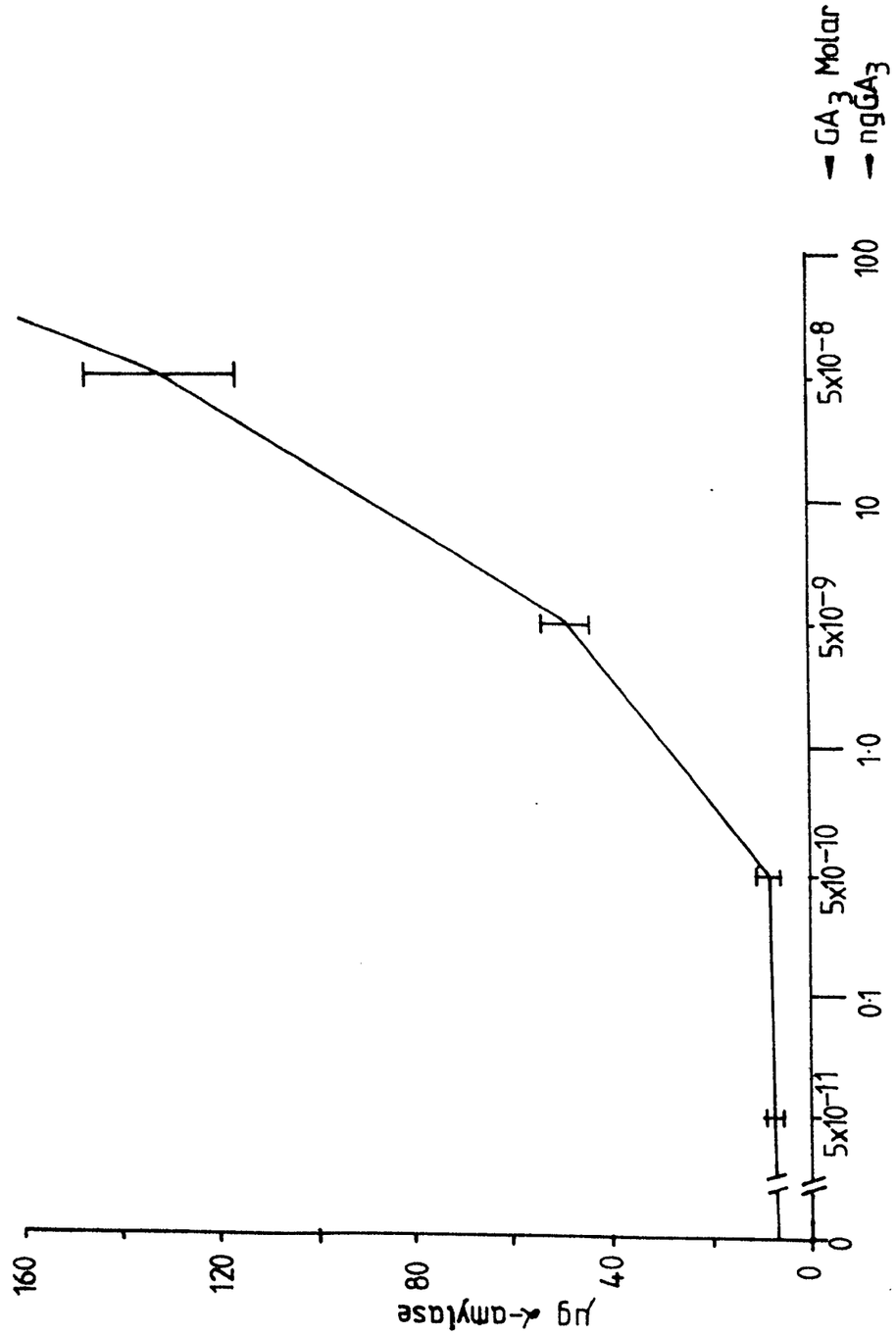


Figure 5.3.

Dose/response curve for the Amaranthus betacyanin bioassay (Biddington & Thomas, 1973) BAP as test solution

OD 620/542 = OD at 620 nm minus OD at 542 nm

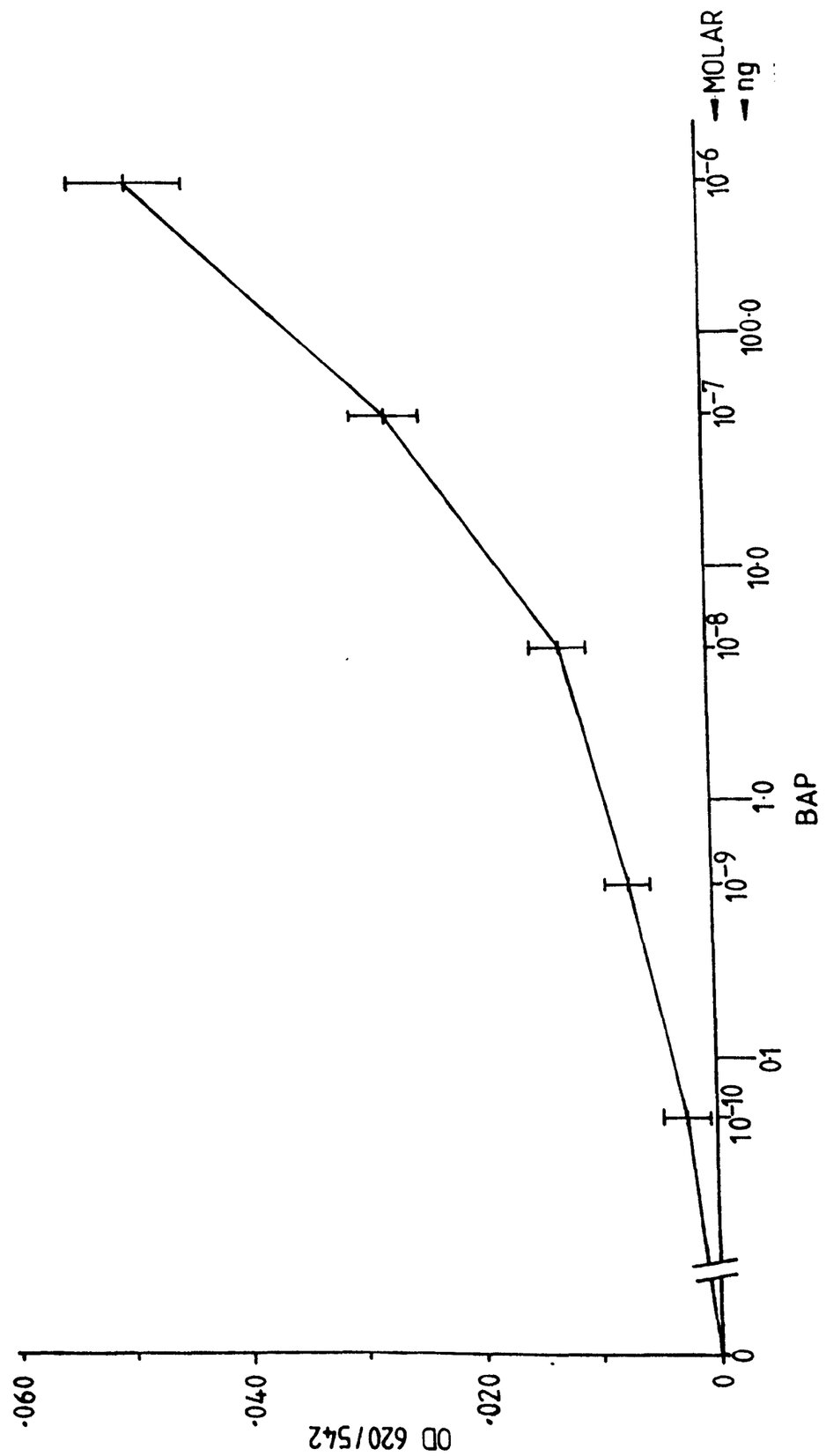


Figure 5.4. a, b.

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Levels of GA-LS showing biological activity in the barley half-seed bioassay, extractable from floral stem explants

▨ - Significant promotion at p=0.05 level

a) Extract No. 1

b) Extract No. 2

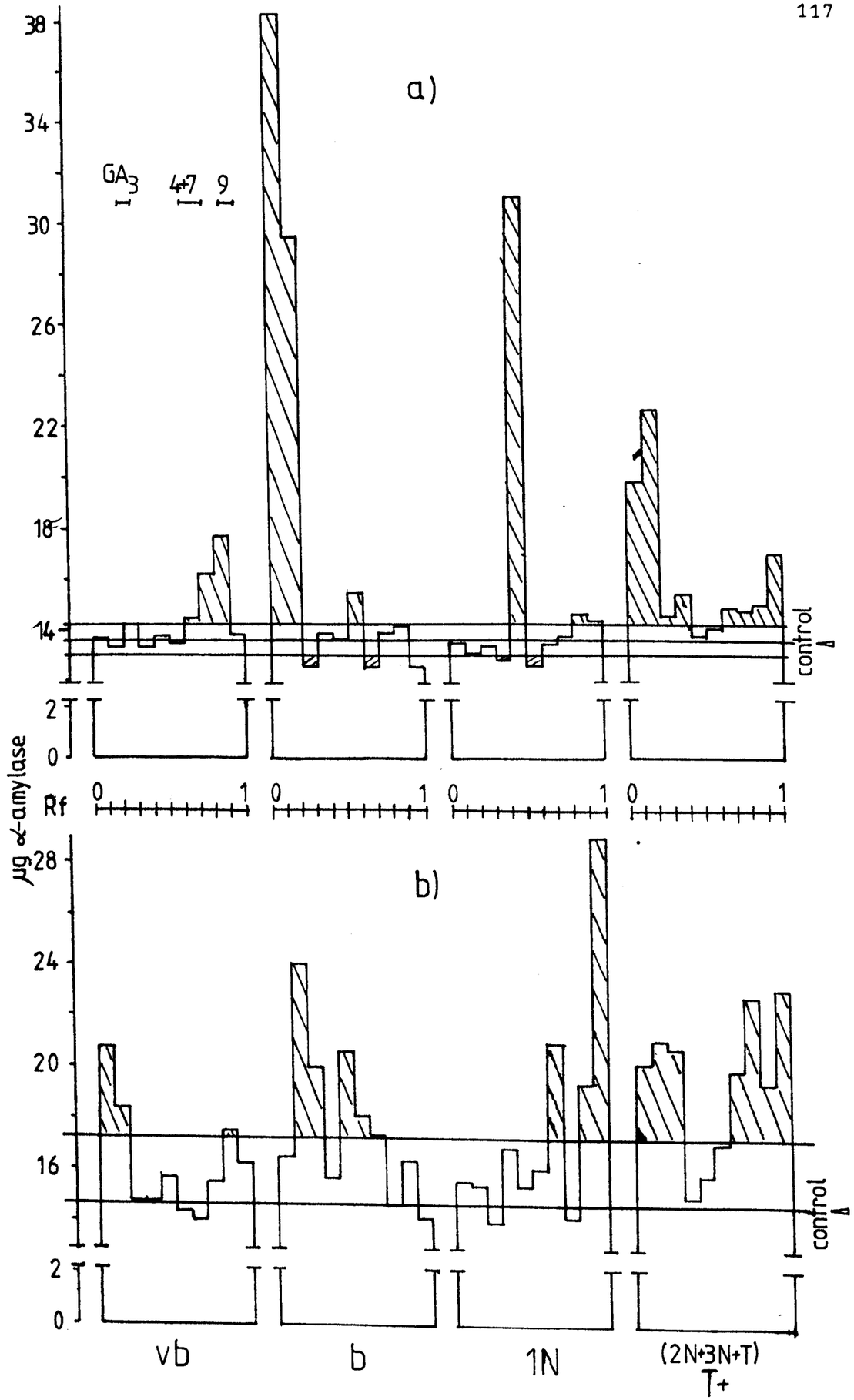


Figure 5.5. a, b.

Levels of C-LS showing biological activity in the Amaranthus betacyanin bioassay, extractable from floral stem explants

OD 620/542 = OD at 620 nm minus OD at 542 nm

▨ - Significant activity at p=0.05 level

a) Extract No. 1

b) Extract No. 2

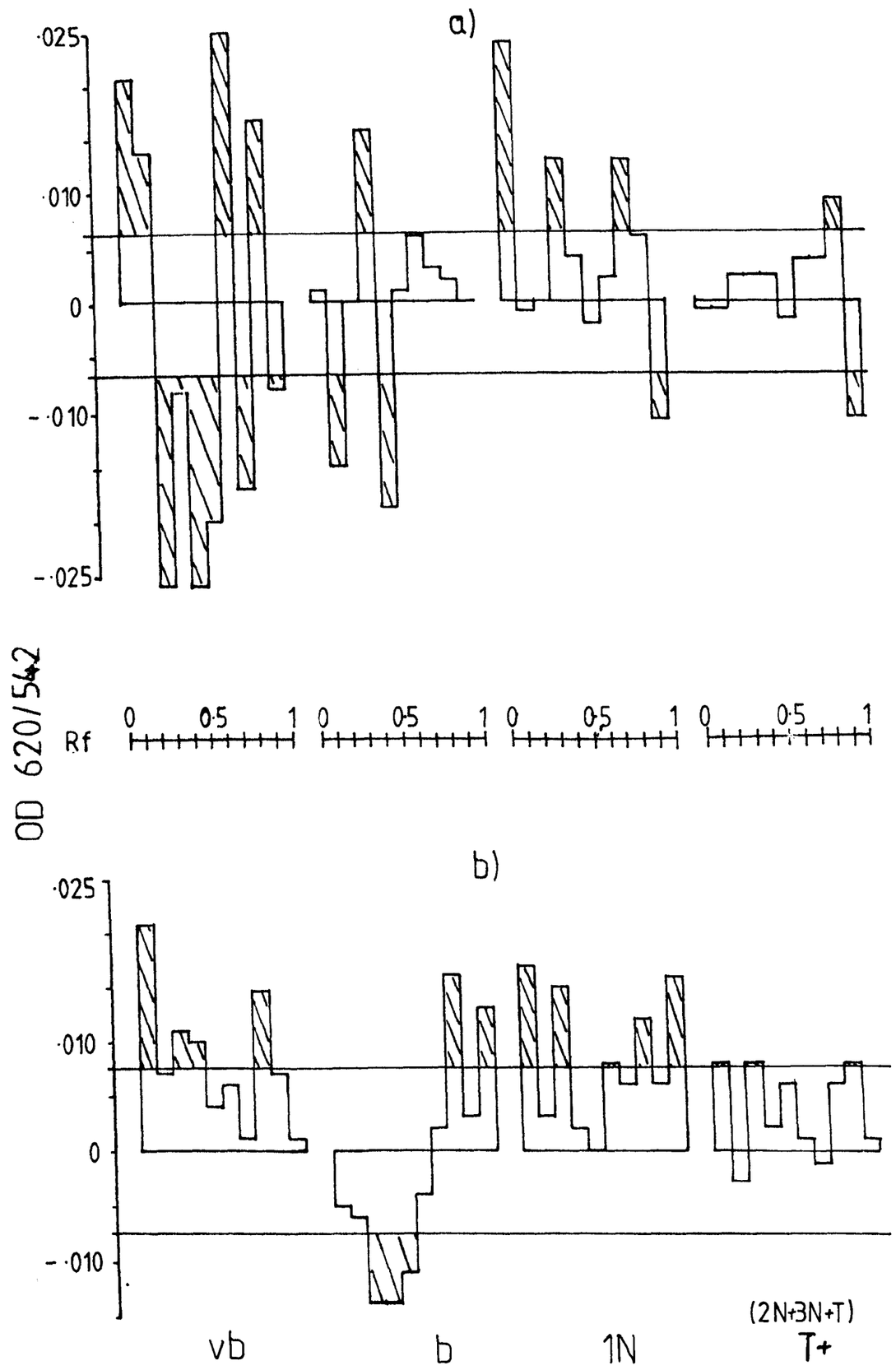


Figure 5.6.

Gas Liquid Chromatography (GLC) traces of typical extracts of tulip, samples of approximately 0.1 gram fresh weight

a) Scale tissue extract

b) Floral stem tissue extract

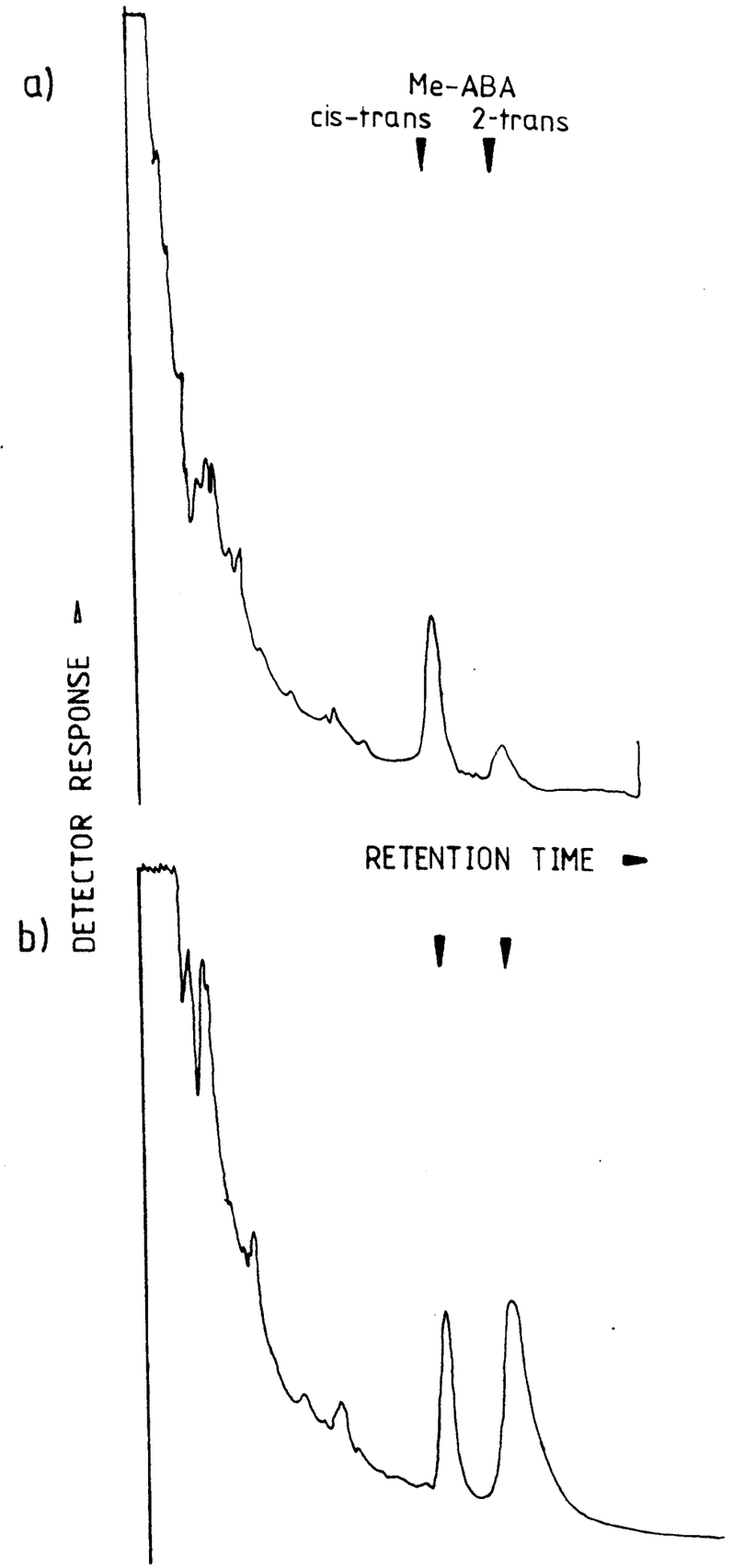


Figure 5.7. a, b, c, d.

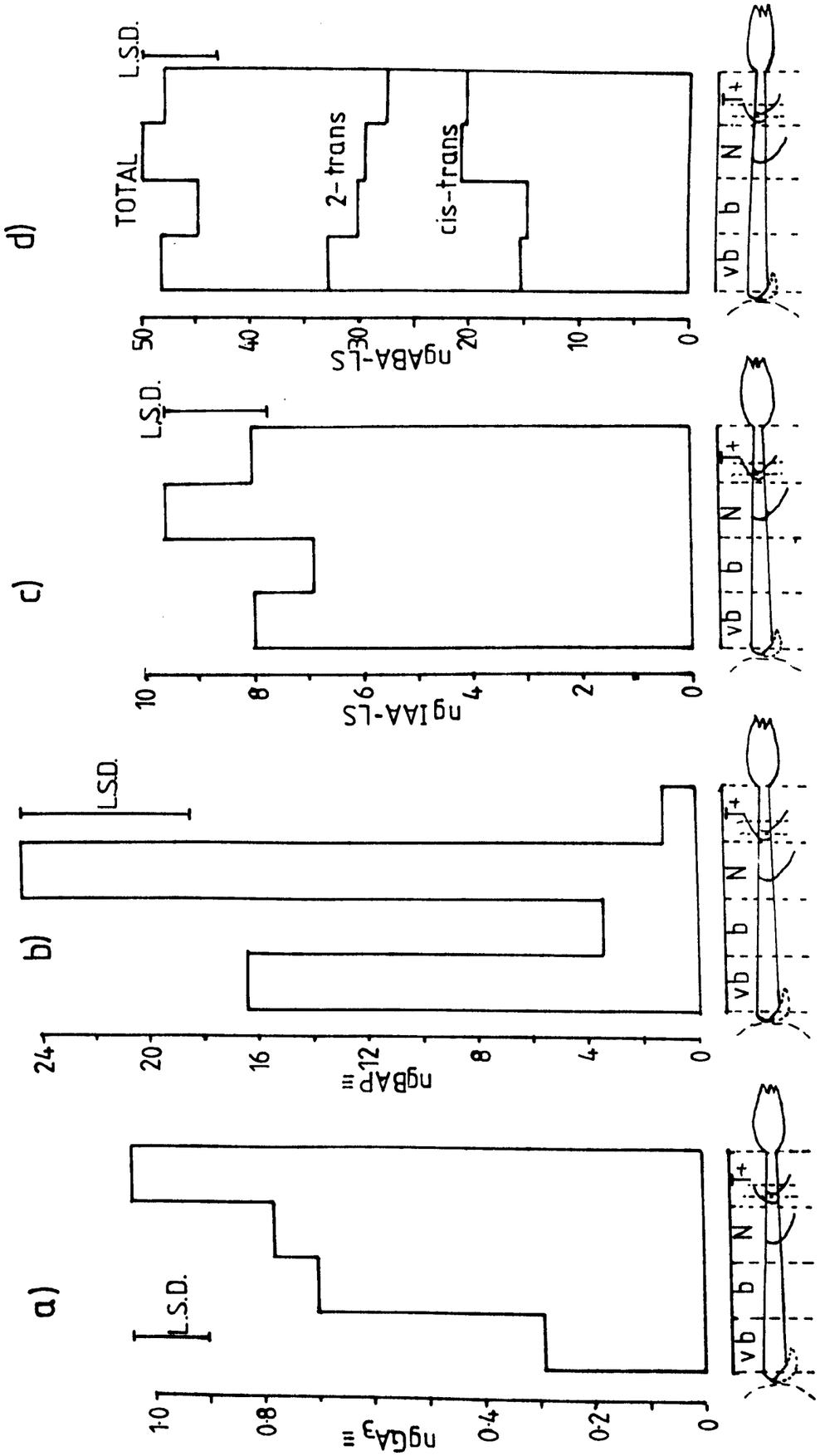
PGR levels extractable from floral stem explants
Mean of two experiments in each case

a) ng$GA_3$ equivalents per gram fresh weight

b) ngBAP equivalents per gram fresh weight

c) ngIAA-LS per gram fresh weight

d) ngABA-LS per gram fresh weight



Chapter 6

# Morphological and Histological Studies

Bancillion (1974) showed that shoots could be obtained on tulip floral stem 'fragments', after a six month culture period, but no bulb or root formation was reported. A histological study was therefore conducted to establish whether the cultures produced in the present investigation (Chapter 4) had the potential to develop into whole plants.

Hussey (1976b) showed that plantlets of Ornithogalum which had arisen from the surface cell layers of parent tissues, consisted of diploid cells. Conversely, callus of shoots arising from cortical cells contained tetraploid cells. He proposed that the cortical cells contained polysomatic cells whereas those of the surface were strictly non-polysomatic. If shoots formed on floral stem tissues of tulip arise from parenchymatous, cortical cells the resultant plants could vary in ploidy thus yielding 'non-true-to-type' plants. It was necessary therefore to determine the cellular origin of the shoots in order to assess whether the procedure developed for shoot formation (Chapter 4) was likely to result in the formation of propagules of variable ploidy.

## 6.1. Materials and methods

Floral stems were fixed in formalin-acetic acid-alcohol (FAA) at two week intervals during their growth and development *in vitro*. After a minimum fixation period of 24 hours the tissues were dehydrated in an ethanol series, and embedded in polyester wax, or dehydrated in ethanol, transferred to chloroform and subsequently embedded in paraffin wax (Figure 6.1, page 128). Embedding was carried out as described by Berlyn & Miksche (1976). In brief, wax was melted in an oven at 5°C above its m.p. (m.p. polyester wax 37°C, paraffin wax 57°C) and subsequently poured into 10 ml or 50 ml glass jars. The solvent containing the sample tissue was added and allowed to mix with the wax whilst it was being re-melted. Following infiltration for a minimum of 24 h in two changes of pure wax the explants were cast into a mould and stored until required for sectioning. The blocks of wax were trimmed, attached to oakwood blocks and mounted on a Cambridge Rotary Microtome type 52164. Serial sections 7 µm thick were cut, attached to glass slides using a gela tine adhesive and stained according to the procedure described in Figure 6.2 (page 129). If required, sections were made permanent using D.P.X mountant (BDH Chemicals Ltd.) and cover slips (glass size 1).

A minimum of three explants were sectioned serially at each of the ten stages of development, yielding approximately 15,000 sections.

Sections were examined using a Vickers M17 photomicroscope, and observations were recorded on Kodak plus-X pan film.

At different stages of development *in vitro* representative explants were photographed using a 35 mm camera system to record the gross morphology of cultured tissues.

Photography was performed under aseptic conditions in the laminar flow clean air cabinet in order to maintain the cultures in a viable state.

## 6.2. Results and discussion

Expansion and extension growth of explants occurred within a few days of culturing. During a similar period starch grains, which had been present in large numbers in the cells of the original explant, disappeared (Plate 6.1.a page 130 cf. Plate 6.1.b page 130). Their disappearance could be explained by their utilization for expansion and extension growth of the explants. When placed in culture the explants were yellow in colour, however, by the second week of incubation some explants were green, i.e. had formed considerable amounts of chlorophyll; the upper stem explants having developed the darkest green colour. In contrast the mid/lower stem explants remained yellow. Explants from the base of the stem were either yellow or had turned white. Chloroplasts were observed in the outer cell layers of the green explants (Plate 6.1.c page 130).

The onset of organogenesis in floral stem explants was indicated by the breakdown of the ordered palisade layer of the epidermis. This appeared to be caused by both periclinal and anticlinal cell divisions which occurred during the first six weeks in culture (Plate 6.2.a page 131). By the tenth week meristematic protuberances had developed (Plate 6.2.b page 131) some of which were visible macroscopically (Plate 6.3.a page 132).

Observation of the further growth of shoots at two stages of development (Plate 6.3.b & c page 132) indicated that the

number of shoots developing was only a small proportion of the shoot initials on each explant, a phenomenon observed by Hussey (1976a) with Iris stem in vitro. There would appear to be a dominance mechanism; when a few shoots have developed, other surrounding shoot initials are inhibited from further development. This type of inhibition follows one of the theories of apical dominance (Phillips, 1975). It may be possible therefore to obtain outgrowth of a larger proportion of shoot initials by the early sub-culture of explants onto fresh media, i.e. by replenishing the supply of cytokinin. Results of shoot sub-culture are discussed briefly in Chapter 7.

After approximately 16 weeks in culture the shoot-like structures appeared to be only loosely associated with the original explants (Plates 6.3.d, page 132; 6.4.a & b, page 133) and distinct growing centres were visible at their basal ends (Plate 6.3.d, page 132). These meristematic growing centres may mark the onset of bulb formation. It was clear that these shoot-like structures formed on floral stem tissues had become, or had the potential to become individual plants. The loose association with the original explant tissues suggests that they may have been formed by embryogenesis, as shown for Iris & Asparagus callus (Reuther, 1977a) and stem (Reuther, 1977b).

Further evidence to suggest that the shoot-like structures formed on floral stem explants have the potential

to develop into complete plants was obtained from extended incubation of some morphogenic cultures. After periods of approximately six months in culture, bulb-like structures developed (Plate 6.4.c & d, page 133) from the base of some of the shoots whilst the associated green leaf senesced.

In Chapter 3 basal enlargement of the explants was described as basal expansion and not as basal callus in order to distinguish between loose, friable callus and the expansion enclosed within an epidermal cell layer (i.e. with a smooth surface). Plate 6.5.a & b (page 134), show that the large basal expansion of these explants was caused by cell divisions in the cortical cells i.e. well under the epidermal cell layer. Since these cell divisions occur in parenchymatous cells the new tissue produced is callus as defined by Thorpe (1980), even though it is contained within the epidermal cell layer.

On some of the media containing high levels of auxin (Chapter 4 - media M[4:4], M[4:1], , M[4:0.25]) large amounts of callus were formed on some of the floral stem explants (Plate 6.5.c, page 134). This callus formation appeared to be a continuation of the basal expansion cell division described above, except that the epidermal layer was no longer intact.

It was observed that explants showing a large basal expansion did not develop any shoots. However, the production of callus in large quantities on floral stem explants usually resulted in the later formation of roots. Occasionally, root-producing cultures also initiated shoots (Plate 6.6.a, page 135) however, there was no vascular connection between the shoots and the roots, as the root arose from the cortical cells (Plate 6.6.b, page 135) and the shoots arose from the epidermal cell layer, as shown above.

It is important that the adventitious shoots arise from the epidermal cell layer because these cells contain fewer polyploid cells (D'Amato, 1975) than those of the cortex. Consequently, as discussed in Chapter 1, the resulting propagules are more likely to be genetically stable.

Figure 6.1.

Procedure for the dehydration and embedding of tissues used in histological studies

Polyester Wax

1) Sample fixed in FAA:

5% (v/v) formaldehyde solution 40% (w/v)
45% (v/v) ethanol 100% (v/v)
5% (v/v) acetic acid glacial A.R.
45% (v/v) water distilled

2) Dehydration through ethanol series

| | | | |
|---|---|---|---|
| 50% (v/v) | ethanol:water | 1 h | |
| 60% (v/v) | " " | 1 h | |
| 70% (v/v) | " " | 2 h | |
| 80% (v/v) | " " | 2 h | |
| 90% (v/v) | " " | 2 h | |
| 100% (v/v) | ethanol | 2 h | 2 changes |

3) Infiltration in polyester wax

Paraffin Wax

1) As for Polyester Wax

2) As for Polyester Wax

| | | | |
|---|---|---|---|
| 3) | 2:1 ethanol:chloroform | 2 h | |
| | 1:2 " " | | |
| | 100% (v/v) chloroform | 2 h | 2 changes |

4) Infiltration in paraffin wax

Figure 6.2.

Procedure for de-waxing and staining thin sections for histological study as described by Sass (1958)

Polyester Wax

1) De-wax in 100% (v/v) ethanol

2) Hydrate in 70% " ethanol:water
50% " " "
30% " " "
Water distilled

Stain aqueous safranin (12-24 h)
Water change until colourless
30% (v/v) ethanol:water
50% " " "
70% " " "
95% " " "

Stain fast green in 95% (v/v) ethanol (5-30s)
100% (v/v) ethanol - 3 changes
Carbol-xylene 25% (v/v) melted c.p. phenol in xylene
100% (v/v) xylene - 3 changes

3) Make permanent with D.P.X. mountant

Paraffin Wax

1) De-wax in 100% (v/v) chloroform

2) Transfer to ethanol
2:1 (v/v) chloroform:ethanol
1:2 " " "
100% " ethanol

- continue as for polyester wax 2) & 3)

Plate 6.1

Photomicrographs of transverse sections of floral stem explants

Bar represents 100 μm

a) (i & ii) At culture initiation

Note: Many starch grains

b) (i & ii) After incubation for 2 weeks

Note: Few starch grains

c) After incubation for 4 weeks

Note: Chloroplasts in outer cell layers

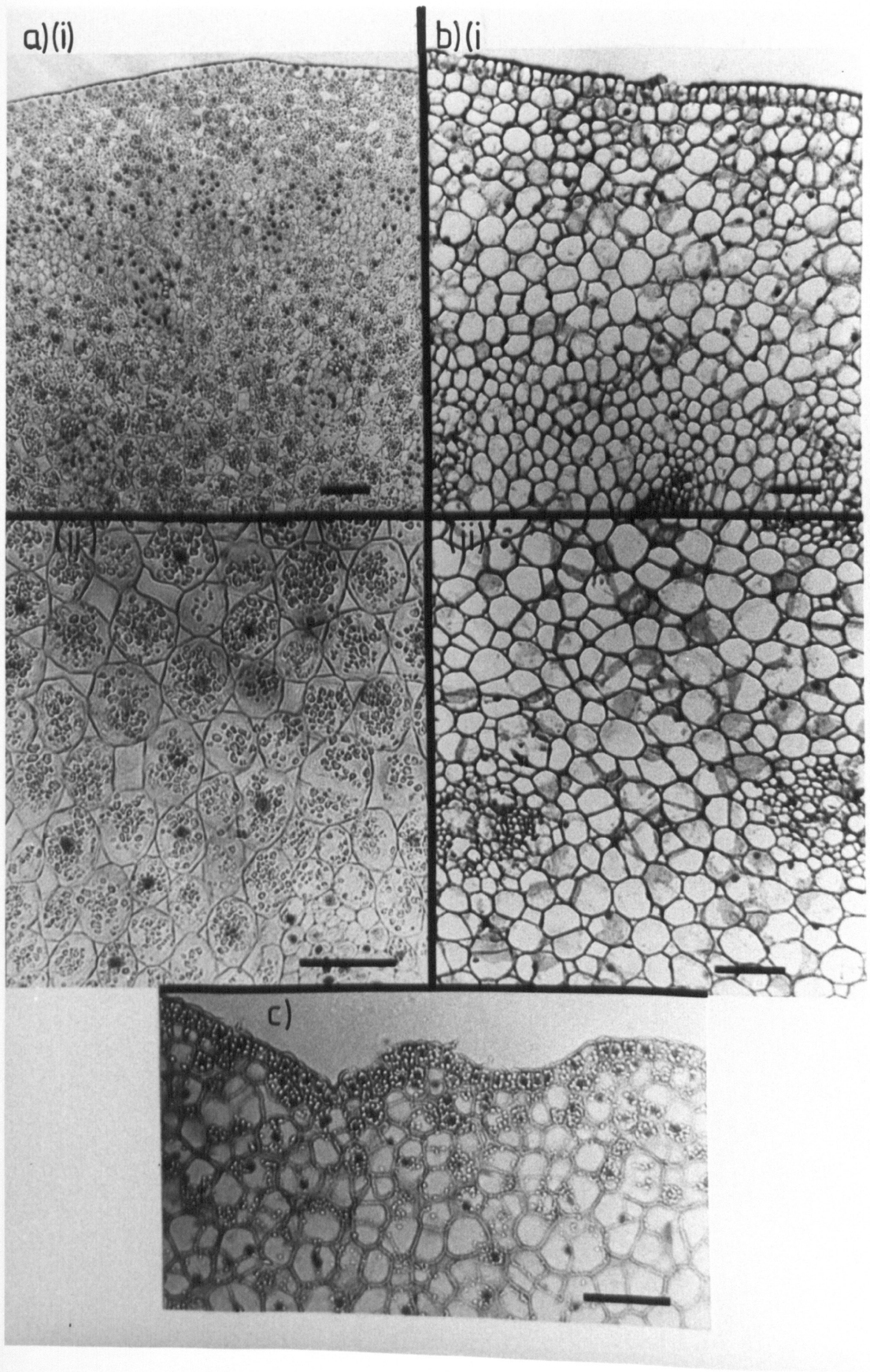


Plate 6.2

Photomicrographs of transverse sections of floral stem explants

a) After incubation for 6 weeks

(i) Bar represents 50 μm

(ii) Bar represents 100 μm

b) After incubation for 10 weeks

(i) Bar represents 100 μm

(ii) Bar represents 50 μm

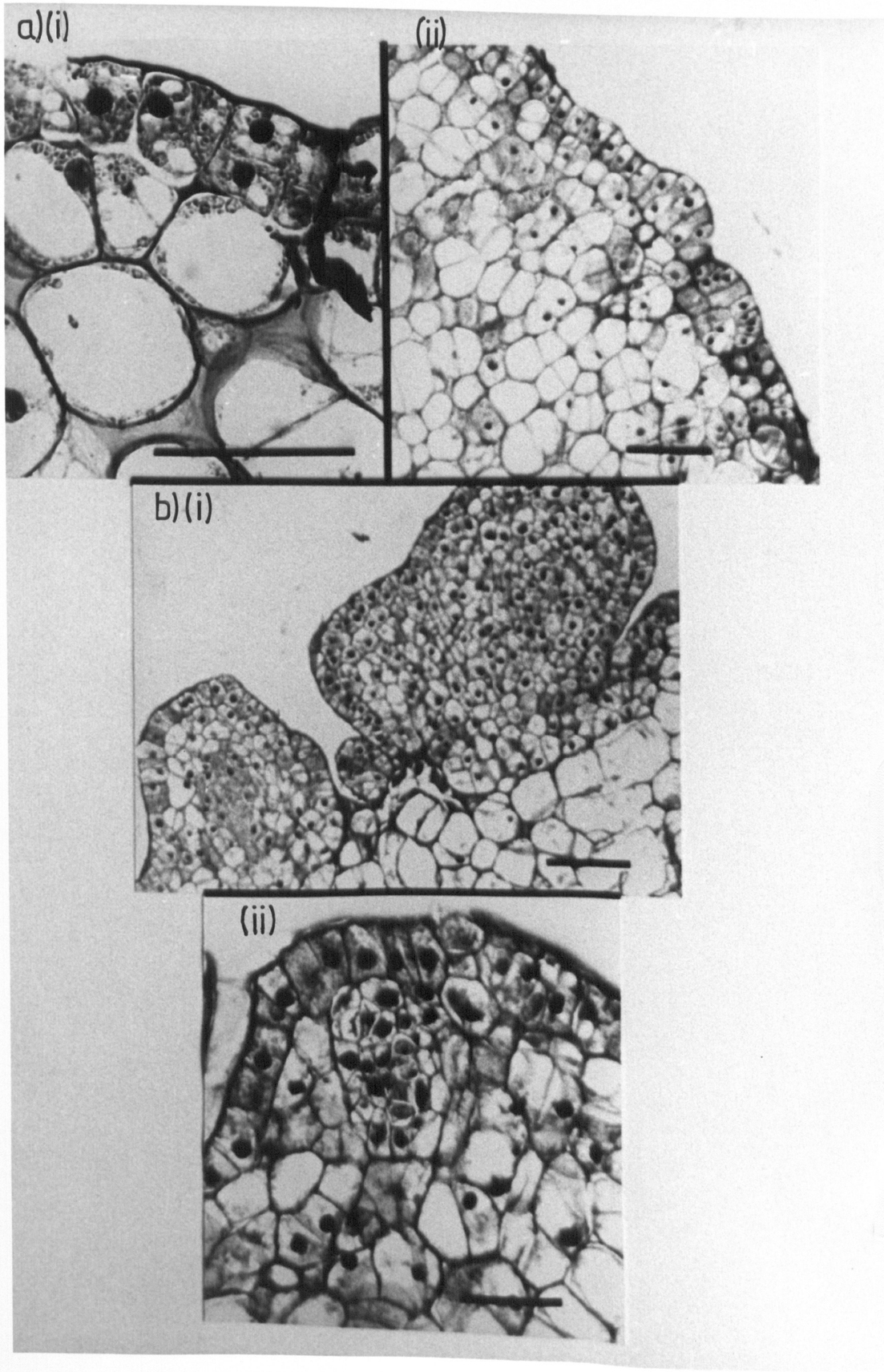


Plate 6.3

Photographs of floral stem explants:

a) After incubation for 10 weeks

Bar represents 0.5 cm

b) After incubation for 12 weeks

Bar represents 1 cm

c) After incubation for 16 weeks

Bar represents 1 cm

d) Photomicrograph of a transverse section of a floral stem explant after incubation for 16 weeks

Bar represents 100 μm

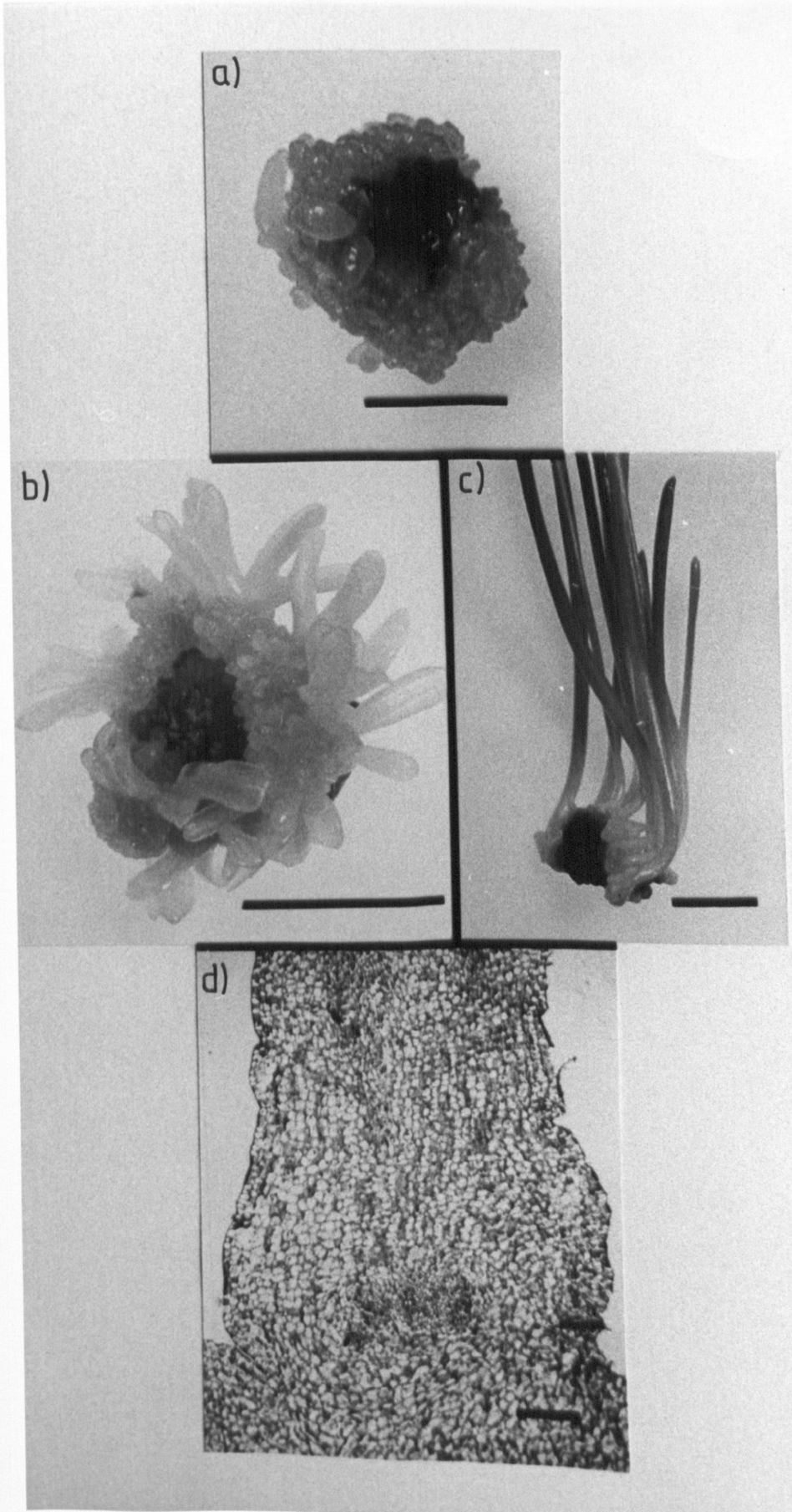


Plate 6.4

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Loosely associated shoots on floral stem explants

a) Photograph after incubation for 14 weeks

Bar represents 1 cm

b) Photomicrograph - transverse section

Bar represents 100 µm

Bulb-like structures formed on floral stem explants after incubation for approximately 6 months

c) Photomicrograph - transverse section

Bar represents 100 µm

d) (i & ii) Photographs

Note: Senescence of leaf-like structure

Bar represents 1 cm

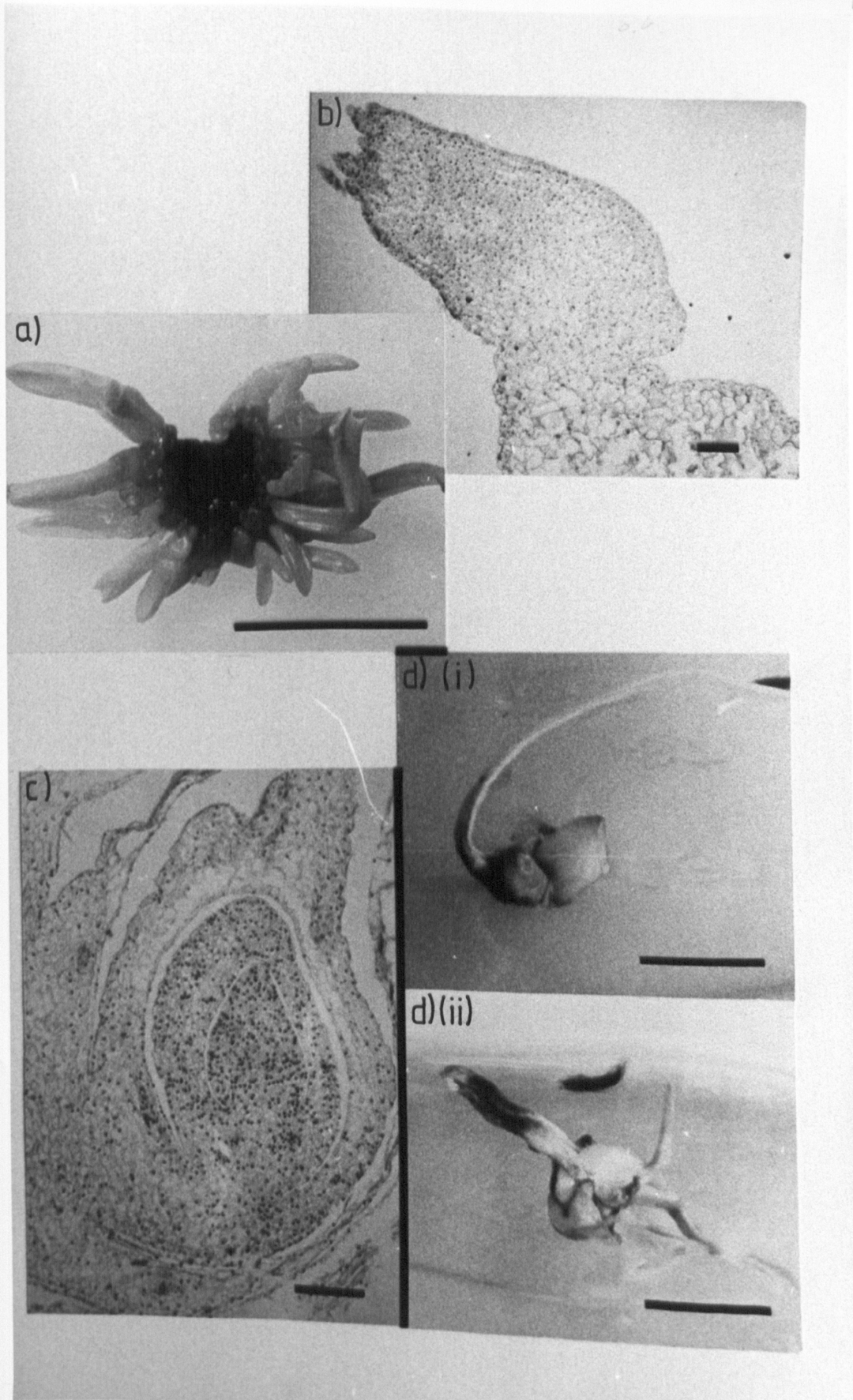


Plate 6.5

a) Photographs of basal expansion occuring on large floral stem explants (Chapter 4)

Bar represents 0.5 cm

(i) After 6 weeks incubation
(ii)After 12 weeks incubation

Note: Large explant - no shoot production

b) Photomicrograph of a transverse section of a floral stem explant undergoing basal expansion after incubation for 6 weeks

Bar represents 100 μm

c) Photograph of callus formed on a floral stem explant incubated on a high auxin medium for 16 weeks

Bar represents 0.5 cm

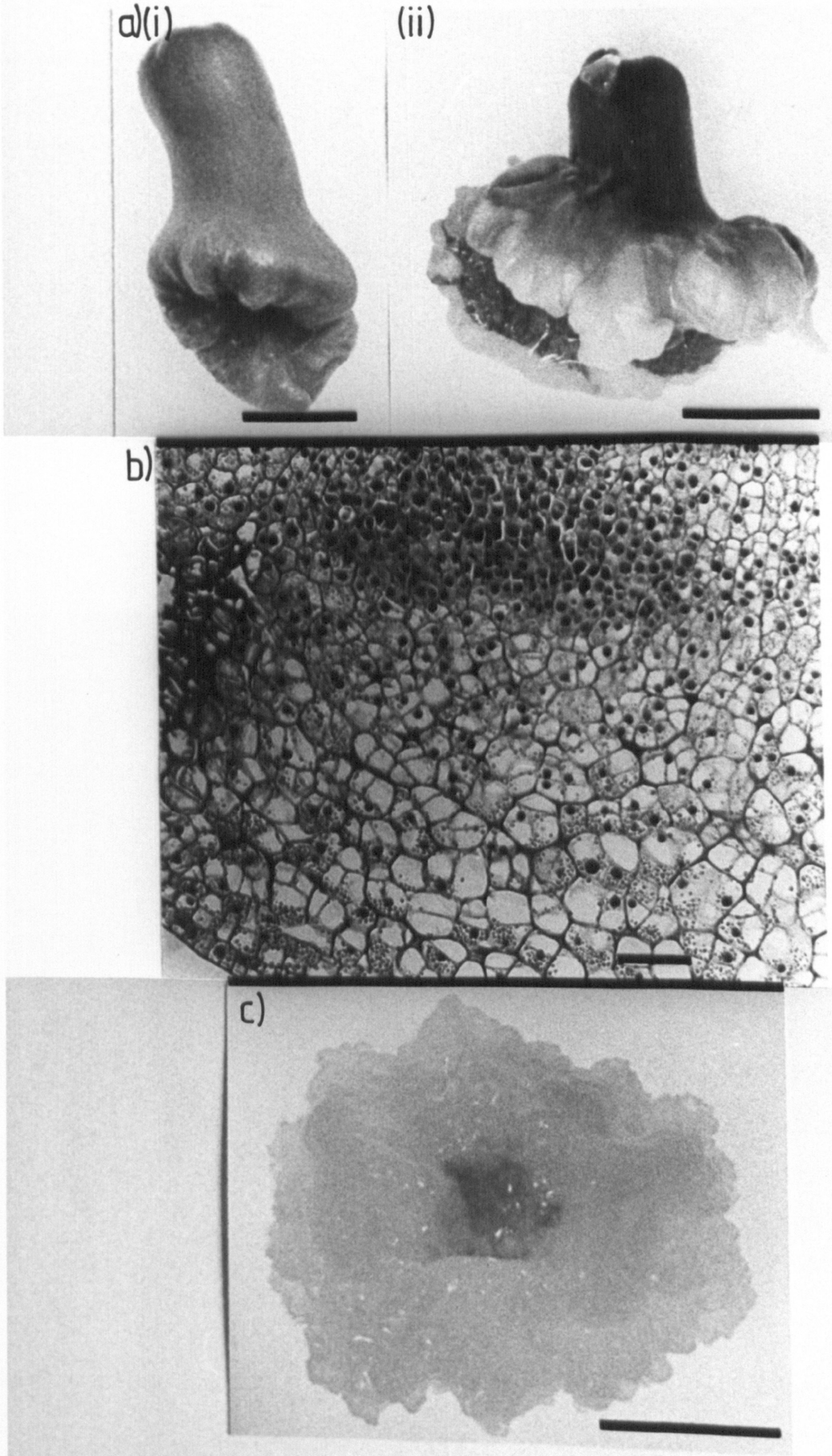


Plate 6.6

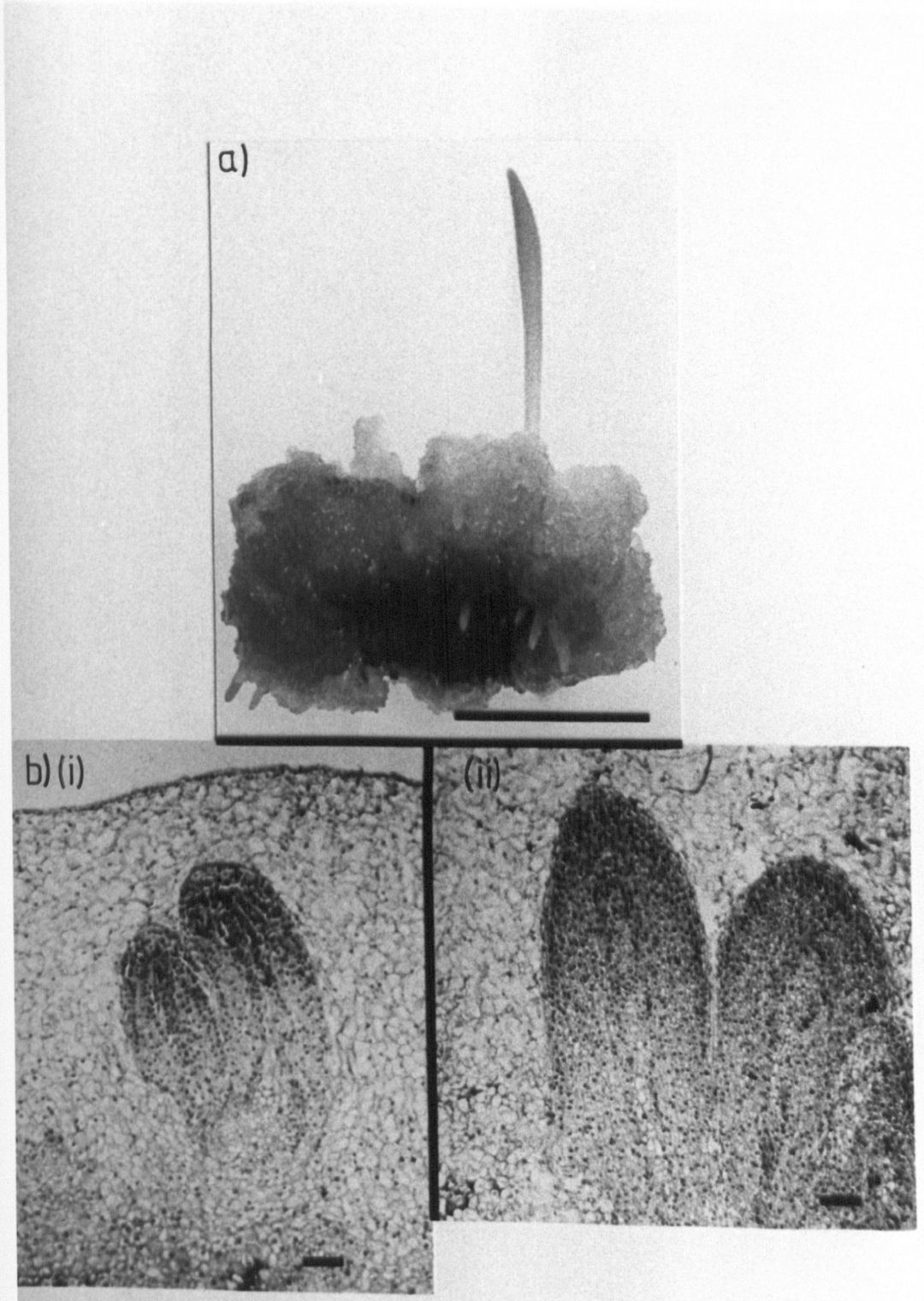
a) Photograph of callus, root and shoot production on a floral stem explant incubated on a high auxin medium

Bar represents 1 cm

b) (i & ii) Photomicrographs of transverse sections of floral stem explants incubated on a high auxin medium

Bar represents 100 μm

Note: Roots arise from the cortex



# Chapter 7

## General Discussion

A high level of shoot production on the majority of floral stem explants was obtained on medium M [1:1], irrespective of the stage of development of the dry bulb (Chapter 4). In contrast to other bulbous plants (Hussey, 1976 a & b) the presence of a cytokinin in the medium was found to be essential for the production of shoots on tulip. However, Ziv et al. (1970) reported that 0.5 mg $l^{-1}$ kinetin was necessary for optimum shoot production in Gladiolus. Seabrook et al. (1976) showed that 10 mg $l^{-1}$ BAP in the presence of 1 mg $l^{-1}$ NAA was necessary for optimum shoot production on floral stem explants of an unnamed cultivar of Narcissus, whereas the cultivars Geranium & Fortune only require 5 mg $l^{-1}$ in combination with 1 mg $l^{-1}$ NAA (Hosoki & Asahira, 1980). Similarly, Iris has a requirement for the presence of 5 mg $l^{-1}$ PBA, in the presence of 1 mg $l^{-1}$ NAA, for optimum shoot production (Pierik & Steegmans, 1975).

The requirement of tulip floral stem tissue for the presence of cytokinin in the medium could partially explain the high shoot production by 1N explants, when compared to that of other stem explants. The results presented in Chapter 5 show that the levels of C-LS extractable from 1N stem sections were higher than those extractable from any other floral stem section. However, the presumed deficit in cytokinin cannot be replaced by exogenous application of cytokinin, and the above hypothesis does not account for the low shoot production on vb stem

sections. The vb sections produce few shoots despite the level of endogenous C-LS being only slightly lower than that of stem section 1N. It appears therefore that the shoot forming properties of stem explants are determined by a balance of the endogenous and exogenous PGR's.

The levels of both ABA-LS and IAA-LS extractable from floral stem were found to be similar in all explant types tested (Chapter 5). This leads to the conclusion that the levels of GA-LS (in conjunction with C-LS) are involved in the regulation of shoot initiation in tulip. But, even though GA-LS stimulate the growth of organs, they can also inhibit organ initiation (Murashige, 1963; 1964; Schrandolf & Reinert, 1959). Thus the higher concentration of GA-LS reported in Chapter 5 for explant 1N (compared to vb) is unlikely to be responsible for the higher shoot production of explant 1N.

Thorpe (1980) proposed that the inhibition of organ formation in tobacco callus by exogenously applied GA's resulted from the presence of supraoptimal levels; the callus itself being able to synthesize or produce sufficient for the organogenetic processes. Tobacco (Thorpe & Murashige, 1970) rice (Thorpe, 1974) and tomato (Bonnett & Torrey, 1965) all accumulate starch in their tissues during shoot induction, which is subsequently utilized during organ formation. The inhibitory effects of $GA_3$ on organogenesis in all these tissues have been cor-

related with the reduction in starch accumulation (Murashige, 1974). Conversely, in Chrysanthemum (Earle & Langhaus, 1974) and Arabidopsis (Negrutiu et al., 1978), exogenously applied GA was shown to stimulate organ formation. One hypothesis proposed by Coleman & Greyson (1977) to explain these results was that $GA_3$ may stimulate IAA biosynthesis. This hypothesis was based on data showing that $GA_3$ can stimulate rooting of tomato leaf discs in the presence of presumed IAA precursors.

Lance et al. (1976) reported that both the level and the spectrum of endogenous GA-LS change during shoot formation, and they suggested that GA-LS are involved not only in normal growth of tissues but also in their differentiation.

The results presented in Section 4.1.d show that the addition of exogenous $GA_3$ did not result in any increase in shoot production on floral stem explants of tulip. However, these experiments were conducted using only one type of explant, so in order to fully elucidate the role of GA-LS in the organogenesis of tulip floral stem explants, it would be necessary to conduct experiments using a greater number of explant types.

Although the methods used for the extraction, purification and assay of PGR's are well documented and widely used, serious criticisms of them have been made (e.g. Letham et al., 1978). These include the inadequacy of

extraction procedures, the lack of measurement of losses occurring during purification, and the specificity of the various bioassay systems. For example Browning & Saunders (1977) reported the extraction of 1000 times more GA-LS when extracting with a detergent than by the'accepted' method using methanol. Throughout this work, internal radioactive standards were used (where available) for the calculation of PGR recoveries, and physico-chemical methods of detection were used, in an attempt to reduce the errors which may result from PGR extraction and assay. However, for the reason stated above and those discussed in Chapter 5, the results of PGR analysis and the conclusion drawn from them can be only very tentative.

There are many other factors which may interact with PGR levels in determining the response of plant tissue to 'organogenetic' stimuli. These may include media constituents (amino acids, carbohydrates, vitamins, charcoal, etc.), the genetic determinant, the physiological stage of the mother plant, and the intertissue and intercellular correlations (Tran Thanh Van, 1980).

The effects of explant dissection (Section 4.3) on the response of tissues <u>in vitro</u> may be caused, at least in part, by the release of compounds into the culture medium from damaged cells. The formation of IAA at the cut surfaces may be responsible for initiating cell division, as suggested by Yeoman (1970), from the evidence of Sheldrake & Northcote (1968). Some support to this hypothesis

was gained from the results of experiments conducted with large explants. When explants were damaged by scoring with a scapel blade, those scored vertically produced more expansion 'callus' growth, and those scored horizontally more shoots than control cultures. A similar response to wounding was observed by Hackett (1969) using Lilium scale explants. However, the scoring procedure used above was very tedious and of little practical value.

Tran Thanh Van (1980) has shown a strong correlation between both intercellular and intertissue cell-to-cell contact and the control of morphogenesis. He suggested that gradients of stimulatory and/or inhibitory substances at the intra-cellular level may be a determining factor in morphogenesis. The techniques required to test this hypothesis are not yet available, and although radioactive tracer and autoradiographic procedures have been used (Stodart, 1981; Venis, 1981) their use was considered to be beyond the scope of this thesis.

The influence of season on the response of tulip bud, scale and floral stem in vitro (Chapter 3) is similar to that observed by Robb (1957) and Takayama & Misawa (1980) using Lilium. These effects may be the result of variations in endogenous PGR levels, as it has been shown that these can vary considerably during the seasonal growth of tulip bulbs (e.g. Hanks & Rees, 1980).

Gilford & Rees (1973) reported that stem and leaf extension after planting the bulb were almost entirely due to the elongation of cells produced from earlier cell divisions. Active cell division occurring in floral stems during bulb storage may account for their responsiveness *in vitro* during this period. The reported marked rise in the endogenous level of GA-LS (Hanks & Rees, 1980) in the tulip stem after planting could be the reason for the lack of reaction from floral stem tissues *in vitro* (i.e. corresponding to the supraoptimal levels of GA-LS as discussed above). However, the decline in GA-LS reported in later stem growth was not reflected by an increase in response *in vitro* (Section 3.4). More information on the changes in endogenous PGR's and the occurrence of cell division within the floral stem *in vivo* may reveal whether they are causally related, and may lead to the induction of cell division *in vitro* beyond the 'responsive period'.

Another explanation of the sudden cut-off in shoot production after bulb planting may be the greening of floral stems. This occurs rapidly after planting and has considerable effects at the biochemical and cytological levels (Smith, 1975). Preliminary experiments conducted to examine this hypothesis have revealed that floral stem explants, from etiolated tulip bulbs, are very responsive *in vitro*. Many of these explants from 20-25 cm stems produced large quantities of yellow callus and some sub-

sequently produced shoots. The results from these preliminary experiments showed that much larger floral stems than those used in Chapter 4 can be induced to respond *in vitro* and that this could be a rewarding area for further research.

Pierik & Steegmans (1975) have shown that the light environment has a considerable effect on regeneration from floral stem explants of *Freesia in vitro*. In the present study, dark-incubated tulip explants, initiated shoots 1-2 weeks earlier than those incubated in the light but they were less productive in terms of the number of shoots. Optimum light intensities and qualities have been reported (Nebel & Naylor, 1968; Weis & Jaffe, 1969) but a wide range of light intensities have been used to obtain regeneration *in vitro* (Murashige, 1974) and consequently light intensity and quality were not examined in the present study.

Murashige (1974) suggested that differences in regenerative capacity of stem explants could be due to differences in the physiological age of explants. This hypothesis was based on observations that stem explants of *Nicotiana tobaccum* from the apical region regenerated shoots and roots more readily than those from the basal region. Raju & Mann (1970) found a similar correlation using *Echeveria* leaves. Conversely Robb (1957), Hackett (1969) and Takayama & Misawa (1979) observed the highest

regeneration from the outer, older scales of Lilium. Variation in age of stem explants of tulip is very small; the entire stem being initiated within a period of a few weeks (Gilford & Rees, 1974). Consequently, physiological age is unlikely to have any significant effect on regeneration in vitro, however, it may be one of the contributing factors involved in determing the response of explants.

In the experiments reported in Chapter 3 floral stem was shown to be the most responsive tissue in vitro. However, recently there have been reports of shoot production from axillary bud and scale explants of tulip. Hussey & Hilton (1979) using axillary buds 3-5 mm high on an M & S medium with 4 mg $l^{-1}$ BAP & 0.12 mg $l^{-1}$ NAA were able to produce proliferating clusters of shoots, although growth occurred very slowly.

Since the completion of the work reported in Chapter 3 Nishiuchi (1979) has reported the production of adventitious buds on scale explants of Apeldoorn on an M & S medium with kinetin at 'about 3 mg $l^{-1}$' and 2.4-D at 1.0 - 1.4 mg $l^{-1}$. However, success was very limited as 'bulb-like bodies' were not formed until 6 months after the cultures were set up and a maximum of 2.6 shoots per explant were produced.

Riviere & Muller (1979) have also reported the induction of buds on scale explants, in this case on tissue of the

cultivar Paul Richter. Explants were taken from partly-formed daughter bulbs and placed on a modified M & S medium supplemented with NAA and BAP. Budding was described as 'risky'; the number of half-scales budding representing only 25% of the explants placed in culture, and an average of only 'half a dozen' buds were produced on each half-scale.

The shoot-like structuresproduced on tulip floral stem explants were shown (Chapter 6) to have the potential to develop into whole plants, and that regeneration may have been via embryogenesis. If this is correct it follows that the shoot-like structuresmay respond similarly to isolated embryos in vitro. Niimi (1978) reported that tulip embryos require a period of cold before germination will occur and also a minimum of ten weeks at 5°C before 'normal' bulblet production will occur. He also reported that embryos grown at high temperature (24°C) produced distorted structures lacking bulbs. In the present study similar distorted structures were observed at the base of shoots produced on floral stem explants (Chapter 6). Preliminary experiments designed to study the effects of a cold treatment on shoots were performed, but because of the limited amount of uniform material available, the results obtained were inconclusive and are therefore not presented in this thesis.

Bulblets have been produced on floral stem explants incubated for a number of months (10-15) after transfer to

fresh media. Bulblets have also been produced from subcultured single shoots within a six month culture period, thus confirming the observations made in Chapter 6, namely that the shoot-like structures have the potential to produce whole plants.

Ideally for an efficient system of clonal propagation, a continuous multiplication process is required. Hussey (1980) has reported techniques for continued axillary branching and adventitious bud production for some monocotyledons. In the present study of tulip, subcultured shoots and parts of shoots have continued to proliferate, thus indicating that a technique could be developed for the continued adventitious bud production on tulip tissues.

In conclusion, the results presented in this thesis describe in detail techniques for the production of large quantities of shoots, and indicate the feasibility of the rapid propagation of tulip by tissue culture from floral stem tissues. Also presented are the results of a study of plant growth regulators within the floral stem designed to elucidate their role in the organogenetic processes occurring <u>in vitro</u>.

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a) Composition of Murashige & Skoog (1962) medium

Inorganic salts

| | | |
|---|---|---|
| Ammonium nitrate | $NH_4NO_3$ | 1650 mg $l^{-1}$ |
| Potassium nitrate | $KNO_3$ | 1900 " |
| Calcium chloride | $CaCl_2\ 2H_2O$ | 440 " |
| Magnesium sulphate | $MgSO_4\ 7H_2O$ | 370 " |
| Potassium dihydrogen phosphate | $KH_2PO_4$ | 170 " |
| Disodium EDTA | $Na_2$ EDTA | 37.3 " |
| Ferrous sulphate | $FeSO_4\ 7H_2O$ | 27.8 " |
| Potassium iodide | KI | 0.83 " |
| Boric acid | $H_3BO_3$ | 6.2 " |
| Manganese sulphate | $MnSO_4\ 4H_2O$ | 22.3 " |
| Zinc sulphate | $ZnSO_4\ 4H_2O$ | 8.6 " |
| Zinc sulphate | $ZnSO_4\ 7H_2O$ | - |
| Sodium molybdate | $Na_2MoO_4\ 2H_2O$ | 0.25 " |
| Copper sulphate | $CuSO_4\ 5H_2O$ | 0.025 " |
| Cobalt chloride | $CoCl_2\ 6H_2O$ | 0.025 " |

Organic constituents

| | |
|---|---|
| Casein hydrolysate (optional) | 1 g $l^{-1}$ |
| myo-inositol | 100 mg $l^{-1}$ |
| glycine | 2.0 " |
| pyridoxine HCl | 0.5 " |
| nicotinic acid | 0.5 " |
| thiamine HCl | 0.1 " |
| BAP | 0-10 mg $l^{-1}$ |
| IAA | 0-10 " |
| Sucrose | 30 g $l^{-1}$ |
| Agar | 6.75 " |

Preparation of Stock solutions

A. Macronutrients (x 10) - to prepare 500 ml
(Store in fridge, 2 month maximum)

| | | |
|---|---|---|
| Ammonium nitrate | $NH_4NO_3$ | 8.25 g |
| Potassium nitrate | $KNO_3$ | 9.50 g |
| Calcium chloride | $CaCl_2\ 2H_2O$ | 2.20 g |
| Magnesium sulphate | $MgSO_4\ 7H_2O$ | 1.85 g |
| Potassium dihydrogen phosphate | $KH_2PO_4$ | 0.85 g |

## Preparation of Stock solutions

A. Macronutrients (x 10) - to prepare 500 ml
(Store in fridge, 2 month maximum)

| | | |
|---|---|---|
| Potassium chloride | KCl | 3.75 g |
| Sodium nitrate | $NaNO_3$ | 3.0 g |
| Magnesium sulphate | $MgSO_4\ 7H_2O$ | 1.25 g |
| Sodium phosphate dihydrogen | $NaH_2\ PO_4$ | 1.25 g |
| Calcium chloride | $CaCl_2\ 2H_2O$ | 0.375 g |

B. Micronutrients (x 100) - to prepare 500 ml
(Store in fridge, 2 month maximum)

| | | |
|---|---|---|
| Ferric chloride | $FeCl_3\ 6H_2O$ | 0.05 g |
| Zinc sulphate | $ZnSO_4\ 7H_2O$ | 0.05 g |
| Boric acid | $H_3BO_3$ | 0.05 g |
| Manganese sulphate | $MnSO_4\ 4H_2O$ | 0.005 g |
| Copper sulphate | $CuSO_4\ 5H_2O$ | 0.0015 g |
| Aluminium chloride | $Al\ Cl_3$ | 0.0015 g |
| Nickel chloride | $NiCl_2\ 6H_2O$ | 0.0015 g |
| Potassium iodide | KI | 0.0005 g |

B. Micronutrients (x 10) - to prepare 500 ml
(Store in fridge,2 month maximum)

| | | |
|---|---|---|
| Boric acid | $H_3BO_3$ | 0.031 g |
| Manganese sulphate | $MnSO_4\ 4H_2O$ | 0.112 g |
| Zinc sulphate | $ZnSO_4\ 4H_2O$ | 0.043 g |
| Sodium molybdate | $Na_2\ MoO_4\ 2H_2O$ | 0.0013g |
| Copper sulphate | $CuSO_4\ 5H_2O$ | 0.0001g |
| Cobalt chloride | $CoCl_2\ 6H_2O$ | 0.0001g |
| Potassium iodide | Kl | 0.0042g |

C. Iron source (x 10) to prepare 500 ml
(Store in fridge, 2 month maximum)

| | | |
|---|---|---|
| Sodium ethylenediamine-tetraacetate | $Na_2$ EDTA | 0.187 g |
| Ferrous sulphate | $FeSO_4\ 7H_2O$ | 0.139 g |

D. Vitamins (x 1000) to prepare 25 ml
(Freeze in 1 ml aliquots)

| | |
|---|---|
| Meso-inositol | 2.50 g |
| Glycine | 0.050 g |
| Pyridoxine - HCl | 0.0125 g |
| Nicotinic acid | 0.0125 g |
| Thiamine - HCl (aneurine - HCl) | 0.0025 g |

E. NAA - naphthalene acetic acid (0.1 mg $l^{-1}$)
(Store in fridge, 1 month maximum)

Dissolve 0.01 g in 2 ml ethanol - warm slightly - dilute slowly to 100 ml with dist. $H_2O$

F. BAP (0.1 mg $l^{-1}$)
(Store in fridge, 1 month maximum)

Dissolve 0.01 g in approximately 2 ml 0.1 N HCl - heat slightly - dilute slowly to 100 ml with dist. $H_2O$

b) Composition of Heller (1953) medium

Inorganic salts

| | | | |
|---|---|---|---|
| Potassium chloride | KCl | 750 | mg $l^{-1}$ |
| Sodium nitrate | $NaNO_3$ | 600 | " |
| Magnesium sulphate | $MgSO_4$ $7H_2O$ | 250 | " |
| Sodium phosphate dihydrogen | $NaH_2PO_4$ | 125 | " |
| Calcium chloride | $CaCl_2$ $2H_2O$ | 75 | " |
| Ferric chloride | $FeCl_3$ $6H_2O$ | 1 | " |
| Zinc sulphate | $ZnSO_4$ $7H_2O$ | 1 | " |
| Boric acid | $H_3BO_3$ | 1 | " |
| Manganese sulphate | $MnSO_4$ $4H_2O$ | 0.1 | " |
| Copper sulphate | $CuSO_4$ $5H_2O$ | 0.03 | " |
| Aluminium chloride | $AlCl_3$ | 0.03 | " |
| Nickel chloride | $NiCl_2$ $6H_2O$ | 0.03 | " |
| Potassium iodide | KI | 0.01 | " |

Organic constituents

As for M & S medium

Interaction between pH and PGR concentration in the medium in terms of shoot production (shown diagrammatically in Figure 4.2, page 64).

| Medium PGR concentration mg $l^{-1}$ | | Medium pH | | |
|---|---|---|---|---|
| NAA | BAP | 4.7 | 5.4 | 6.1 |
| 4 | 4 | 8.0 | 2.80 | 5.70 |
| 4 | 1 | 8.50 | 14.70 | 6.00 |
| 4 | 0.25 | 4.10 | 6.89 | 7.25 |
| 1 | 4 | 5.10 | 5.30 | 6.00 |
| 1 | 1 | 13.50 | 20.50 | 28.10 |
| 1 | 0.25 | 11.00 | 13.70 | 11.67 |
| 0.25 | 4 | 0.00 | 0.00 | 2.00 |
| 0.25 | 1 | 10.50 | 8.50 | 7.38 |
| 0.25 | 0.25 | 3.80 | 9.11 | 18.40 |

L.S.D. at p = 0.05 level = 9.5

Section A3

Interaction between explant origin and medium at stage 1 of floral stem development in terms of the mean shoot production per culture (shown diagrammatically in Figure 4.5, page 84)

| Medium PGR concentration mg $l^{-1}$ | | Explant type | | | | | |
|---|---|---|---|---|---|---|---|
| NAA | BAP | T | 3N | 2N | 1N | b | vb |
| 4 | 4 | 2.10 | 6.90 | 7.20 | 2.40 | 0.80 | 0.10 |
| 4 | 1 | 10.80 | 5.80 | 24.67 | 28.10 | 24.80 | 9.90 |
| 4 | 0.25 | 5.60 | 6.70 | 9.80 | 34.20 | 9.40 | 0.80 |
| 1 | 4 | 6.80 | 6.70 | 12.30 | 28.10 | 13.50 | 10.67 |
| 1 | 1 | 5.40 | 8.50 | 16.40 | 29.11 | 10.30 | 6.56 |
| 1 | 0.25 | 5.60 | 13.80 | 16.70 | 20.90 | 15.20 | 13.30 |
| 0.25 | 4 | 1.40 | 2.30 | 3.80 | 1.20 | 0.40 | 0.00 |
| 0.25 | 1 | 2.80 | 3.70 | 9.80 | 20.40 | 14.20 | 9.30 |
| 0.25 | 0.25 | 8.00 | 9.80 | 13.00 | 19.11 | 6.70 | 4.44 |

L.S.D. at p = 0.05 level = 9.4

Section A4

Interaction between explant origin and medium at stage 2 of floral stem development in terms of the mean shoot production per culture (shown diagrammatically in Figure 4.7, page 86)

| Medium PGR concentration mg $l^{-1}$ | | Explant type | | | | | |
|---|---|---|---|---|---|---|---|
| NAA | BAP | T | 3N | 2N | 1N | b | vb |
| 4 | 4 | 2.56 | 9.00 | 23.00 | 29.40 | 4.00 | 6.30 |
| 4 | 1 | 0.40 | 6.10 | 13.10 | 30.40 | 13.00 | 10.60 |
| 4 | 0.25 | -0.00 | 3.90 | 9.30 | 31.40 | 6.30 | 1.90 |
| 1 | 4 | 4.20 | 8.50 | 14.80 | 25.70 | 8.90 | 1.70 |
| 1 | 1 | 1.50 | 5.22 | 10.80 | 24.30 | 3.10 | 1.80 |
| 1 | 0.25 | -0.00 | 3.50 | 4.60 | 21.40 | 3.50 | 5.60 |
| 0.25 | 4 | 0.90 | 2.60 | 8.30 | 3.60 | 5.30 | 1.11 |
| 0.25 | 1 | 1.10 | 1.70 | 6.10 | 13.50 | 6.80 | 5.70 |
| 0.25 | 0.25 | 0.00 | 1.90 | 3.40 | 7.70 | 0.60 | 1.10 |

L.S.D. at p = 0.05 level = 7.8

Section A5

Interaction between explant and medium at stage 2 of floral stem development in terms of mean fresh weight gain per culture
(shown diagrammatically in Figure 4.11, page 90)

| Medium PGR concentration mg $l^{-1}$ | | Explant type | | | | | |
|---|---|---|---|---|---|---|---|
| NAA | BAP | T | 3N | 2N | 1N | b | vb |
| 4 | 4 | 51.04 | 56.95 | 55.78 | 37.14 | 30.39 | 28.13 |
| 4 | 1 | 41.43 | 58.78 | 62.99 | 39.70 | 40.18 | 32.13 |
| 4 | 0.25 | 47.09 | 57.56 | 54.30 | 57.28 | 46.45 | 31.61 |
| 1 | 4 | 42.93 | 55.02 | 47.77 | 32.52 | 23.49 | 14.88 |
| 1 | 1 | 42.68 | 52.43 | 49.06 | 44.65 | 21.70 | 13.11 |
| 1 | 0.25 | 32.99 | 48.38 | 36.16 | 45.56 | 25.64 | 26.08 |
| 0.25 | 4 | 47.06 | 41.98 | 47.39 | 20.52 | 18.11 | 10.78 |
| 0.25 | 1 | 46.72 | 38.46 | 34.94 | 37.99 | 15.76 | 12.90 |
| 0.25 | 0.25 | 36.64 | 37.90 | 32.46 | 39.30 | 12.33 | 7.03 |

L.S.D. at p = 0.05 level = 13.7

# The growth of tulip tissues *in vitro*

# THE GROWTH OF TULIP TISSUES IN VITRO

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## Abstract

Large numbers of adventitious shoots have been induced to form on floral stem sections of tulip cv. Merry Widow cultured i n v i t r o, using a modified Murashige & Skoog medium containing inorganic salts and vitamins at full strength and supplemented with casein hydrolysate, benzylamino-purine (BAP) and naphthyl acetic acid (NAA). Between 70% and 90% of floral stem explants regularly produced shoots on this medium. In comparison with other explants from the bulb (scale and axillary bud), floral stem tissue showed the greatest potential for shoot production.

The stage of development of the bulb was found to be an important factor in determining the ability of explants to regenerate shoots. Shoot producing potential of floral stem explants was greatest during dry storage of the bulbs at 17°C and 5°C, but the ability to produce shoots was lost once rapid extension growth and greening of the floral stem had commenced.

Small explants, 1mm in thickness, gave rise to many adventitious buds, but larger explants (3-5mm) were optimal for maximum shoot production. There was an interaction between floral stem explant size and origin as regards shoot production.

The results of a histological study of tulip shoot production i n v i t r o are discussed.

## Introduction

The first report of the culture of tulip tissue i n v i t r o was by Bancillion in 1974, in which a complex temperature pre-treatment was used to induce bud formation on various parts of the tulip bulb. Callus cultures have been induced on tulip scale pieces by Nishiuchi and Myodo (1976), however shoots were not produced from the callus of any of the 11 cultivars tested and only some regenerated adventitious roots. In 1979 Nishiuchi was successful in inducing buds on scale explants. Riviére and Muller (1976) produced bulbs from axillary buds but this represented only a slight increase above the natural propagation rate of daughter bulbs. In 1979 Riviére and Muller reported the formation of buds on some scale explants from daughter bulbs within a culture period of 6 months.

We have successfully induced adventitious bud formation on floral stem explants during an 8 week culture period and we report here the factors affecting the formation of these adventitious shoots.

## Materials and Methods

Tulip bulbs cv. Merry Widow (12/13cm) were obtained from Kirton Experimental Horticulture Station and stored dry at 17°C. Bulbs

Acta Horticulturae 109, 1980
Flower bulbs

used in the study of the stage of development were subjected to a double cooling treatment of 6 weeks dry storage at 5°C followed by 3 weeks at 5°C after planting. The bulbs were subsequently grown in a growth room at 9°C for 1 week, 13°C for a further week and then 17°C until flowering, under HLRG lamps with a 10 hour photoperiod and light intensity of 100 ± 10 $\mu Em^{-2}s^{-1}$.

Bulbs with their outer papery scale removed were surface sterilised in 20% (v/v) 'Domestos' (a commercial bleach containing 2% available chlorine, Lever Bros., England) for 30 min and washed 3 times in sterile distilled water. The outer fleshy scale and apical half of the bulb were removed and the remaining part of the bulb was sterilised for a further 20 min in 20% (v/v) 'Domestos' and washed 3 times in sterile distilled water. Explants of scale tissue (approximately 5 x 10mm) and of axillary buds were excised to include some basal plate tissue. Floral stems were removed from non-sterile bulbs, immersed for 10-15 min in 10% (v/v) 'Domestos' and washed 3 times in sterile distilled water. Surface sterilisation and subsequent dissections were carried out in a laminar flow clean air cabinet.

The basic medium contained Murashige & Skoog (1962) macro-elements, micro-elements, vitamins and casein hydrolysate (500 mg $l^{-1}$). Agar (6.75g $l^{-1}$), BAP and NAA were added before the media were adjusted to pH 6.1 (except where stated) with 0.1N KOH. The media were autoclaved at 121°C for 5 min to dissolve the agar and either 10 ml aliquots were dispensed into 25 x 75mm glass tubes with foil caps or 20 ml into 40 x 75mm screw top glass jars. The culture vessels containing the media were then sterilised by autoclaving for a further 15 min at 121°C.

In the first experiment explants of scale, bud and floral stem were placed on basic medium supplemented with either NAA (0.0, 0.5 and 5.0 mg $l^{-1}$), BAP (0.0, 1.0 and 10.0 mg $l^{-1}$) or NAA and BAP at all concentrations.

The cultures were maintained at 20° ± 1°C with a photoperiod of 16 hours and a light intensity of 30 ± 10 $\mu Em^{-2}s^{-1}$ at the culture level.

Specimens were fixed in formalin-acetic acid-alcohol, dehydrated through an ethanol series and embedded in polyester wax. Sections 7μ thick were cut using a Cambridge Rotary Microtome. The sections were stained in Safranin/Fast Green (Berlyn & Miksche, 1976), and examined with a Vickers M17 Photomicroscope.

## Results

Comparison of the different types of explant on the most suitable media for each type revealed that, although a large proportion of the cultures of both scale and floral stem produced callus, 90% and 95% respectively, 50% of the floral stem explants produced shoots in contrast to 5% for scale and 20% for bud explants (20 replicates).

The stage of bulb development was found to be a critical factor in determining shoot regeneration and callus production from floral stem explants. There was an almost total loss of ability to produce shoots once the bulbs had entered the "active growth" stage (figure 1). Callus production continued beyond this stage, but it too declined to zero once rapid extension growth of the floral stem was proceeding.

Preliminary work suggested that an auxin: cytokinin ratio of approximately 1:1 was optimum for shoot production from floral stem explants. Data from a more detailed study of plant growth regulator

(PGR) levels on non-nodal explants (figure 2) indicated that although a wide range of media gave some shoot production, the highest mean number of shoots per culture was obtained on medium E (1 mg $l^{-1}$ NAA and 1 mg $l^{-1}$ BAP). However, 4 mg $l^{-1}$ of the cytokinin BAP (medium C) was almost totally inhibitory to shoot production (medium C) unless present in combination with concentrations of NAA greater than 1 mg $l^{-1}$ (media F and I). Such concentrations of BAP were apparently toxic as death of cultures was common within 2-3 weeks of inoculation. Medium E was therefore used in subsequent experiments.

The pH of the medium affected shoot production from floral stem explants cultured on medium E (figure 3). Shoot initiation was obtained over the pH range tested (pH 4.7 - 6.8), but the greatest mean number of shoots per culture occurred when the pH was 6.1

The effect of explant origin on shoot production was apparent when floral stems, which contained 3 nodes, were sectioned into 1mm pieces. The mean number of shoots produced per culture (figure 4) was highest in the region of the lower 2 nodes, i.e. sections 9 & 10 and 13 & 14. In contrast there was no apparent effect of the 3rd node, but there was a significant decrease in regeneration towards the top of the stem. It was also noted that virtually no shoot production occurred at the basal end of the stem. However, when the floral stem was dissected into 6 pieces according to the position of the nodes (figure 5), the basal segment b1 regenerated more shoots than the top segment of the stem T, but the nodal regions 1N and 2N still produced a significantly larger number than both T and b1. A comparison of each region of flower stem, whole and divided into 1mm sections, showed a correlation with distance along the stem from T to b1. At region T the mean number of shoots produced by the region as a single explant was less than the number produced from the region as 1mm sections. In contrast, region b1 as a single explant gave rise to many more shoots than the number produced by 1mm sections.

The total number of shoots produced per stem was greatest when the stem was divided into 24, even though on average the number of shoots per culture was greater when divided into 6 (Table 1). When the stem was divided into 2 explants, only one shoot was produced from each of the nodal regions, whereas when it was divided into 6 an average of 13.3 shoots were produced per culture.

Results of an histological study of floral stem explants at different stages of development suggest that the adventitious shoots originated from the outer cell layers (figure 6A). Cell division in these layers started immediately prior to the 6th week in culture and by the 8th week meristematic protuberances were apparent (figure 6B). After 16 weeks in culture large adventitious shoots had developed, which were associated with the meristematic growing centres (figure 6C). Shoot initials were macroscopically visible after 8 weeks in culture (figure 6D), and were approximately 0.5 cm in length by 12 weeks (figure 6E). After 16 weeks in culture they had undergone considerable development and were approximately 5 cm in length (Figure 6F).

## Discussion

The low rates of shoot regeneration obtained from bulb scale and daughter bulb explants confirm the findings of Riviere & Muller (1976 and 1979): only one bulb was produced from each axillary bud, and bud

formation occurred on only 25% of scale explants during a 6 month culture period.

The high regeneration rate obtained on floral stem explants was similar to that reported by Hussey (1975) for several species in the Liliaceae family (*Hyacinthus*, *Muscari*, *Ornithogalum* and *Scilla*), although he reported no plantlet regeneration for tulip. This negative report for tulip may have been because of the absence of cytokinin in the culture medium, as we have found it necessary to include cytokinin for shoot production. Assuming shoot formation follows the classical model of Skoog & Miller (1957), the requirement of cytokinin in the medium for shoot initiation indicates that the endogenous levels of cytokinin are inadequate. In comparison with *Hyacinthus*, *Muscari*, *Scilla* and *Ornithogalum* tulip is unusual in its requirement for cytokinin. However, it is similar to *Brassica napus* (Kartha *et al*, 1974) which also depends on applied cytokinin for shoot formation.

The large number of adventitious shoots formed on nodal tissue is similar to that reported by Custers (1978) who induced adventitious bud formation between the axils of opposite leaves of carnation *in vivo*. The lack of apparent effect of the third node on shoot production from 1mm stem sections was probably because of its variation in position within different stems (sections 16 to 19).

The decrease in shoot production at low pH may reflect the natural preference of the tulip for high pH (above 6) when grown in soil. The low regeneration at pH 6.8 may have been because of reduced availability of salts or PGR's. A study of the interaction of medium pH with PGR levels in terms of shoot production may reveal whether these effects are due to the availability of PGR's.

The formation of indole acetic acid (IAA) at cut surfaces may be responsible for triggering the initial cell division as indicated in a review by Yeoman (1970) on the role of the wound reaction in the early stages of callus formation, and from the evidence of Sheldrake and Northcote (1968) on auxin production by autolysing tissues. The release of IAA may be responsible for the differences in shoot production when comparing a stem divided into 6 sections with one divided into 24 sections. The differences between the regions of stem could be explained by a gradient of some PGR along the stem, giving an optimum interaction at the nodal regions. In order to test this hypothesis PGR's are being assayed from divided and whole regions of the floral stem.

An interaction may also exist between the effects of PGR levels in the medium and the stage of bulb development in terms of shoot production. A study of this interaction may provide information to enable regeneration of shoots from floral stem tissue at later stages of development. In this way many more explants could be excised from a single bulb, thereby increasing the potential for tulip multiplication.

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Table 1 - Effect of explant size on shoot regeneration

| Number of explants per stem | Number of shoots per culture | Number of shoots per stem |
|---|---|---|
| 1 | 0.0 | 0 |
| 2 | 1.5 | 3 |
| 6 | 13.3 | 80 |
| 24 | 7.8 | 185 |

Figure 6 - A - C. Photomicrographs : A. Cell division in outer cell layers, 6 weeks in culture (x 175). B. Meristematic protuberances, 8 weeks in culture (x 175). C. Adventitious shoots from the outer cell layers associated with meristematic centres, 16 weeks in culture (x 70).
D - F. Stages of shoot development on floral stem. D. 8 weeks in culture. E. 12 weeks in culture. F. 16 weeks in culture. (Bar represents 1 cm).

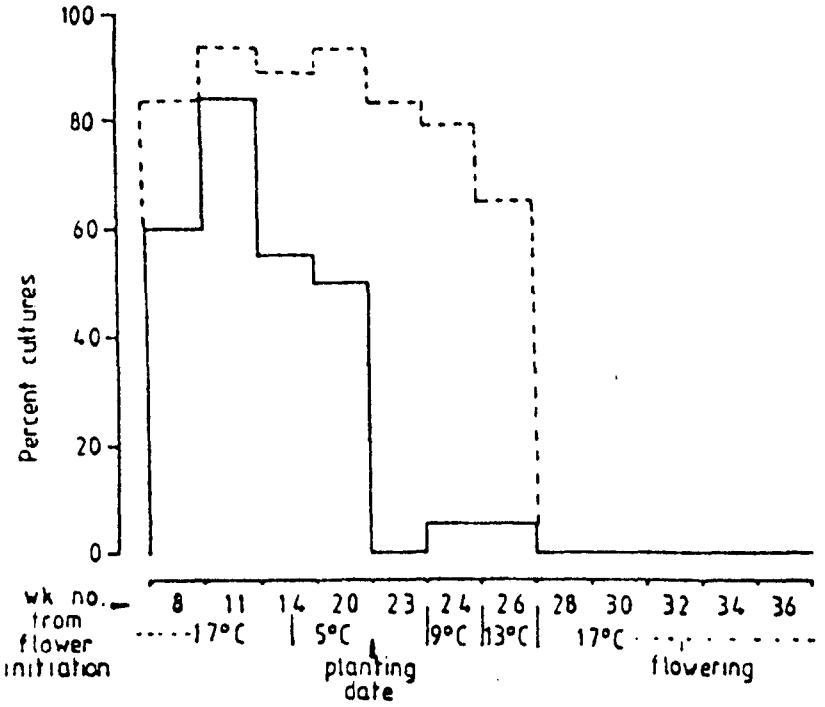


Figure 1 - Effect of stage of bulb development on regeneration (20 replicates)
-------- Basal expansion 'callus' production
———— Shoot production

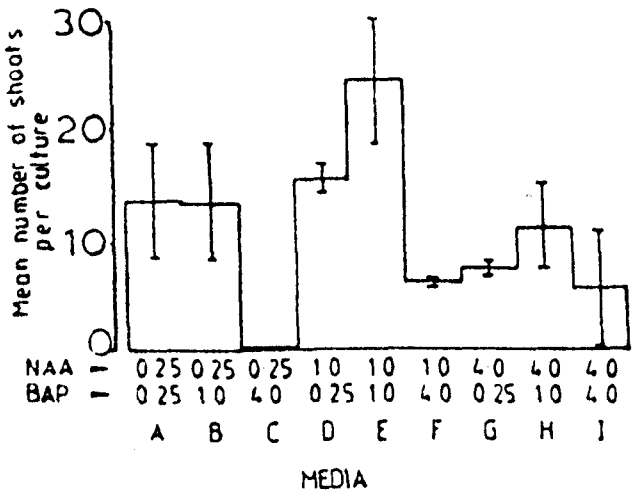


Figure 2 - Effect of auxin/cytokinin levels in the medium on shoot production from non-nodal floral stem explants

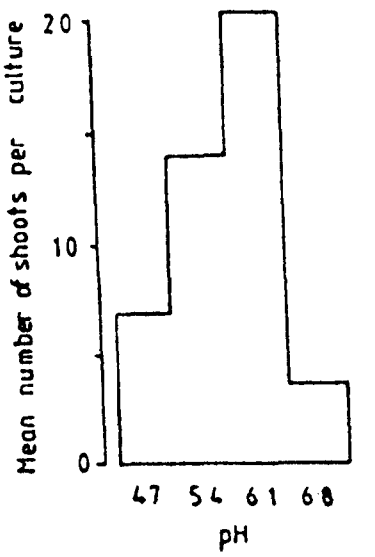


Figure 3 - Effect of medium pH on shoot production (mean of 30 cultures)

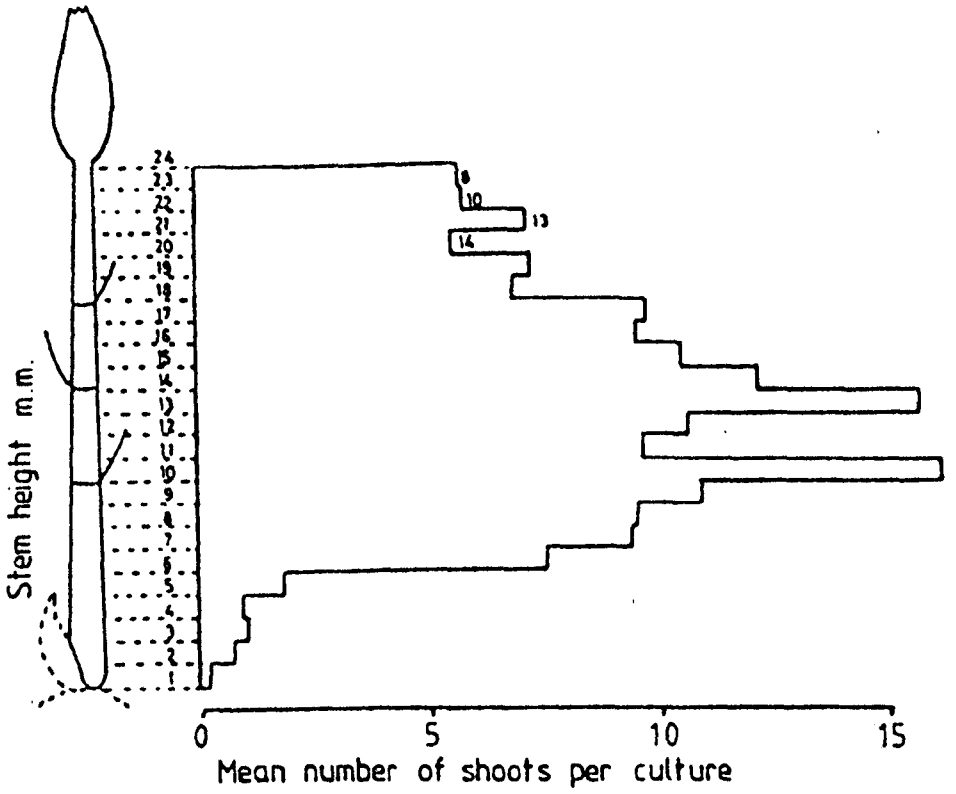


Figure 4 - Mean shoot production per culture from 1 mm sections of floral stem (mean of 20 cultures except where stated).

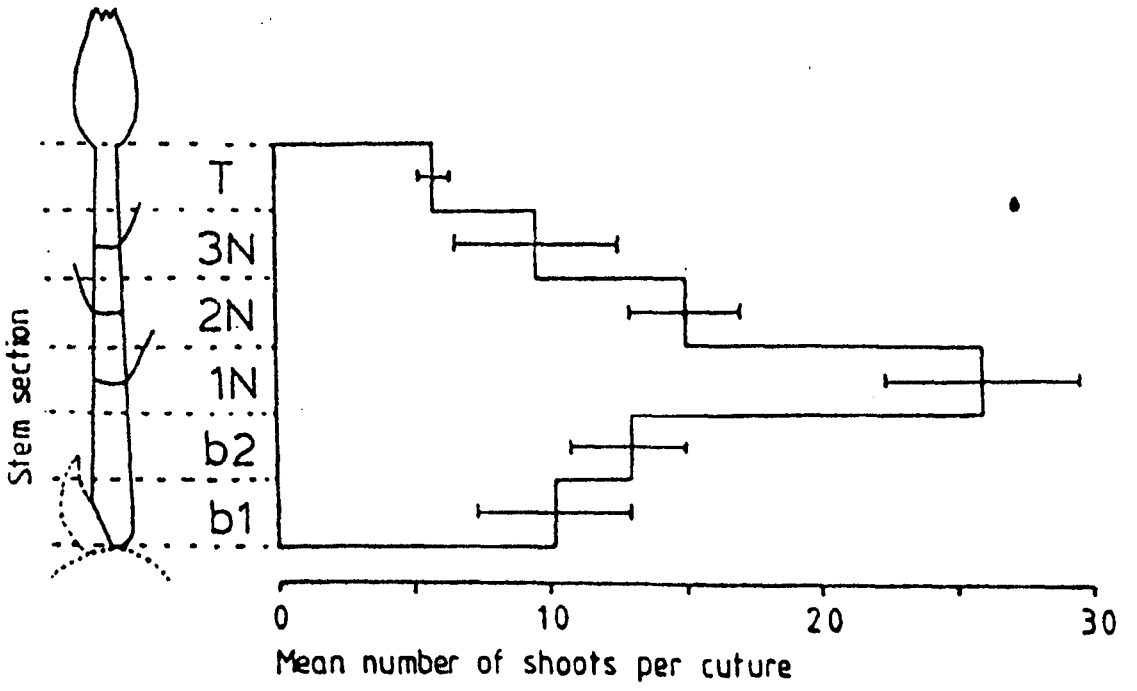


Figure 5 - Mean shoot production per culture from floral stem divided into 6 regions