RAPID MULTIPLICATION OF CHINCHERINCHEE (*ORNITHOGALUM* SPECIES) BY TISSUE CULTURE.

by

PENELOPE ANNE LANDBY.

SUBMITTED AS PARTIAL FULFILMENT FOR THE DEGREE

MASTER OF SCIENCE.

IN THE FACULTY OF NATURAL SCIENCES
DEPARTMENT OF BOTANY
UNIVERSITY OF PRETORIA
PRETORIA.

SEPTEMBER, 1988.

PROMOTER; PROF. H. A. van de VENTER.

TABLE OF CONTENTS.

CHAPTER 1.

INTRODUCTION.

1.1. Motivation for the study.

The aim of clonal propagation is to establish plants in large numbers that are uniform and predictable for certain qualities (Murashige, 1977).

This study was undertaken to satisfy the following requirements of the Ornithogalum breeding programme, which began at the Vegetable and Ornamental Plant Research Institute in 1974:-

1. A technique for fast and timely establishment of new cultivars.

2. A technique for bulking up breeding material for selection purposes.

1.2. The economic importance of the genus Ornithogalum in South Africa.

In South Africa, sixteen species of Ornithogalum are horticulturally important as cut flowers and garden plants (van Niekerk, 1965). For the year ending February, 1987, the Ornithogalum cut flower turnover on the local market was:- (information supplied by Multiflora).

Quantity	76 190	(no offers for 2706 bunches)
Value	R42 064,00	
Average Price	R0,55 / bunch.	
Highest Price	R5,99 / bunch.	

No figures concerning the export market are available. Export arrangements are directly negotiated by growers.

Hadeco is believed to be the main exporter, but declined to comment when approached for details.

For the year ending February, 1987, the sales of Ornithogalum constitute the following percentages of the total turnover of cut flowers in South Africa (information supplied by Multiflora).

Month (ending 30/31st)	Percentage of turnover.
January	0,03
February	No figures available.
March	0,05
April	0,05
May	0,01
June	No flowers marketed.
July	0,02
August	0,29
September	0,61
October	0,25
November	0,47
December	0,41

Considerable scope exists for expanding the commercial exploitation of the genus Ornithogalum, both in South Africa and on the overseas market. The breeding programme at the Vegetable and Ornamental Plant Research Institute is aimed at improving the quality and diversity of both the cut flowers and the plants.

1.3. Selection of Experimental Material.

The plant selected on which to test the effect of cultural parameters was the Ornithogalum `Rollow'. It is hoped to extend the results of this study to other selections in the breeding programme. The use of `Rollow' was suggested towards this end because of the broad genetic background of

the cultivar. Limited comparisons (due to the scarcity of donor plants) were drawn between the requirements of Ornithogalum 'Rollow' and those of the species Ornithogalum maculatum. The genetic isolation of this species found in the breeding programme is supported by the uniqueness of its in vitro requirements.

1.4. Description of the experimental material.

The family Liliaceae sensu lato comprising 250 genera and 3700 species, 1000 of which are represented in South Africa, was restructured by Dahlgren & Clifford in 1982 (Perry, 1985). It has been divided into two orders: the Liliales and Asparagales. The latter order comprises six families, distinguished between on habit, underground parts, inflorescence and fruits (Perry, 1985). Members of the family Hyacinthaceae to which the genus Ornithogalum L. belongs are characterised as:

> "Geophytes with underground parts a bulb. Leaves basal. Inflorescence a simple raceme. Perianth parts simple, or united at base. Fruit a capsule" (Perry, 1985).

The name of the genus, Ornithogalum is derived from the Greek, ornithogalen meaning bird's milk. The genus is distributed throughout Europe, Western Asia and South and South West Africa, with one species in Madagascar. A revision of South African members of the genus was published by Obermeyer (1978). Fifty-four species comprising three subgenera are recognized:-

subgenus Aspasia (Salisb) Oberm.
subgenus Urophyllon (Salisb) Bak.
subgenus Osmyne (Salisb) Bak.

The subgenera Aspasia and Urophyllon do not interbreed and are considered karyotypically distinct (de Villiers Pienaar in Obermeyer, 1978). Members of the subgenus Osmyne also

form a natural group, distinguished by sweet scented flowers with characteristic long, exserted deflected styles and pom-pon like stigmas (Obermeyer, 1978).

Three groups are recognized within the subgenus Aspasia, the distinctions being made by Obermeyer (1978) on the basis of bract characteristics. The Aspasiae and Hispidae are Cape plants, while Angustifoliati are widespread, mostly in summer rainfall areas.

The species Ornithogalum maculatum Jacq. (Fig. 1) belongs to the large flowered Aspasiae where it constitutes a cenospecies (van Niekerk & de Villiers Pienaar, 1968) failing to set fertile seed as a result of cross pollination with other members of the subgenus. The varieties of the species, speciosum, concolor and splendens are not upheld by Obermeyer (1978) as these are merely larger or spotted forms.

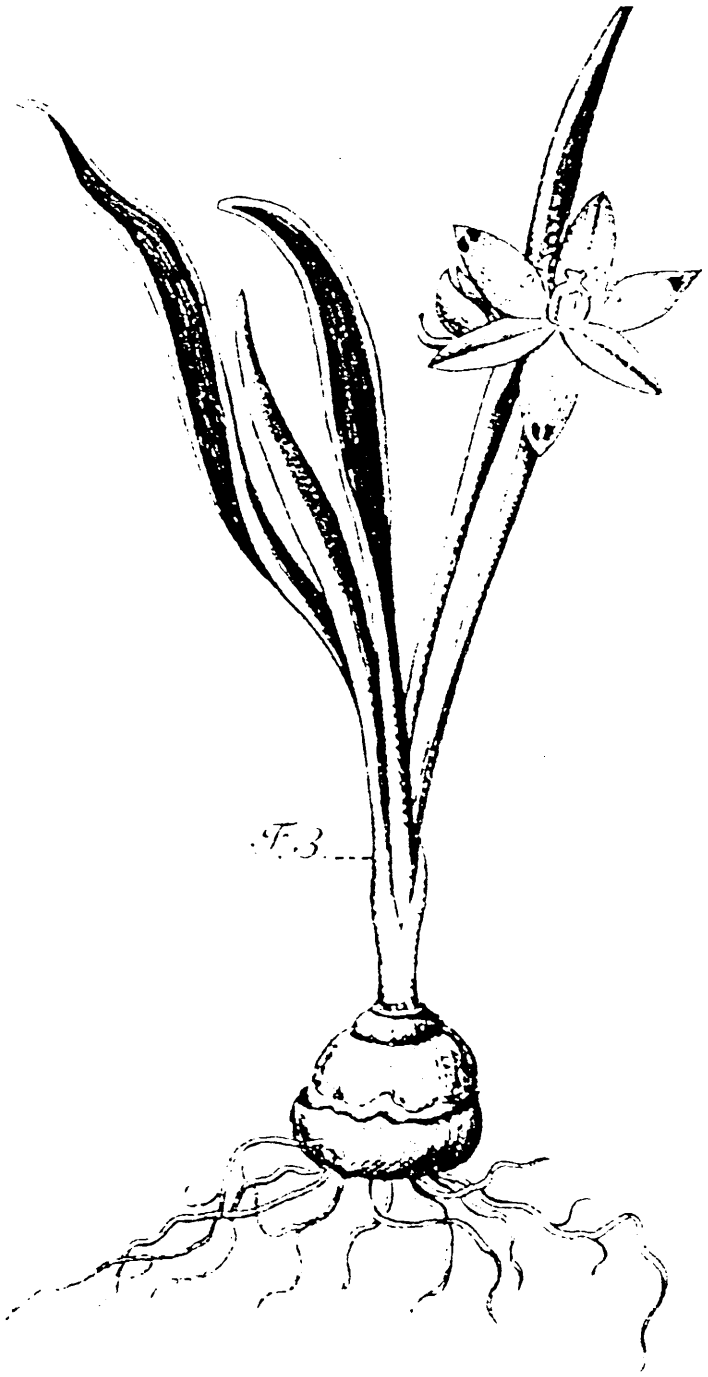


Figure 1. Ornithogalum maculatum; Jacq., Coll.2, t. 18, f. 3 (iconotype). (from Obermeyer, 1978).

The following description of the species maculatum appears in Obermeyer:- (Obermeyer, 1978: page 340).

"Plants 0,08-0,5 m tall. Bulb obturbinate up to about 30 mm in diam. soft and juicy, with white (or brown when dry) tunics; roots many, thin, radiating from a basal disk. Leaves 2-5, usually synanthous, linear to oblong or narrowly ovate, up to 150 mm long, 20 mm broad, apex acute to rostrate, rarely cirrhose, bases usually clasping, folded, erect, glabrous, glaucous, somewhat fleshy. Raceme 1-8 flowered, sub-corymbose; peduncle up to 450 mm long, firm; bracts usually longer than, and clasping pedicels in lower half, broadly ovate-acuminate, dry, papery; pedicels 10-30 mm long, arched. Flower with spreading, yellow, golden orange to bright orange-red perianth segments, the outer usually with a black spot or transverse, wavy line or transparent blotch at or near apex; segments elliptic to obovate, 10-25 mm long. Stamens erect, about half as long as segments; filaments subulate, inner often wider. Ovary oblong-globose, obtusely angled, pale yellow or green, abruptly narrowed into a very short style and capitate stigma with 3 decurrent papillate lobes; ovules multisterate. Capsule ellipsoid; seeds minute, 1 mm, cuneiform, somewhat tongue-shaped below, densely wrinkled, black, shiny. Chromosomes: 2n=12.

Recorded from the western Cape, Namaqualand to Malmesbury, Montagu and Laingsburg, in shallow, sandy rock pockets. Flowering September-October."

A description of the Ornithogalum `Rollow' was prepared based on a minimum of thirty observations per characteristic, unless otherwise stated:-

Plants 366 +/- 92 mm tall (n = 9). Bulbs very widely ovate to depressed ovate, mass 6,4 +/- 2,4 g in the second season of flowering, carnose, with

brown semi-folded tunics. Roots numerous, radiating from central basal disc. Leaves 9,6 +/- 1,5 per plant, protantherous, linear to narrowly oblong, 235,5 +/- 33,6 mm in length, 23,2 +/- 3,4 mm broad, inclined, straight to adaxially conduplicate, glaucous, carnose, yellow-green (RHS 144 A); apex acute, involute; bases clasping; margins ciliate. Raceme 34,4 +/- 10,4 flowered (n = 9); peduncle 227,6 +/- 23,5 mm long (n = 20), yellow-green (RHS 144 A); bracts 19,9 +/- 4,5 mm long and 10,3 +/- 1,2 mm wide, lower half clasping the pedicel, ovate, yellow-green (RHS 144 C); pedicels 13,6 +/- 4,4 mm long circular, straight, yellow-green (RHS 144 A). Flower 35,5 +/- 4,5 mm in diameter, unscented; tepals 18,0 +/- 2,2 mm long and 9,05 +/- 1,0 mm broad, rhombic to obtrullate, yellow-green (RHS 152 B) tepal base, blending to yellow (RHS 8a/8b); stamens 6,7 +/- 1,2 mm long, inclined straight, terete, yellow (RHS 8a); ovary oblong-globose, obtusely angled, yellow-green (RHS 152 A); style 3,1 +/- 0,5 mm long, 3 decurrent papillate lobes, yellow (RHS 12 A); ovules multiseriate. Capsule ellipsoid. Seeds 1 mm long, cuneiform, somewhat tongue shaped below, densely wrinkled, black, sclerous. Chromosomes: 2n = 18.

The genetic background to the cultivar appears in the Appendix. Flowering from October to approximately 7 December. (RHS refers to the colour chart of the Royal Horticultural Society).

1.5. Experimental Approach.

Towards clonal propagation of the study material, procedures were developed to optimise initial explant characteristics, nutrient formulations as well as the cultural environment.

The most important determinant of the rate of *in vitro* increase and the quality of the regenerated plants is the initial explant. In this study the influence of physical characteristics of *Ornithogalum* `*Rollow*'explants on explant viability were examined.

Generally, extant media are adequate for plantlet initiation *in vitro* (Gamborg *et al.*, 1976). However, growth yield is determined by the chemical composition and the physical qualities of the medium. The influence of phytohormones, carbohydrates, agar and pH on growth yield are discussed.

In practice, it has been observed that the optimum cultural environment is the one that most closely simulates the plant's natural habitat. The effect of culture temperature on growth yield is assessed.

The propagation method used was the establishment of adventitious shoots and bulbs directly on the explant. Organogenesis was stimulated using moderate amounts of auxin and cytokinin. A drawback of this technique is the increasing genetic variability and diminished multiplication rates associated with repeated subcultures (Hussey, 1976). An advantage of the method over other techniques is that success is more consistently achieved than in the cell embryogenesis method and it is more rapid than the enhanced axillary branching method (Murashige, 1977).

In analyses of growth yield, the relationship between the plant and its environment are investigated (Hunt, 1978). In the process, however, little information is gained about the fundamental physiological processes which govern the reaction of plants to their environment. Despite this drawback, valuable clues, although not detailed explanations may sometimes emerge (Hunt, 1978).

CHAPTER 2.

MATERIALS AND METHODS.

2.1 The donor plants.

2.1.1 The bulbs.

Bulbs of Ornithogalum `Rollow' and Ornithogalum maculatum were used in the study. Ornithogalum `Rollow' bulbs were clonally derived through tissue culture from the Ornithogalum breeding programme. Ornithogalum maculatum bulbs were collected from shallow, sandy, rock pockets southwards from Springbok and also in the vicinity of Calvinia, Laingsburg and Malmesbury.

The bulbs were stored in an incubator at 22°C +/- 2°C in sterilized vermiculite after being treated for 30 minutes with an aqueous solution comprising 0.2 % Benlate (a.i. 50 % benomyl) and 1.5 % Difolatan (a.i. 48 % captafol), to prevent bulb rot. The bulbs were stored from lifting (mid January) to planting.

The bulbs did not require vernalization in order to grow, although overseas workers report a high temperature requirement to break dormancy in Ornithogalum thyrsoides bulbs (Tompsett,1985; Shoub & Halevy,1971).

2.1.2 The plants.

Ornithogalum bulbs were planted in March, although planting from mid-February to mid-May is feasible (Eliovson,1957). A potting mix of two parts sandy loam and one part equal portions of leaf mould, peat and sand was used, good drainage being essential. Bulbs were placed in 15 cm diameter plastic pots at a depth of approximately 5 cm. The bulbs were grown in a commercial glasshouse covered

with 40% shade cloth at a temperature of 22°C +/- 2°C. The bulbs were watered once a week at the beginning of the growing season, and 2-3 times a week when they were established. When the foliage began turning yellow, water was gradually withheld. Growth stimulants at half the recommended dosage were applied once a month while the bulbs were in full growth.

2.2 Selection of donor material.

Healthy, mature donor organs were selected from donor plants prior to anthesis (unless otherwise stated). Senescent organs were avoided although this usually happened after anthesis. Dyer (1976), observed that the onset of senescence after anthesis varied among species. Some species, therefore, probably yield viable explant material after anthesis.

2.3 Sterilizing donor material.

Donor organs were sterilized to remove surface contaminants. The sterilizing technique used was only slightly modified from that described by Nel (1981). The donor material was rinsed in running tap water and blotted dry to remove any soil particles. All water used for subsequent sterilization steps was distilled and deionized. Donor organs were dipped in 70% ethanol. The material was soaked in a 1,5% (w/v) sodium hypochlorite solution for 15 minutes. Five drops of Tween 80 were added to this solution as a wetting agent to reduce the surface tension of the aqueous phase and increase its contact with the explant material. The donor organs were then rinsed three times in water, and left to soak in the last rinse for 15 minutes.

2.4 The explant.

Leaf explants were divided into one square centimetre sections. Unless otherwise stated, explants from the basal quarter of leaves were used. Inflorescence stalks were sectioned into cylinders 1 cm in length. Inflorescence bracts were detached and used as explants after trimming the site of detachment.

2.5 The basal medium.

The medium of Nel (1981) was used (Table 1). This is based on the medium of Murashige & Skoog (1962) as amended by Linsmaer & Skoog (1965).

Table 1. Constituents of Nel's (1981) growth medium.

Macro-elements.	Concentration.	
$NH_4\ NO_3$	1650 mg. dm^{-3}	20,60 mmol. dm^{-3}
KNO_3	1900 mg. dm^{-3}	18,80 mmol. dm^{-3}
$CaCl_2\ 2H_2O$	440 mg. dm^{-3}	2,99 mmol. dm^{-3}
$MgSO_4\ 7H_2O$	370 mg. dm^{-3}	1,50 mmol. dm^{-3}
$KH_2\ PO_4$	170 mg. dm^{-3}	1,25 mmol. dm^{-3}
NaFeEDTA	25 mg. dm^{-3}	68,00 µmol. dm^{-3}
Micro-elements.		
H_3BO_3	6,200 mg. dm^{-3}	100,00 µmol. dm^{-3}
$MnSO_4\ H_2O$	22,300 mg. dm^{-3}	100,00 µmol. dm^{-3}
$ZnSO_4\ 7H_2O$	8,600 mg. dm^{-3}	29,90 µmol. dm^{-3}
KI	0,830 mg. dm^{-3}	5,00 µmol. dm^{-3}
$Na_2\ MoO_4\ 2H_2O$	0,250 mg. dm^{-3}	1,03 µmol. dm^{-3}
$CuSO_4\ 5H_2O$	0,025 mg. dm^{-3}	0,10 µmol. dm^{-3}
$CoCl_2\ 6H_2O$	0,025 mg. dm^{-3}	0,11 µmol. dm^{-3}
Organic Constituents.		
Sucrose	30,0 g. dm^{-3}	87,60 mmol. dm^{-3}
Agar	7,0 g. dm^{-3}	7,00 g.dm^{-3}
Thiamine.HCl (Vitamin B1)	0,5 mg. dm^{-3}	1,36 µmol. dm^{-3}
Meso-Inositol	100,0 mg. dm^{-3}	555,00 µmol. dm^{-3}
Napthaleneacetic Acid(NAA)	0,1 mg. dm^{-3}	0,57 µmol. dm^{-3}
Benzylaminopurine (BAP)	2,0 mg. dm^{-3}	8,88 µmol. dm^{-3}

In place of a combination of sodium EDTA and ferrous sulphate, as suggested by Linsmaer & Skoog (1965), NaFeEDTA was used, thus reducing the concentration of sodium ions in the medium.

The level of thiamine.HCl (vitamin B1) in the Linsmaer & Skoog (1963) medium was raised from 0,1 to 0,5 mg.dm^{-3} in Nel's (1981) medium. The requirement for an increased thiamine.HCl concentration in the medium probably stems from

the higher pH (6,2) of Nel's medium as opposed to that of Linsmaer & Skoogs (pH 5,6). In the autoclaving process, thiamine.HCl is increasingly inactivated with increasing pH (Linsmaer & Skoog, 1965).

For experimental purposes, certain constituents of the above media were modified. In these instances, modifications are noted in the introduction to the relevant chapters. In all instances the medium as specified was used to serve as the control. Plant hormones were added to the media at the concentrations stated unless otherwise specified.

2.6 Media preparation.

All media constituents were added prior to autoclaving. Media were dispensed in 10 ml volumes into rimless borosilicate test tubes (12,5 x 160 mm). The tubes were plugged using non-absorbent cotton wool and capped using heavy duty aluminium foil (lighter grades develop pinholes). The foil caps were necessary, as medium condenses on the cotton wool during the autoclaving process, and fungal growth occurred on these during subsequent storage and incubation. The tubes were autoclaved at 103 kPa and 121^{o}C for 20 minutes (Dodds & Roberts, 1982).

2.7 Sterilization of equipment and work surfaces.

Instruments, flasks and other materials which were autoclaved were wrapped in paper. Airborne contaminants were eliminated using a laminar flow cabinet (Fibatron, SA). The work area was subjected to UV radiation for 15 minutes before use, and sprayed with 70 % EtOH at frequent intervals. Instruments in contact with explant material were dipped in 80 % EtOH and flamed at regular intervals during use to surface sterilize them.

2.8 Culture conditions.

Explants were cultured in growth chambers or cabinets under the conditions recommended by Nel (1981), unless otherwise stated. The temperature was a constant 22°C +/- 2°C. Lighting was supplied for a 16 hour photoperiod using Phillips 'TL' 40W/33 fluorescent lamps with an irradiance at explant level of 24 070 $mW.m^{-2}$ and an illuminance of 8 300 $lm.m^{-2}$.

Temperature experiments were conducted in growth cabinets where the temperature was monitored using a thermocouple. One of the problems that occurs in growth chambers is that rapid temperature fluctuations are underestimated when using thermographs or laboratory thermometers. Although if calibrated accurately these instruments are accurate to 0,5 °C, the slow response time of the instrument may result in erroneous recordings.

Under *in vitro* conditions, temperatures monitored seldom correspond to those of the plant tissue (Downs & Hellmers, 1975). Plant tissue acts a black body, absorbing heat from its environment at temperatures below 28°C (Downs & Hellmers, 1975). Little heat is lost through conduction and convection and if the walls of the tube or cabinet are warmer than the tissue they will add to the radiant energy absorbed by it. Fluorescent tubes, rather than incandescent lamps, were used in this experiment as they emit less energy in the infrared than in the visible spectrum. Despite discrepancies which occur, temperature measurements provide a rough guide to the growth conditions suitable for rapid propagation of plant material.

2.9 Experimental design.

Factorial experimental designs were used in this study. This experimental design is appropriate to exploratory studies, where a number of factors are involved in determin-

ing the observed response and where results must lead to recommendations that have application over a wide range of conditions.

According to Cochran & Cox (1957), factorial analyses have the advantage of allowing the study of factors, which may or may not be independent of one another, over a sufficiently wide range of combinations to form a basis for understanding their interaction (or lack of it), while obtaining as much information about each factor as if it had been studied separately.

The disadvantages of factorial analyses are their size, which makes them labour intensive and time consuming, and leads to a bewildering array of possible treatment comparisons, which may be difficult to interpret (Cochran & Cox, 1957).

Before deciding on the factor combinations (called treatments) to be used, it is important to assess the use to which the results are to be put. Treatments not relevant to experimental objectives should be excluded.

Including a control in the experiment provides a more accurate estimate of the average treatment effect and reveals, by comparison, the effects of the treatments, at the expense of some loss of accuracy between the treatment comparisons (Cochran & Cox, 1957). If the effectiveness of the treatment has been demonstrated previously, the control may be omitted. In this study, the culture conditions of Nel (1981) served as the control.

Incorporated into the experiment must be some method for eliminating experimental bias. This occurs when one treatment is successively handicapped or favoured in an experiment by some source of extraneous variation, known or unknown. In this study, complete randomization of explants

between treatments was achieved by shaking explants prepared for culture in a bottle before subculture.

The number of replicates should be as large as is practically possible, as this determines the accuracy with which treatment effects can be determined. All treatments used in the study comprised twenty replicates. However, unforeseen losses through contamination and test tube breakage often reduced the number of replicates involved.

2.10 Experimental parameters.

The data collected concerning the effect of an experimental variable may be qualitative or quantative, and will vary depending on:

1. The use to which the experimental results are to be put. Experiments aimed at increasing the number of plants derived from a given explant for rapid multiplication purposes emphasize the number of individuals produced as well as the biomass per individual. The quality of these individuals is also important.

2. Practical considerations. Factorial analysis aims at assessing the effects of experimental variables on explant productivity over a wide range of conditions. The type of data that can be collected depends on the amount of labour involved in collecting the data, available equipment, the size of the experiment and the need to work under sterile conditions where repetitive measurements are involved.

Generally, it is necessary to use more than one parameter when assessing the effect of an experimental variable as the influence of the variable need not be the same on all parameters. Quantative and qualitative data were considered during this study.

2.10.1 Qualitative Data.

Qualitative data is subjectively determined. Because of its inherent inaccuracy, a minimum of subjective data was accumulated.

Chlorophyll Content. The presence or absence of the chlorophyll pigment in explants was successfully used by Yamada et al. (1978) as a marker in selecting for photoautotrophism. Chlorophyll content appears related to explant viability in terms of plantlet production in Ornithogalum.

2.10.2 Quantitative data.

Three categories of quantitative data were collected in this experiment; demographic data, data concerning growth, and data on the water potentials of nutrient media. Demography is the study of population dynamics - the emphasis is on describing and interpreting changes that occur in the number of individuals. Growth analysis is concerned with changes that occur in the biomass per individual (Hunt, 1978).

2.10.2.1 Demographic Data.

a.) The number of plantlets per explant. These provide valuable information where the experimental emphasis is on maximising the number of individuals per explant. In instances where the plantlets were too numerous to count, the incidence of plantlets was calculated as the percentage of the explant surface they occupied. To avoid variation between estimates, these were made by the same person for the entire experiment.

b.) Shoot length. This was determined to the nearest millimetre, using a ruler.

c.) Root counts. In some instances the presence of roots

on *in vitro* plants determines their ability to survive *in vivo*. Unrooted *in vitro* plants have, however, been shown to survive *in vivo* subculture.

d.) Contamination. The presence of contaminating microorganisms on explants was noted.

2.10.2.2 Growth Analysis Data.

Explant mass.

Mass determinations are more rapidly arrived at than plantlet counts. Furthermore, they are mechanically determined and thus less subjective.

As the large numbers of plantlets and their small masses precluded individual mass determinations, mass changes were calculated as the rate of biomass accumulated per explant.

The mean absolute growth rate ($\bar{G}$) was used to monitor the change in explant mass over time (Hunt, 1978):-

$$1\text{-}2^{\bar{G}} = \frac{{}_2M - {}_1M}{{}_2T - {}_1T}$$

$$\text{Units} = \text{mass.time}^{-1}$$

Where ${}_2M$ is the mass of the explant determined at the end of the specified culture period (${}_2T$)

${}_1M$ is the initial mass of the explant prior to culturing (${}_1T$)

Where the parameter, ${}_1M$ was unknown, mass changes over time were calculated as:-

$$\text{Percentage change in fresh mass} = \frac{({}_2M - {}_3M)}{{}_3M} \times 100\%$$

Where ${}_3M$ is the explant mass measured first during a specified culture period (${}_2T$).

To determine the efficiency with which explants accumulated biomass *in vitro*, the relative growth rate was calculated (Hunt, 1978):-

$$1\text{-}2\bar{R} = \frac{(\log_e {}_2M - \log_e {}_1M)}{{}_2T - {}_1T}$$

This formula was used only to a limited extent as it has to be assumed that all biomass is equally capable of producing more biomass. Furthermore, log masses do not lend themselves to easy interpretation.

Of the three calculations, only $\bar{R}$ supplies information concerning the relative efficiency of biomass accumulation by the explant relative to the initial mass of the explant. The other calculations monitor the change in biomass over time (Hunt, 1978).

i) Fresh mass determinations.

During experiments fresh mass was measured at predetermined intervals to three decimal places, using a top loading balance.

ii.) Dry mass determinations, and calculations of the percentage moisture content of explants.

In order to determine whether increases in the fresh mass of explants resulted from growth processes, or from an increase in the water content of the tissues, the percentage moisture content of the explants was calculated on a fresh mass basis as:-

$$\text{Water content} = 100\% \times \frac{M_f - M_d}{M_f}$$

Where M_f = Tissue fresh mass

M_d = Tissue dry mass

To dry the explants, the microwave drying technique of Ferreira (1982) was used. This technique has the ad-

vantage of being more rapid than conventional oven drying techniques. The minimum drying time was found to be 40 minutes in a 2450 MHz microwave oven (Table 2).

Table 2. The effect of drying time on the moisture content of Ornithogalum 'Rollow' leaf explants.

Drying time (minutes).	Mean moisture content (%) $= 100 \times \frac{M_f - M_d}{M_d}$	θ_{n-1}
30	619	198
35	719	80
40	772	62
45	780	63
n=5		

On Nel's (1981) medium, the moisture content of Ornithogalum 'Rollow' explants was 98 +/- 1,5% of the total fresh mass (n = 10).

2.10.2.3. The water potential (ψ) of the culture medium.

The water potential of a solution depends on the total number of solute particles (molecules or ions) in solution, rather than their kind, or their charge (Salisbury & Ross, 1985). In a solution which consists of different solutes, the water potential is the sum of the individual water potentials contributed by each of the solutes.

The water potential of the medium is measured using the vapour equilibration method, the pressure of water vapour in equilibrium with the water in a medium sample enclosed in a small chamber is measured with a screen cage thermocouple psychrometer. This method is generally regarded as the most accurate of all methods for measuring water potential.

When isothermal conditions are achieved (the temperature of

the air, medium and psychrometer are all equal), a current (5 mA) is passed through the chromel junction of the chromel-constantin thermocouple for about 15 s, cooling it to about Ø,6 ^{o}C. If the thermocouple is cooled to below the dewpoint of the atmosphere surrounding it, water will condense on the junction. Following a specified cooling period, the current is switched off, and the water condensed onto the junction begins to evaporate back into the surrounding atmosphere.

When the water evaporates from the sensing junction, the junction will be cooled. The rate of evaporation of water from the sensing junction is determined by the vapour pressure in the chamber and hence the water potential of the medium. An electromotive force proportional to the difference in temperature between the sensing and reference junction is generated. This is known as the Peltier Effect.

Where the atmosphere adjacent to the thermocouple is at saturated vapour pressure, ambient and dew point temperature will be identical and water will not evaporate from the sensing junction. The situation corresponds to Ø kPa water potential (Ø microvolt readout).

Samples of the culture media were introduced into the screen cage thermocouple (Merrill, Utah) on 1 x 2 cm strips of Whatman # 1 filter paper (Whatman Ltd., England) and the chamber was allowed to equilibrate in a water bath overnight at 3Ø^{o}C before readings were taken.

2.11 Statistical analysis of results.

Variability is characteristic of experimentation. Because of this, the problem of drawing conclusions from an experiment is one of induction from the sample to the population. The statistical theories of estimation and hypothesis test-

ing provide answers which have a known and controlled probability of being correct (Cochran & Cox, 1957). This information allows the experimenter to make decisions based on experimental results.

A statistical hypothesis is one that asserts something about the form of the population being sampled (non-parametric) or about the parameters of the population, the form being assumed (parametric). The distribution of data used in these experiments was assumed to be normal, or checked for normality using histograms and univariate plots from Biomedical Data Processing's P-series (BMDP) 5 D programme (Dixon, 1985).

There are two major reasons why data is assumed to be normally distributed (Stuart, 1967); firstly, according to the central limit theorem, the majority of statistics (any function of experimental observations) in common use have sampling forms which tend to normal forms as the sample size increases. Secondly, the applications of "normal theory" tend to be relatively insensitive to departure from normality. This feature is known as robustness.

Missing data was estimated using BMDP's AM programme (Dixon, 1985). Although estimation techniques can be used to substitute values for data missing as a result of contamination and test tube breakage, the percentage of estimated data should be minimal to ensure accuracy of results. In this study, not more than 20% of the total observations were estimated while not more than 50% of any given case (the measurements made on a given replicate in a treatment) was estimated.

Factorial analysis was used to study the effect variables had on a given parameter and the interaction between these variables. A casual explanation of a hypothesis could be destroyed by empirical evidence, but it could not be proved.

The fact that two variables had apparently similar effects, did not logically imply that there was any causal relationship, direct or indirect, between them.

The majority of estimation problems are approached and solved by using test statistics (a function of a sample of observations that provides a basis for testing a statistical hypothesis) based on maximum likelihood estimates (which maximise the ratio of likelihood estimates under the conditions of the null hypothesis to the absolute maximum of the function). These test statistics are always distributed in Fischer's variance ratio form (Stuart, 1967).

Regression analysis was used to study the dependence of variables on one another. Linear regressions were carried out according to Brown & Hollander (1977). Curves establishing the quadratic relationship between medium water potential and pH were established using Genstat V (copyright Lawes Agricultural Trust, 1984).

Analyses of variance and covariance are distinguished on the basis of the variables involved. In the analysis of variance, the variables are called attributes (the data specifies whether or not a particular event occurred during the culture period). In an analysis of covariance, one or more of the variables is numerically specifiable (e.g. the explant mass).

Analyses of variance and covariance were performed using BMDP's 2 V and 7 D programmes (Dixon, 1985). The omnibus hypothesis that there were no differences between sample means was tested. For treatments, the omnibus hypothesis was:-

$$H = \mu_1 = \mu_2 = ... = \mu_k$$

Where μ_1 = The mean of the first treatment

μ_k = The mean of the k^{th} treatment.

Where differences between means were detected, these differences were quantified using multiple comparison tests. The tests used include the t-tests of Bonferroni & Sidak (Dixon, 1985) and the multiple range test of Student-Newman-Keuls.

The test of the omnibus hypothesis has the disadvantage of being very conservative. It is equivalent to the Scheffe procedure, guarding against error in all possible contrasts (Brown & Hollander, 1977). When a particular small set of contrasts was of special interest, the omnibus hypothesis was disregarded and tests of the specific contrasts were done, adjusting the level of significance to take into account only the small number of inferences that were of interest (Brown & Hollander, 1977).

In this study, the technique for calculating contrasts as supplied by Brown & Hollander (1977) was used. Where controls were not suggested by the experimental design, the Scheffe procedure for calculating confidence intervals was used.

Chi squared tests were also used in this study (Dixon, 1985). The sum of squares of k independent normal observations where each observation emanates from a normal distribution with mean zero and standard deviation one, is the Chi squared distribution with one degree of freedom (Brown & Hollander, 1977).

In this study, BMDP Chi square tests were used. Where significant differences between the treatment means occurred, the significance of these was further assessed, adjusting the results to take into account only the treatment comparisons of interest. Where sample sizes involved were borderline, Yates' continuity correction was used (Brown & Hollander, 1977).

Where variables interacted in a puzzling way, prolonged study of the results or additional experiments was necessary. The problem being that the phenomena involved were complex, not that the experimentation was faulty.

CHAPTER 3.

THE INFLUENCE OF PHYSICAL CHARACTERISTICS OF *ORNITHOGALUM* `ROLLOW' EXPLANTS ON EXPLANT VIABILITY.

The initial explant determines the quality of regenerated plants. In the absence of a suitable explant, success is limited, in spite of optimum provisions of nutrient formulations or culture environment.

This chapter is divided into five sections, each of which assesses the impact of some explant characteristic on explant viability.

Section 1. Growth and regeneration of explants from various *Ornithogalum* `*Rollow*' organs at anthesis.

Section 2. The optimal explant site on *Ornithogalum* `*Rollow*' leaves in terms of regenerative ability *in vitro*.

Section 3. The effect of explant orientation relative to the culture medium on growth yield *in vitro*.

Section 4. The influence of wounding, a mechanical stress on plantlet regeneration from *Ornithogalum* `*Rollow*' explants *in vitro*.

Section 5. The influence of explant size on the productivity of *Ornithogalum* `*Rollow*' explants.

3.1 SECTION 1. Growth and regeneration of explants from various *Ornithogalum* 'Rollow' organs at anthesis.

3.1.1 Introduction.

Selections of *Ornithogalum* plants for breeding purposes can only be made during anthesis. At anthesis, *Ornithogalum* 'Rollow' leaves began to senesce, reducing their regenerative potential. To prevent the loss of a season between plant selection and clonal propagation, alternative explant sources to leaves were sought at anthesis.

After a third of the flowers on the inflorescence opened the regenerative ability of 1,0 x 1,0 cm leaf base explants, 1,0 cm long inflorescence stalk explants and the bracts, in the axils from which branches of the raceme arise were compared. Arbitrarily, the inflorescence stalk below the branches was divided in half. The proximinal end, closest to the bulb is referred to as downstalk and the distal end, closest to the flowers is referred to as upstalk.

Nel's medium (1981) containing 0,1 mg.dm^{-3} NAA and 2 mg.dm^{-3} BAP was used in the experiment. The duration of the culture period was six weeks. After three and six weeks, fresh masses were determined. At the end of the culture period, the number of plantlets per explant and the number of explants lacking chlorophyll were noted. In total, 80 explants were involved in the experiment.

The number of plantlets produced per hundred explants was extrapolated from the number of plantlets produced.

Absolute growth rates ($\overline{G}$) were calculated from the fresh mass data (Hunt, 1978). The distribution of the absolute growth rate data was checked for normality using BMDP programme 5D (Dixon, 1985). A parametric variance

analysis was carried out using BMDP programme 2V (Dixon, 1985). The significance of the treatment effects was assessed using pairwise t-tests.

Absolute growth rates depend on initial explant mass. As explant masses differed significantly, $\bar{G}$ can not be considered an adequate basis for comparing different explant sources. For this reason, the relative growth rate ($\bar{R}$) was calculated, to supply information about the relative efficiency with which different explant sources accumulate fresh mass.

The number of explants lacking chlorophyll was extrapolated from the number of explants from each donor organ lacking chlorophyll.

The incidence of contaminated explants was recorded as a percentage of the total number of explants used for each treatment.

3.1.2 Results.

3.1.2.1. The effect of the donor organ of *Ornithogalum* 'Rollow' used as a source of explants on adventitious shoot formation in vitro.

At anthesis, the bracts of the inflorescence when used as explants are the most prolific source of adventitious shoots (Table 3).

Table 3. Expected number of adventitious shoots per hundred Ornithogalum `Rollow' explants after six weeks in vitro.

Site of explant on the donor organ.	Number of Adventitious shoots.
Bract	569
Leaf	5Ø
Upstalk	Ø
Downstalk	Ø

Adventitious shoots began to differentiate asynchronously on the explants after three weeks in culture. Inflorescence stalks could not be considered satisfactory explant sources, as these failed to differentiate plantlets in vitro (Table 3).

3.1.2.2. The effect of the Ornithogalum `Rollow' donor organ used as an explant source on the rate of fresh mass accumulation in vitro.

A check on the normality of the experimental data showed that, after three weeks in culture, the mean absolute growth rates of the inflorescence stalks were sufficiently distinct from those of the leaves and bracts to be considered a distinct population. After six weeks in culture, explants from all organs showed similar absolute growth rates (Figure 2).

Despite the initial lack of normality in the data (Figure 2), a non-parametric variance analysis was performed which makes no assumptions based on the distribution of the data (Table 4). The F-value obtained is robust for lack of normality.

Table 4. Results of a non-parametric analysis of covariance to determine the effect of the donor *Ornithogalum* 'Rollow' on the absolute growth rate of explants.

Absolute Growth Rate.	$3\text{-}\emptyset^{\bar{G}}$ Variance Ratio.	$6\text{-}\emptyset^{\bar{G}}$ Variance Ratio.
Treatment	22,16 **	1,55
Equality of means test, variances not assumed equal.		
Welch	24,49 **	2,30
Brown-Forsythe	23,61 **	1,57
Levene's test for equality of variance	5,44 **	0,80

** : Highly significant result, Reject H^{O} at p = 0,01

Where $_{3\text{-}\emptyset}\bar{G}$ = (Fresh mass after 3 weeks - fresh mass initially) = Time interval.

$_{6\text{-}\emptyset}\bar{G}$ = (Fresh mass after 6 weeks - fresh mass initially) = Time interval.

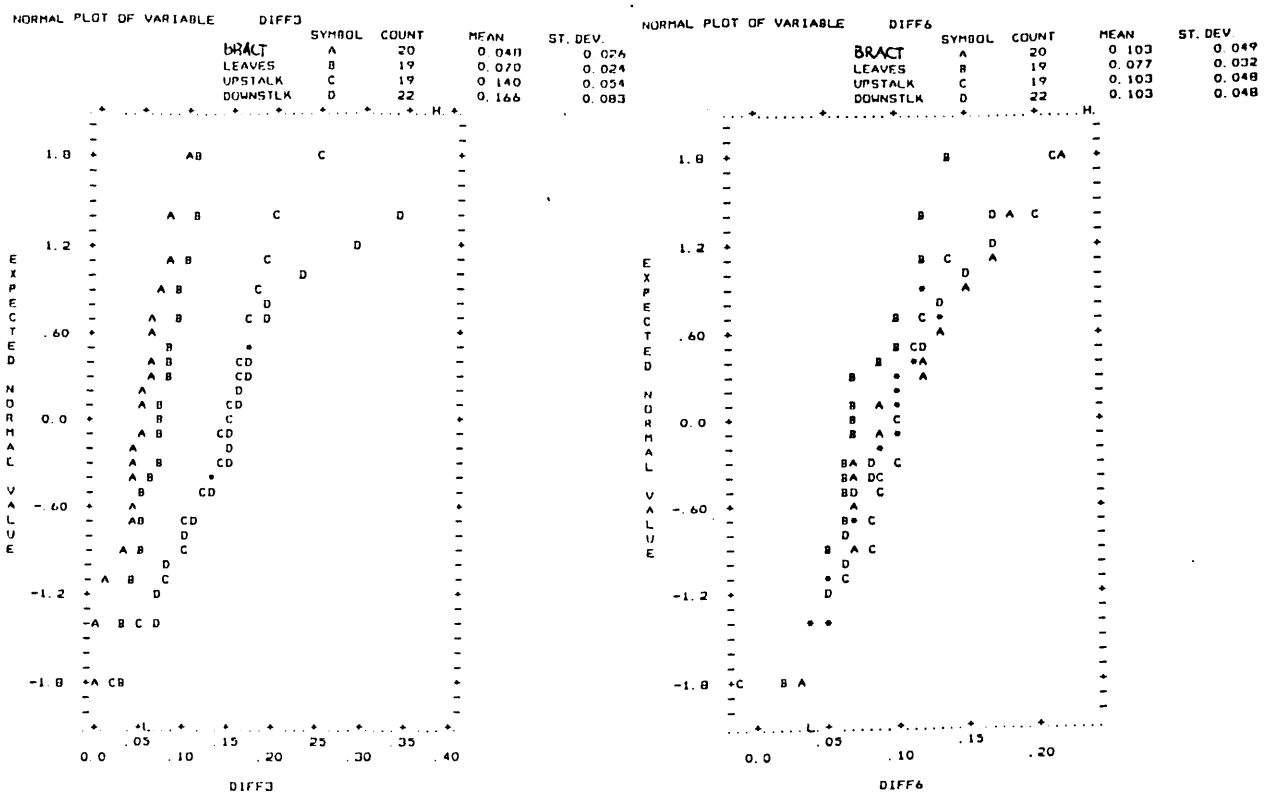
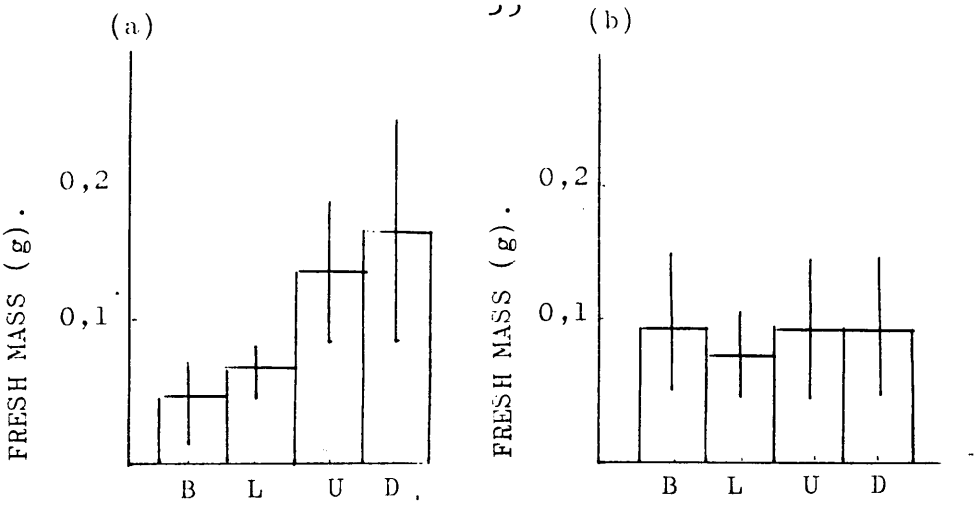


Figure 2. Normal plot of the absolute growth rates of explants from four organs on Ornithogalum 'Rollow' plants.

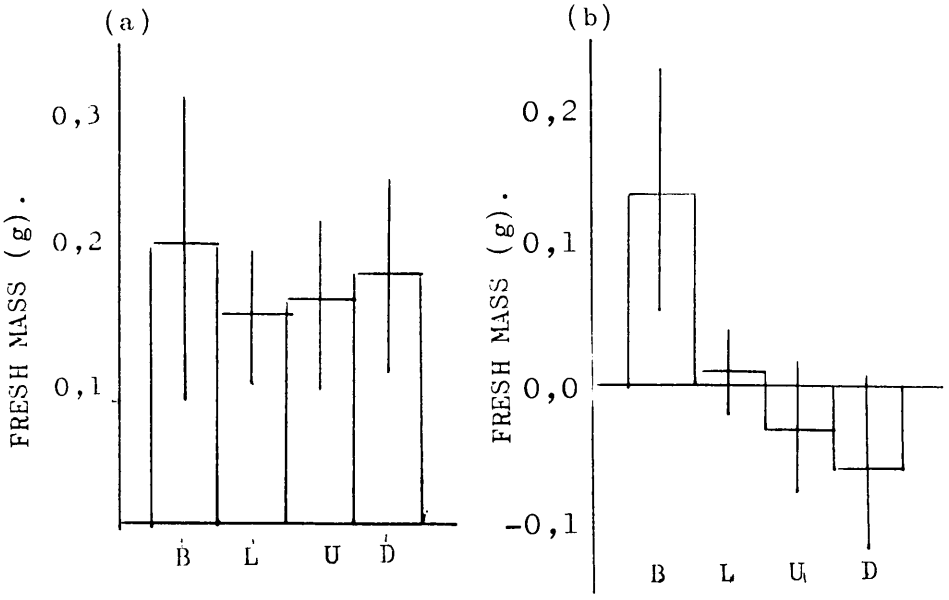
The variance analysis showed that the absolute growth rate of explant sources differed significantly during the first three weeks of the culture period only (Table 4). These differences may be attributed directly to the different initial masses of the explants used in this experiment. Pairwise t-tests ($p = Ø,Ø1$) reveal that inflorescence stalk explants with the highest initial masses (Figure 3) show the greatest fresh mass accumulation during this period (Figure 3.1). After three weeks in culture no distinction between the explant sources on the basis of their absolute growth rates could be made (Figure 3.1).

Absolute growth rates supply no information about the relative efficiency of fresh mass accumulation (Figure 3.1). This information is supplied by mean relative growth rates (Figure 3.2).

During the first three weeks in culture, the relative growth rates of all explants were similar (Figure 3.2). After three weeks in culture, plantlets started to differentiate on leaf and bract explants. The accumulating biomass of developing adventitious plantlets resulted in an increase in the relative growth rates of these explants (Figure 3.2) which was proportional to the number of plantlets initiated (Table 3).



3.1 EXPLANT POSITION.



3.2 EXPLANT POSITION.

Figure 3. The absolute (3.1) and relative (3.2) growth rates of Ornithogalum 'Rollow' explants after three (a) and six (b) weeks in culture. Bract (B); Leaf (L); Upstalk on peduncle (U); Downstalk on peduncle (D).

3.1.2.3. How the chlorophyll content of explants from different organs of Ornithogalum 'Kollow' affects in vitro growth and regeneration.

Leaf explants contained more chlorophyll than explants from other organs (Table 5), despite the onset of leaf-senescence at anthesis.

Table 5. Expected number of explants lacking chlorophyll per hundred Ornithogalum 'Kollow' explants cultured in vitro for six weeks.

Site of explant on donor organ.	Number of explants lacking chlorophyll.
Bract	59
Leaf	28
Upstalk	64
Downstalk	64

No relationship was observed between the chlorophyll content of explants and the regenerative ability of organs from which they were derived (Table 5). However, once chlorophyll was lost, explants no longer differentiated plantlets. The chlorophyll content of explants was not altered by subculturing, although previously differentiated plantlets contain chlorophyll and continue developing.

3.1.2.4. The visible contamination rates observed in explants taken from various plant organs.

The rate of visible contamination of the different explant sources was recorded after six weeks in culture (Table 6).

Table 6. Visible Contamination rates for explants taken from various organs of Ornithogalum `Rollow'.

Site of explant on the donor organ.	% Contaminated explants.
Bract	9
Leaf	14
Upstalk	14
Downstalk	Ø

3.1.3. Summary of results.

- At anthesis, in terms of efficiency of fresh mass accumulation ($\bar{R}$) and plantlet initiation, inflorescence bracts were the most productive source of explants.

- Leaves and inflorescence stalks at anthesis were unsuitable explant sources and failed to differentiate sufficient plantlets for rapid multiplication purposes.

- The number of plantlets produced *in vitro* corresponded well with the efficiency with which fresh mass was accumulated.

3.1.4. Discussion.

The regeneration of members of the genus Ornithogalum through tissue culture from various organs is extensively reported in the literature (Hussey, 1975; 1976; Klesser & Nel, 1976; Nel, 1981; Chung *et al.*, 198Ø). Propagation of these plants from leaves (Nel,1981), bulb scales and inflorescence stalks (Hussey, 1975; 1976) and from stigma, style and ovary tissues (Chung *et al.*, 198Ø) has been reported.

The viability of different organs as sources of explant material differed on Nel's (1981) shoot multiplication medium. At anthesis, the bracts in the axils of the raceme branches were the most prolific explants. Leaf explants were viable but produced fewer plantlets, while inflorescence stalks when used as explant sources failed to produce plantlets.

Plantlets began to differentiate on explants after three weeks in culture. After six weeks in culture, explants differentiating plantlets showed higher relative growth rates than those on which plantlets failed to differentiate.

Absolute growth rates are dependent on initial explant mass. Where these differ significantly when explants from different organs are used, or of different sizes, they supply no information about the efficiency of fresh weight accumulation by explants. A relationship was observed between the chlorophyll content of the explants and the donor organ from which they were derived. No relationship existed between the chlorophyll content of explants and their regenerative ability.

Leaf explants showed the highest incidence of chlorophyll, which may be an indication of a higher cytokinin concentration in these tissues. Cytokinin is known to delay chlorophyll breakdown and senescence (Letham,Goodwin & Higgins, 1978), however, other hormones are also known to be involved in regulating these processes.

The ability of explants to differentiate plantlets represents control at the gene level. Differentiation results from altered enzyme levels in the cells (Bonner in Rawal & Mehta, 1982). The inability of certain organs to differentiate plantlets under media conditions considered optimal in the literature, is attributed to the stage of development of these organs. Scott et al. (1979) noted that under stress

conditions, mature tissues lose their ability to synthesize certain proteins.

Dyer (1976) noted that the onset of senescence among members of the genus relative to anthesis varied. In Ornithogalum `Rollow', leaf senescence was linked to temperature and water stress under glasshouse conditions, while plantlet differentiation by explants stopped with the loss of chlorophyll from explants.

Certain organs appear to have higher contamination loads than others. The contamination load is a function of the disease status of the donor plant, the age of the organ and its exposure to micro-organisms in the soil and air.

3.2. Section 2. The optimal explant site on Ornithogalum `Rollow' leaves in terms of regenerative ability in vitro.

3.2.1. Introduction.

Before anthesis leaves are suitable as sources of explant material for mass propagation of Ornithogalum plants. They are abundant, obtained with minimal damage to the donor plant and are relatively free of micro-organisms (Niimi & Onozawa,1979; Klesser & Nel,1976). Nel (1981) estimates that one leaf of an Ornithogalum cultivar propagated in vitro yields 10^4 plants in a growing season and these flower within ten months.

A decline in the regenerative ability of explants along the length of the explant organ has been noted in Lilium (Niimi & Onozawa,1979) and Lachenalia (Klesser & Nel, 1976) and Narcissus (Denne, 1960). In order to optimise regeneration in vitro, the productivity of explants from four sites along the length of leaves was assessed.

Five mature Ornithogalum 'Rollow' leaves were detached from each of three Ornithogalum donor plants prior to anthesis. Leaves were transversely sectioned into four pieces. The distal portion, closest to the bulb was called the leaf base and sequential portions of the leaf were referred to as midbase, midtip and leaf tip.

Explants 1,Ø x 1,Ø cm were subcultured for two eight week periods on Nel's (1981) basal medium containing Ø,1 mg.dm^{-3} NAA and 2,Ø mg.dm^{-3} BAP. Growth was expressed as the percentage change in mass of the explant over 4 to 8 weeks, 8 to 12 weeks and 12 to 16 weeks, using the formula:

$$\frac{(_2M - _3M)}{_3M} \times 1ØØ = \% \text{ change in fresh mass}$$

where $_3M$ is the mass measured first in time during a specified culture period, and $_2M$ is the mass measured at the end of the specified culture period. This gives the % increase or decrease from $_3M$ to $_2M$. Forty-eight explants were considered.

The number of plants present on the 16Ø explants involved in the study were determined as a percentage of the explant surface occupied by plantlets, at weekly intervals for twelve weeks. This was necessary as differentiating plantlets were too numerous to count in many instances.

Analyses of variance were carried out to assess the relative importance of the experimental variables. The significance of the treatment effects were assessed using the multiple comparison tests of Bonferroni & Sidak which control the frequency of wrong inferences being made from experimental results (Dixon, 1985). In cases where only a specific set of comparisons was of special interest, the tests of Bonferroni & Sidak which guard against the chances of erroneous conclusion being arrived at in all possible contrasts were

set aside. Instead, specific contrasts were made, the confidence limits of which were adjusted (Brown & Hollander, 1977) to take into account only the small number of inferences which were of interest.

Linear regressions (Brown & Hollander, 1977) were used to arrive at relationships between the rate at which plantlet formation and fresh mass accumulation occurred.

3.2.2. Results.

3.2.2.1. The effect of explant site on the donor organ on plantlet initiation.

The site on the leaf from which explants were taken, the donor plant and the length of the culture period all significantly influenced the number of plants produced by *Ornithogalum* 'Rollow' leaf explants *in vitro* (Table 7). Significant interactions between all these factors were also apparent.

Table 7. Results of an analysis of variance showing the effect of donor plant explant site and length of the culture period on *in vitro* plantlet initiation by *Ornithogalum* 'Rollow' explant.

Source of variation.	Variance ratio.
Experimental mean.	349,09 **
Donor Plant	26,70 **
Explant Site	26,64 **
Culture Period	138,19 **
Interactions:-	
Plant x Site	6,80 **
Plant x Culture Period	7,44 **
Site x Culture Period	9,59 **
Plant x Site x Culture Period	3,40 **

** : Highly significant result; Reject H^{O} at p = 0,01.

The leaf base, when compared to the midbase, midtip and tip portions of the leaf was the most prolific source of plants (Table 8). The leaf base was chosen as the control in this experiment, as in the literature it is widely designated as the most prolific source of plantlets (Denne, 1960; Niimi & Onozawa, 1979 and Klesser & Nel, 1976).

Table 8. Results of a contrast in population means showing the influence of explant site on the Ornithogalum 'Rollow' leaf on plantlet formation.

Contrast.	Variance ratio.
Base - midbase	107,95 **
Base - midtip	285,70 **
Base - tip	351,52 **

** : Highly significant result; Reject H^{O} at p = 0,01.

The culture period was the most significant determinant of the number of plants produced per explant (Table 7). The dependence of the number of plants produced on the culture period is given, in this experiment, by the linear relationship y = - 4,59 + 2,86 x (Figure 4). The 95% confidence interval for the slope of the graph is 2,86 +/- 0,23 and 4,59 +/- 1,88 for the intercept. The 95% confidence band for the regression line is an indication of the mean number of plantlets expected after various culture periods. Assuming homogeneity of variance, individual values of y will vary about the regression line with an estimated standard deviation ($\theta_{y.x}$) of 0,92. Thus individual values of y may lie outside the confidence band.

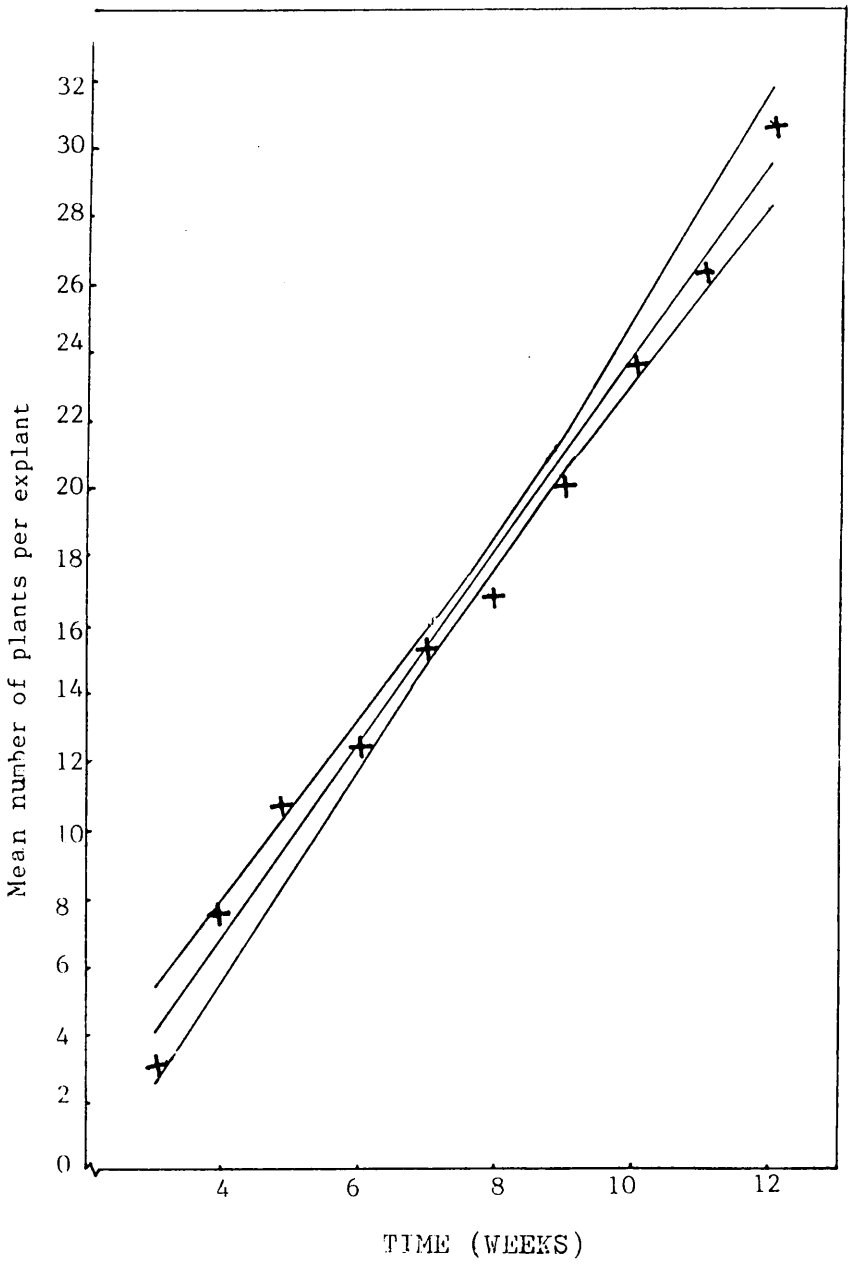


Figure 4. The relationship between plantlet formation on *Ornithogalum* '*Rollow*' explants and the culture period showing the 95% confidence bands for the regression line.

From the analysis of variance (Table 7), it is clear that the site on the leaf from which the explant is removed also influences the rate of plantlet initiation. The rate of plantlet initiation on the explants was found to be a linear function of time for all four explant sites on the leaf (Figure 5).

The intercept of the regression lines did not differ significantly at the 95% confidence limit (Table 9). From the 95% confidence limits for the slope, it is apparent that explants derived from the leaf base have a significantly higher rate of plantlet initiation than those derived from the midbase of the leaf (Table 9). The least satisfactory explants, with the slowest rates of plantlet initiation, were derived from the tip and midtip sections of leaves (Table 9). The standard deviation of individual values from the regression line ($\Theta_{y.x}$) is indicated in Table 9.

Table 9. Linear regression showing the rate of plantlet initiation at various sites on *Ornithogalum* 'Rollow' leaves, and the variance ($\Theta_{y.x}$), slope and intercept for the lines.

Position	$\mu_{y.x}$	$\Theta_{y.x}$	95% c.l. for intercept.	95% c.l.for slope.
Base	y = -2,42 +1,569x	Ø,874	-2,42 +/-Ø,221	1,569+/-1,782
Midbase	y = -Ø,969+Ø,9Ø6x	Ø,512	-Ø,969+/-Ø,129	Ø,9Ø6+/-1,ØØ3
Midtip	y = -1,231+Ø,635x	Ø,282	-1,231+/-Ø,Ø71	Ø,635+/-Ø,574
Tip	y = -1,412+Ø,568x	Ø,223	-1,412+/-Ø,Ø58	Ø,568+/-1,31Ø

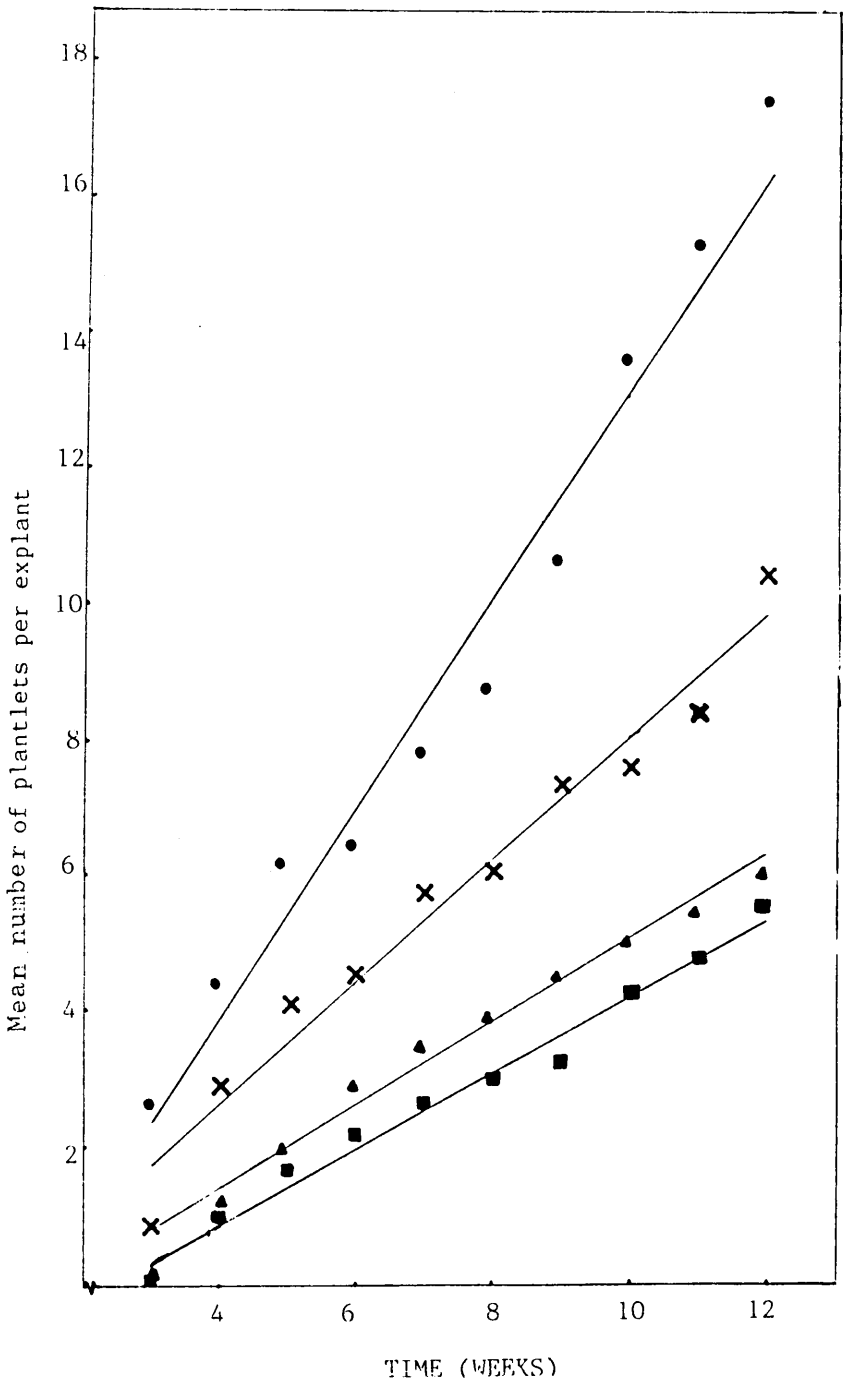


Figure 5. The relationship between plantlet formation on *Ornithogalum* 'Rollow' explants and culture period at different sites on the leaf. Base (•); Midbase (×); Midtip (▲); Tip (■).

3.2.2.2. The effect of explant site on the donor organ on fresh mass accumulation.

In Ornithogalum `Rollow' the site on the leaf from which the explant is derived and the length of the culture period both alone, and through their interaction, determine the efficiency with which fresh mass is accumulated by the explant (Table 10).

Table 10. Results of an analysis of variance to determine the effect of explant site and culture period on fresh mass accumulation as a percentage of initial explant mass.

Source of variation.	Variance ratio.
Experimental mean	195,04 **
Explant site	10,10 **
Culture period	6,68 **
Interaction: Explant site x culture period	2,23 *

** : Highly significant result ; Reject H^{O} at p = 0,01
* : Significant result ; Reject H^{O} at p = 0,05

The greatest increases in fresh mass were associated with explants from the base and midbase of leaves (Table 11). As these were also the sites of the most prolific plantlet initiation in vitro it would appear that biomass accumulation by explants is linked to plantlet initiation in vitro.

Table 11. Results of multiple comparison tests to determine the effect of explant source on the donor organ or change in explant mass (as a percentage).

Explant site	Mean.
Midtip	34,34 %
Tip	41,40 %
Midbase	62,40 %
Base	92,92 %

L.S.D. = 31,40% ; p = 0,01

The rate of fresh mass accumulation by explants was constant, and did not differ significantly over the first and second subculture periods. However, after twelve weeks in culture, explants accumulated fresh mass at significantly higher rates than during the first subculture period (Table 12).

Table 12. Results of multiple comparison tests to determine the effect of culture period on the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants taken from different sites on the leaves in vitro (as a percentage).

Culture period	Mean rate of fresh mass accumulation
4 - 8 weeks	43,56 %
8 - 12 weeks	59,34 %
12 - 16 weeks	70,39 %

L.S.D. = 17,56 % ; p = 0,01

3.2.2.3. The relationship between fresh mass of explants and the incidence of plantlets on explants.

Plantlet formation is strongly time dependent (Table 7). The relationship between these two variables is linear (Figure 5). The rate of change in fresh mass over the culture period is constant (Table 12). This implies the existence of a linear relationship between the fresh mass of the explant and the length of the culture period. Given that plantlet formation and fresh mass accumulation are both linearly related to time, it follows that a linear relationship exists between fresh mass of the explant and the number of plantlets present on the explant.

Knowing the rate at which fresh mass accumulation and plantlet formation occur, it is possible to calculate the relative time dependence of these two growth parameters, as:-
$\tan \alpha = m$, where m is the rate of either parameter. The slope of the graph for the rate of plantlet formation is approximately 45^{o}, while for fresh mass accumulation it is approximately 89^{o}.

This emphasises the strong time dependence of plantlet formation, and explains the relatively low level of significance observed for the interaction of time and fresh mass (Table 7). It appears that, during the first twelve weeks of the culture period, fresh mass is dependent on the number of plantlets on the explant, which, in turn, is time dependent.

3.2.3. Summary of results.

Explants from the base of the leaf have the highest yields in terms of plantlets and fresh mass, making them the most suitable sources of explant material for

rapid multiplication purposes.

During the first 12 weeks, the rate of fresh mass accumulation is linearly dependent on the number of plantlets formed on the explant which in turn is linearly dependent on the length of the culture period.

3.2.4. Discussion.

In order to maximise explant productivity and reduce cost, for mass multiplication purposes, it may be necessary to confine sources of explant material to areas of high regenerative ability on the leaves of donor plants.

In this experiment, explants from the leaf base were found to be the most prolific sources of plantlets, with a decreasing incidence of plantlet regeneration from the base of the leaf to its tip.

Similar gradients in regenerative ability of explants taken from different sites along the length of donor organs have been noted in Lilium (Niimi & Onozawa, 1979), Lachenalia (Klesser & Nel, 1976) and Narcissus (Denne, 1960).

The rate of fresh mass accumulation by explants was related to the number of plantlets initiated by them during the first twelve weeks in culture. Despite the variation to which they are subject, both fresh mass data and counts of plantlet incidence provide information concerning the viability of explants in terms of their ability to produce plantlets.

Both plantlet formation and fresh mass accumulation are linear functions of the culture period. Of the two, fresh mass accumulation is markedly less time dependent. The rate of fresh mass accumulation is linearly dependent on

the number of plantlets formed on the explant, which in turn depends on the culture period.

The degree of regenerative success has been related to the stage of development of the donor organ (Section 1). Gradients in the regenerative ability of explants from different sites on the same donor organ have been explained in the literature in terms of the distribution of chemical substances within the tissues (Stimart & Ascher, 1978; Takayama & Misawa, 1980).

Stimart & Ascher (1978) observed an asymmetric hormone distribution in Lilium tissues associated with a gradient in regenerative potential, while Takayama & Misawa (1980) noted that higher nitrogen and lower sucrose concentrations occurred in the more prolific inner bulb scales of Lilium.

3.3. Section 3. The effect of explant orientation relative to the culture medium on growth yield in vitro.

3.3.1. Introduction.

Reported effects of explant orientation relative to the culture medium on plantlet initiation and growth yield differ considerably in the literature.

In order to assess the importance of this factor in the tissue culture of Ornithogalum cultivars, so as to maximise explant productivity, four orientations of 1,0 x 1,0 cm Ornithogalum `Rollow' leaf base explants were compared. Horizontally orientated explants were placed on the nutrient medium with their abaxial or adaxial epidermis in contact with the medium. Vertically orientated explants were embedded to a depth of half their vertical height in nutrient medium with either the morphological tip (polar orientation) or base (apolar orientation) of the section

uppermost.

Leaves from three different plants were used in the experiment. It was necessary to analyse the results for each plant separately as all four treatments were not applied to each plant. The number of plantlets per explant was determined together with the fresh masses of the explant initially and at four week intervals for twelve weeks. The absolute growth rate ($\overline{G}$) was determined as the difference between the initial mass and each succeeding mass. One hundred and forty-two replicates were involved in this experiment.

The data was analysed using BMDP - P series programmes (Dixon, 1985). Estimation of missing values was made using the AM programme and the within factor analysis of variance using the 2 V programme. The multiple comparison tests of Bonferroni and Sidak were used to assess the significance of differences between the treatment means where these occurred.

3.3.2. Results.

3.3.2.1. The effect of *Ornithogalum* 'Rollow' explant orientation relative to the culture medium on plantlet initiation *in vitro*.

The length of the culture period significantly affected the number of plantlets present on the explant (Table 13). The effect of explant orientation relative to the nutrient medium on plantlet initiation differed between the plants used in the experiment (Table 13). Where the effect of explant orientation was significant, significant interactions occurred between length of culture period and explant orientation (Table 13).

Table 13. Results of an analysis of variance to determine the effect of the explant orientation and the length of the culture period on *in vitro* plantlet initiation for each of the three *Ornithogalum* 'Rollow' donor plants

Source of variation	Variance ratio.		
	Plant # 1	Plant # 2	Plant # 3
Experimental mean	275.53 **	140,42 **	157.06 **
Orientation	20.65 **	13.16 **	2.49
Time	101.24 **	51.26 **	44.83 **
Interaction, Orientation x time	6.62 **	4.25 **	1.39

** : Highly significant result ; Reject H^{O} at p = 0,01.

The multiple comparison tests of Bonferroni and Sidak showed that the least satisfactory orientation in terms of plantlet initiation was the vertical apolar orientation of the explant (Table 14). Explants with polar vertical orientation performed significantly better than vertically orientated explants with apolar orientation.

Table 14. Results of a multiple comparison test showing the effects of different explant orientations on plantlet initiation in vitro for two Ornithogalum 'Rollow' plants.

Orientation	Plant 1 number of replicates	Plant 1 mean	Plant 2 number of replicates	Plant 2 mean
Vertical, apolar	16	6,49	9	4,6
Vertical, polar	17	19,25	11	18,4
Horizontal, abaxial epidermis uppermost	22	18,30	-	-
Horizontal, adaxial epidermis uppermost	-	-	34	17,92

NOTE 1: L.S.D. (least significant difference) for plant 1: varied between 11,76 and 12,68 depending on the sample sizes being compared.

NOTE 2: L.S.D. for plant 2 : The multiple comparison tests of Bonferroni and Sidak did not indicate a significant difference between treatments. As these tests are conservative when compared to the more robust F test, the biggest and smallest means can be considered to differ significantly.

Analysis of the interaction between the effects of length of the culture period and explant orientation showed that for two of the three plants tested, explants with vertical, apolar orientation showed the slowest rate of plantlet initiation in vitro (Figures 6.1 & 6.3). The relative rate of plantlet production peaked between the fourth and fifth weeks in culture for all orientations, except for explants with vertical apolar orientation, the relative rate of plantlet production for which peaked between the fifth and sixth weeks in culture (Figures 6.2 & 6.4). The adverse effect of this orientation appeared to be through its

delaying effect on plantlet initiation and the resultant fresh mass accumulation. At the end of the culture period, the relative growth rates of explants with different orientations were similar, even though the mean number of plantlets per explant differed significantly (Figure 6).

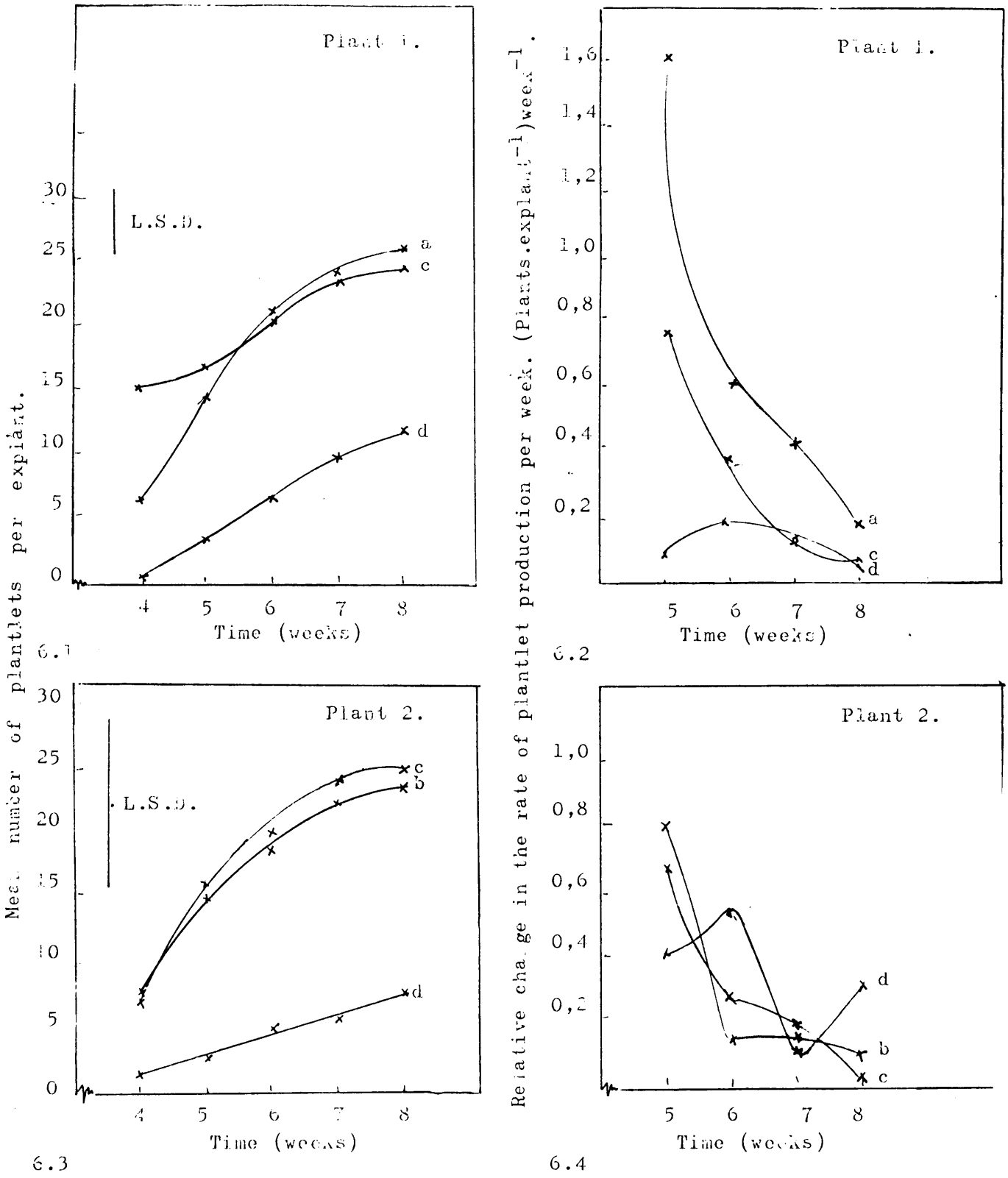


Figure 6. The mean (6.1; 6.3) and relative (6.2; 6.4) rates of plantlet initiation on Ornithogalum 'Rollow' as affected by the orientation of the explant with respect to the culture medium and period. Explant horizontal, abaxial surface uppermost (a); Explant horizontal, adaxial surface uppermost (b); Explant vertical, polar orientation (c); Explant vertical, apolar orientation (d).

3.3.2.2. The effect of explant orientation on fresh mass accumulation in vitro.

Both the length of the culture period and the explant's orientation on the nutrient medium significantly affected fresh mass accumulation. A significant interaction occurred between these two factors (Table 15).

Table 15. Results of an analysis of variance showing the effect of explant orientation and the length of the culture period on fresh mass accumulation by Ornithogalum 'Rollow' explants in vitro.

Source of variation	Variance ratio.
Mean	534,32 **
Orientation	12,58 **
Time	319,Ø **
Interaction of Orientation x Time	14,Ø **

** : Highly significant result ; Reject H^{O} at p = Ø,Ø1

Of the treatments, explants orientated with vertical, apolar orientation, performed significantly worse than those orientated horizontally with the adaxial leaf surface uppermost (Table 16). No significant differences were found between the other orientations.

Table 16. Results of multiple comparison tests of Bonferroni and Sidak showing the effect of the orientation of Ornithogalum 'Rollow' explants in vitro on fresh mass accumulation.

Orientation.	Number of replicates	Mean mass (g)
Vertical, apolar	27	Ø,11
Vertical, polar	48	Ø,21
Horizontal, abaxial epidermis uppermost	32	Ø,23
Horizontal, adaxial epidermis uppermost	35	Ø,27

L.S.D. values vary, depending on the number of replicates between Ø,11 and Ø,13 g.

Vertical, apolar orientation of explants on the nutrient medium, delayed the formation of plantlets (Table 16) resulting in significantly lower fresh masses being associated with this orientation (Table 17). This effect was significant by the twelfth week in culture (Table 17).

Table 17. Interaction table showing the influence of the culture period on the absolute growth rate ($\bar{G}$) of *Ornithogalum* 'Rollow' for various explant orientations.

Orientation.	Length of culture period		
	4 weeks	8 weeks	12 weeks
Vertical, apolar	Ø,Ø5 g	Ø,Ø8 g	Ø,21 g
Vertical, polar	Ø,Ø7 g	Ø,22 g	Ø,53 g
Horizontal, abaxial epidermis uppermost	Ø,1Ø g	Ø,19 g	Ø,41 g
Horizontal, adaxial epidermis uppermost	Ø,1Ø g	Ø,17 g	Ø,36 g

L.S.D. = Ø,16 g.

3.3.3. Summary of results.

- The donor plant determined the effect explant orientation had on plantlet initiation and the resultant rate of fresh mass accumulation *in vitro*.

- Orientating explants with vertical apolar orientation tended to reduce plantlet initiation and delayed the resultant accumulation of fresh mass by the explant. All other explant orientations were without effect.

3.3.4. Discussion.

Variable responses to explant orientation relative to the basal medium in terms of growth yield are reported in the literature. Paek (1982) found that apolar vertical orientations of *Hyacinthus orientalis* explants stimulated plantlet initiation, while Wietsma (1984) reported that polar or apolar orientations of *Fritillaria persica* explants had no effect on the number of plantlets they initiated *in vitro*.

Of the explant orientations tested in this experiment, only vertical, apolar explant orientation affected growth. In two of the three plants tested, this orientation reduced plantlet initiation and the resultant accumulation of fresh mass by the explant.

Two hypotheses are proposed to explain the adverse effect of vertical, apolar explant orientation on growth yield:-

i. The orientation of the explant affects the distribution of auxin in the tissues. This in turn affects the hormone balance in the explant and their interaction with the growth medium.
ii. Embedding part of the explant in the medium affects the oxygen tension in the tissues.

The adverse effect of vertical, apolar leaf explant orientation on growth yield implicates asymmetrical distribution of some substance in the plant. The polar transport of auxin within plant tissues *in vitro* (Pilet, 1968; Goldsmith, 1977 and Moore, 1979) and its beneficial effect on plantlet initiation *in vitro* implicate the involvement of this growth regulator.

There are two major pathways of auxin transport in plant tissues (Goldsmith, 1977). The first and most important of these is transport via the phloem (Goldsmith, 1977). In explants, callose deposition in sieve tubes (Esau, 1960) as a result of wounding and the small distances involved, would make the gradients involved small. Despite these factors, gradients in regenerative potential in explants are reported (J.G. Janse van Rensburg, pers.comm.), as well as relationships between the site of vascular tissue in explants and the site of plantlet initiation (Yanagawa & Sakanishi, 1980b).

The second pathway of auxin transport is via the epidermis,

cortex, xylem and pith (Goldsmith, 1977). Goldsmith (1977) attributes the polarity of auxin transport in living cells to the asymmetric distribution of membrane bound carriers within cells. Reorientating the explants would affect the distribution of auxin within the tissues. The auxin content of the basal medium would also affect the ability of these explants to initiate plantlets *in vitro*.

The altered distribution of auxin in the cells also affects the levels of other plant growth regulators. Saltveit & Dilley (1978) found that normal, vertically orientated *Pisum sativum* explants produced more ethylene than randomly orientated explants. Ethylene was shown to stimulate cell division in daffodil and hyacinth tissues (Kamp & de Hertogh, 1986). Auxin stimulates ethylene production (Moore, 1979), endogenous and exogenous sources are equally effective. Abeles & Rubinstein (1964) found that the threshold concentration of auxin for ethylene synthesis was $1Ø^{-6}$M. Nel's (1981) medium contains 5,37 x $1Ø^{-7}$M of the auxin napthaleneacetic acid. This concentration is too low to stimulate ethylene synthesis. However, all cells are potentially capable of synthesising auxin from its precursor tryptophan (Letham *et al.*, 1978).

All members of the *Liliacae* tested by Hussey (1976) were capable of producing plantlets without the addition of exogenous auxin to the medium. This implies that the concentration of ethylene in tissues is determined by their endogenous auxin concentration. Abeles & Rubinstein (1964) found that in *Pisum sativum* both synthetic and natural auxins are capable of inducing ethylene formation. The rate of ethylene synthesis increases relative to the auxin concentration up to a threshold value, after which no further stimulation occurs (Moore, 1979).

The second reason proposed for the inhibitory effect of vertical explant orientation on growth was the adverse ef-

fect of reduced oxygen tensions in embedded tissue on growth. The effect of anaerobic conditions on growth depends on the tissue tolerance for low oxygen concentrations and the leaf anatomy, which may prevent the oxygen tension ever becoming low enough to affect growth (Saltveit & Dilley, 1978). In this experiment, oxygen tension did not limit growth, as, although apolar vertical orientation of explants adversely affected growth, polar vertical orientations did not.

It is not known why explant orientations affected plants differently. Robb (1957) working on Lilium speciosum observed that the auxin requirement for plantlet initiation on explants varied, depending on the stage of development of the donor plant. Presumably variations in plant hormone levels during development (Saniewski, Rudnicki & Nowak, 1978) would affect the explant's response to a given orientation. In addition, Saltveit & Dilley (1978) found that ethylene synthesis in response to auxin only occurred in meristematic, but not in fully differentiated tissues.

3.4. Section 4. The influence of wounding, a mechanical stress, on plantlet regeneration from Ornithogalum 'Rollow' explants in vitro.

3.4.1. Introduction.

Van Aartrijk & Blom-Barnhoorn (1983) reported that additional wounding to Lilium speciosum explants increased the incidence of plantlet formation on bulb scale explants. Wounding is known to stimulate certain growth processes (Abeles, 1973). The first record of plant wounding is a frieze, which dates from 1100 B.C. of Egyptians gashing sycamore figs to stimulate ripening.

Wounding is a simple technique to implement, and the plantlets which result are formed with the minimum use of

synthetic agents. Where increases in the number of plantlets per explant are found in response to wounding these must be offset against additional labour involved and possible increases in contamination rates which result from increased handling of the explants.

Explants 1,0 x 1,0 cm in size were obtained from basal sections of the leaves of three different plants. The effect of additional wounding in the form of a grid of nine small squares cut into the adaxial epidermis was tested against an unwounded control. Each treatment comprised 73 replicates.

Nel's (1981) medium containing 0,1 $mg.dm^{-3}$ NAA and 2,0 $mg.dm^{-3}$ BAP was used in this experiment. The duration of the culture period was 12 weeks. The absolute growth rate was calculated by subtracting the initial mass from the mass after 4,8 and 12 weeks in culture.

The effect of wounding on the rate of plantlet formation and fresh mass accumulation was determined using BMDP programme 3 D (Dixon,1985). Separate t-tests were calculated for each time interval. Eighty-four explants were involved in the study.

3.4.2. Results.

3.4.2.1. The effect of additional wounding to the *Ornithogalum* 'Rollow' explants on plantlet initiation *in vitro*.

Wounding the explant significantly reduces the number of plantlets formed on the explants *in vitro* (Table 18). The rate at which plantlet initiation occurs was adversely affected by wounding (Figure 7).

Table 18. Results of t-tests to determine the effect of additional wounding to the Ornithogalum 'Rollow' explants on plantlet initiation on explants in vitro.

Time (weeks)	Test statistic	Mean number of plantlets on wounded explants	on control explants
3	-5,22 *	2,51	8,23
4	-5,74 *	4,88	13,49
5	-5,59 *	6,91	18,49
6	-5,31 *	8,51	19,53
7	-4,83 *	11,07	23,40
8	-4,43 *	13,40	26,40
9	-4,34 *	14,81	32,14
10	-4,79 *	16,19	40,84
11	-4,88 *	19,09	46,05
12	-5,60 *	19,67	52,23

Reject H^{O} at $-2>t>2$:

* Significant result, probability = 0,05.

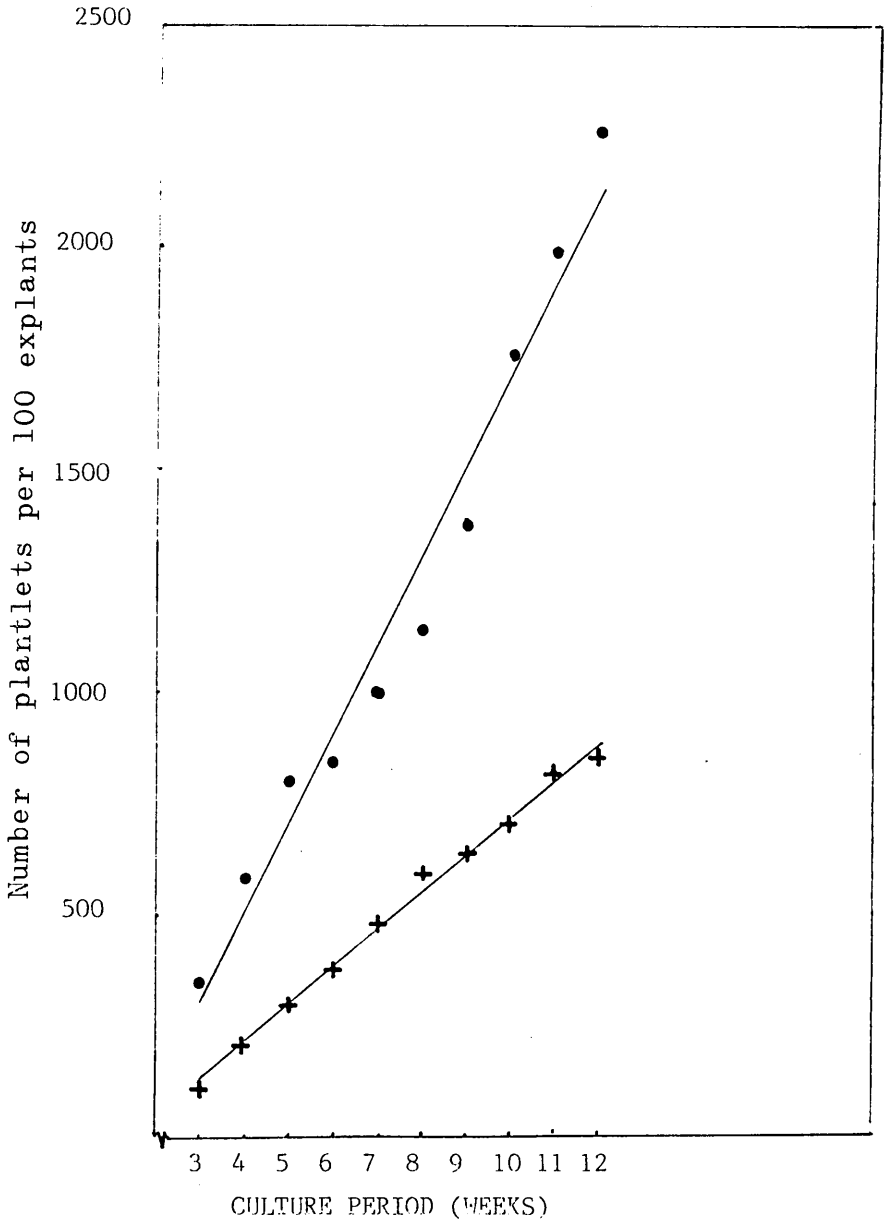


Figure 7. The rate of plantlet initiation *in vitro* on wounded explants and unwounded controls of *Ornithogalum* 'Rollow'. Wounded explants (×); unwounded explants (•).

3.4.2.2. The effect of additional wounding to the *Ornithogalum* 'Rollow' explants on the fresh mass accumulation rate *in vitro*.

Initially, (during the first eight weeks in culture) wounding had no significant effect on the rate of fresh mass accumulation *in vitro* (Table 19). After twelve weeks in culture, the growth rate of the wounded explants was significantly lower than that of unwounded explants. This difference was attributed to the accumulation of biomass by the developing plantlets, which were more prevalent on the unwounded, control explants.

Table 19. Results of t-Test showing the effect of wounding on the growth rate of *Ornithogalum* 'Rollow' *in vitro*.

Time (weeks)	4	8	12
Test statistic (t)	1,12	-0,10	-2,27 *
Mean fresh mass of wounded explants	0,10	0,15	0,24
Mean fresh mass of unwounded explants	0,09	0,15	0,33

* : Significant result ; Reject H^{O} at p = 0,05.

3.4.3. Summary of Results.

- Additional wounding to explants adversely affected initiation of plantlets on the explant. However, the subsequent development of the initiated plantlets was unaffected.

- After twelve weeks in culture, the absolute growth rate of wounded explants was lower than that of unwounded controls. Accumulation of biomass by dif-

ferentiating plantlets was responsible for this difference.

3.4.4. Discussion.

Stimulation of plantlet initiation in response to wounding is extensively reported in the literature; in Ornithogalum thyrsoides (Hussey, 1976), Lilium speciosum (van Aartrijk & Blom-Barnhoorn, 1983), Hyacinthus orientalis (Paek, 198[illegible]) and Populus (Douglas, 1985).

In this experiment, additional wounding to Ornithogalum Rollow explants was found to reduce the incidence of plantlets. The accumulation of biomass by additionally wounded explants was also reduced, as the greatest increases resulted from plantlet development subsequent to differentiation.

Wounding results in a biphasic increase in dark respiration which was proportional to the wound area but independent of the leaf age (Macnicol, 1976). The first phase peaks after 15 - 40 minutes, persisting for 3 hours at most and is the true "wound" respiration. The magnitude of this response bears no relationship to the number of plantlets induced (Brown & Thorpe, 1980 b). The "induced" or "developed" respiration occurs after 1 - 2 hours and may last several days (Macnicol, 1976).

The consistent increase in respiration rates associated with wounding implies metabolic control. The magnitude of the induced respiration depends on RNA and protein synthesis (Macnicol, 1976).

The stimulatory effect of wounding on plantlet initiation is probably linked to the high energy and reducing power requirements of this process. As a result of wounding, there is increased citric acid cycle, pentose phosphate

pathway and glycolytic activity (Macnicol, 1976). The levels of ATP and hexose monophosphates in the tissues also increase (Macnicol, 1976).

Despite the potentially stimulatory effect of wounding the explant on organogenesis, additional wounding to the explant in this experiment proved inhibitory to plantlet initiation.

The effect of additional wounding to explants on organogenesis is determined by the explants ability to recover from membrane damage (van Aartrijk, Blom-Barnhoorn & Bruinsma, 1985 b). The wound effect extends 2 - 5 mm from the cut surface (van Aartrijk & Blom-Barnhoorn, 1983). Free radical mediated peroxidation of fatty acids in the wound-damaged membranes results in ethane (C_2H_6) release (van Aartrijk, Blom-Barnhoorn & Bruinsma, 1985 a & b). These authors found that ethane production is proportional to the amount of membrane damage and is stimulated by high temperature, additional wounding, auxin and aminoethoxyvinylglycine (AVG) - an inhibitor of protein synthesis which prevents the formation of 1 - aminocyclopropane - 1 - carboxylic acid (ACC) synthase in the tissues.

Initially, the synthesis of ethylene in tissues via the pathway (Yu & Yang, 1980):-

> Methionine → S adenosylmethionine (SAM) → 1 - aminocyclopropane - 1 - carboxylic acid (ACC) → ethylene (C_2H_4)

is inhibited by wounding, as the enzyme ACC synthase, responsible for the conversion of ACC to ethylene, is membrane bound. ACC accumulates in the tissues, acting as a free radical scavenger, inhibiting further peroxidation of fatty acids in the membrane and helping to re-establish membrane integrity (van Aartrijk, Blom-Barnhoorn & Bruinsma, 1985 a & b).

Once membrane integrity is re-established, ethylene is synthesized. By competing allosterically with auxin for membrane bound transport sites (Goldsmith, 1977), ethylene may affect auxin distribution within explants and thus their organogenic ability.

Macnicol (1976) observed an inverse relationship between energy charge and distance from the wound surface. The decreased organogenic ability of Ornithogalum `Rollow' explants in response to additional wounding was probably a consequence of the energy required to restore membrane integrity.

3.5. Section 5. The influence of explant size on the productivity of Ornithogalum `Rollow' explants.

3.5.1. Introduction.

The size of the explant determines its ability to survive and the rate at which its derivatives grow and multiply *in vitro* (Murashige, 1977).

Determining the optimal explant size is of interest in mass multiplication of Ornithogalum cultivars, as the yield per explant can be maximized.

The aim of this experiment was to determine whether plantlets arise in association with wound surfaces on the explant as well as to what extent the size of the explant determines its organogenic response.

Leaf explants obtained from three plants of the cultivar Rollow were divided into explants 1,0 x 1,0 cm; 1,0 x 0,5 cm and 0,5 x 0,5 cm in size. The choice of explant size was determined by the culture vessel's diameter (a test tube). One hundred and sixty-one explants were involved

in this study. Twenty-one replicates of each explant size for each plant were used. The explants were cultured on Nel's (1981) medium containing 0,1 $mg.dm^{-3}$ NAA and 2,0 $mg.dm^{-3}$ BAP for 16 weeks. Explants were orientated horizontally on the medium with their abaxial surface in contact with the medium. At four week intervals, fresh mass was determined. The number of plantlets per explant was determined at weekly intervals. Missing values in the data as a result of contamination and test tube breakage were estimated using BMDP's AM programme. Analyses of variance for fresh mass data were made using programme 2V. Chi squared tests were used to assess the impact of different variables on the treatment effect.

3.5.2. Results.

3.5.2.1. The effect of explant size on plantlet initiation in vitro.

Chi squared tests carried out on the data showed that the size of the explant significantly affected the incidence of plantlets (Table 20).

Table 20. Results of a Chi squared analysis of the incidence of plantlets on explants during the culture period.

Time (weeks).	Explant size comparisons (cm).		
	1,0x1,0/1,0x0,5	1,0x1,0/0,5x0,5	1,0x0,5/0,5x0,5
4	19,68*	3.,85*	21,74*
5	19,06*	18,41*	72,84*
6	61,84*	22,39*	49,06*
7	74,37*	20.09*	53,90*
8	68,55*	12,06*	59,50*
9	119,44*	36,22*	70,28*
10	122,87*	42,69*	89,81*
11	118,15*	41,26*	82,48*
12	169,37*	72,81*	105,12*
13	198,39*	123,43*	95,27*
14	247,09*	110,02*	92,36*
15	241,88*	107,51*	83,39*
16	239,22*	130,09*	118,32*

* : Significant result; Reject H^{O} at p = 0,05.

It was hypothesized that the production of plantlets by explants was proportional to the wound surface on the explant. The plantlet production was made comparable by weighting the 1,0 x 1,0, 1,0 x 0,5 and 0,5 x 0,5 cm explants with factors of 1,0, 1,33 and 2,0 respecively.

Table 21. Results of a within-factor anova on data weighted to determine whether plantlets were formed in association with wound surfaces on Ornithogalum 'Rollow' explants.

Source of Variation.	Variance Ratio.
Donor Plant	14,17*
Explant size (weighted)	Ø,48
Interaction of Donor Plant x Explant Size	1,58
Culture Period	22,93**
Interaction of Donor Plant x Culture Period	1Ø,55**
Interaction of Explant size x Culture Period	Ø,65
Interaction of Donor Plant x Explant size x Culture Period	1,39**

** : Highly significant result; Reject H^O at p = Ø,Ø1.

* : Significant result; Reject H^O at p = Ø,Ø5

The analysis of variance results showed that plantlets differentiated in association with wound surfaces on the explants and that the number of plantlets differentiated was constant per unit wound surface area (Table 21).

As a result of the significant effect of donor plants and culture period on plantlet differentiation (Table 21) the anova was repeated (Table 22). The donor plants were considered separately because they differed significantly in the number of plantlets they differentiated (Table 22). As repeated measurements were made on the same set of explants over the culture period, separate analyses of variance were conducted for each time interval (Table 22).

Table 22. Results of parametric and non-parametric anovas for each time interval to determine whether, in terms of the hypothesis, for the three donor plants used in this experiment plantlet initiation is determined by the wound surface on the Ornithogalum 'Rollow' explant.

Time (weeks)	4	5	6	7	8	9	10	11	12	13	14	15	16
DONOR PLANT 1. Analysis of variance.													
Size of explant	0,35	0,89	0,91	0,65	0,85	0,95	1,10	0,89	0,93	1,10	1,55	1,37	0,70
Equality of means test; variances are assumed equal.													
Welch	0,33	0,88	1,05	0,78	1,00	1,20	1,38	1,13	1,20	1,36	1,93	1,59	0,84
Brown-Forsythe	0,37	0,91	0,81	0,57	0,73	0,81	0,94	0,76	0,79	0,93	1,31	1,18	0,59
Levene's test for equality of variance.	0,65	0,92	2,47	2,53	2,62	3,95*	3,89*	4,19*	4,39*	5,36*	5,38*	4,44*	5,10*
DONOR PLANT 2. Analysis of variance.													
Size of explant	2,49	1,76	3,34*	3,07	2,50	3,37*	3,17	2,95	3,40*	4,10*	4,90*	4,65*	4,82*
Equality of means test; variances are assumed equal.													
Welch	0,90	1,10	2,84	3,11	3,31	3,35	3,01	2,39	2,34	2,40	2,86	3,05	2,90
Brown-Forsythe	2,42	1,72	3,27	3,01	2,47	3,30	3,12	2,90	3,34	4,01*	4,79*	4,56*	4,72*
Levene's test for equality of variance.	10,63**	5,67**	23,57**	16,93**	13,66**	13,09**	11,50**	8,86**	11,62**	14,38**	20,32**	19,01**	18,50**
DONOR PLANT 3. Analysis of variance.													
Size of explant	1,55	2,19	2,16	2,30	2,25	2,05	1,75	1,80	1,40	1,35	1,56	1,63	1,62
Equality of means test; variances are assumed equal.													
Welch	0,25	0,77	0,64	0,73	0,81	0,95	0,66	0,58	0,65	1,81	1,98	2,04	2,02
Brown-Forsythe	1,97	3,11	2,96	3,19	3,21	3,09	2,57	2,65	2,19	2,11	2,40	2,46	2,47
Levene's test for equality of variance.	8,73**	12,82**	12,69**	14,49**	13,56**	10,59**	8,10**	8,81**	5,56**	5,34**	6,77**	7,11**	7,28**

** : Highly significant result ; Reject H^0 at $p = 0,01$.

* : Significant result ; Reject H^0 at $p = 0,05$.

The results of the variance analyses (Table 22) support the hypothesis that the number of plantlets formed is determined by the wound surface area on the explant. Over the range of explant sizes tested in the experiment, no decline in the ability of explants to form plantlets was observed using smaller explants (Tables 21 and 22).

3.5.2.2. The effect of explant size on fresh mass accumulation in vitro.

The number of plantlets initiated on a given wound surface area was constant, regardless of explant size. If it can be assumed that fresh mass is accumulated by explants as a result of plantlet formation, then explant fresh mass will be proportional to the cut surface on the explant at any given time. If this hypothesis is correct, then by weighting 1,Ø x 1,Ø cm explants by a factor of one, 1,Ø x Ø,5 cm explants by a factor of one and a third, and Ø,5 x Ø,5 cm explants by a factor of two, the different sized explants could be compared on the basis of wound surface area.

To test this hypothesis, the data was weighted by these factors and an analysis of variance was performed (Table 23). As the experimental error to which the data was subject was not normally distributed, the data were transformed by a factor $y^{Ø,25}$.

Table 23. Results of within-factor analysis of variance on transformed ($y^{0,25}$) fresh mass data weighted on the basis of wound surface area on the Ornithogalum 'Rollow' explants.

Source of Variation.	Variance Ratio.
Donor Plant	16,578**
Size of Explant	5,623**
Interaction; Donor Plant x size of Explant	2,797*
Culture Period	14,581**
Interactions; Donor Plant x Culture Period.	2,767*
Explant size x Culture Period.	2,558*
Donor Plant x Explant size x Culture Period.	1,852*

** : Highly significant result ; Reject H^O at p = 0,01.

* : Significant result ; Reject H^O at p = 0,05.

Although all explants were expected to accumulate fresh mass at the same rate based on the wound surface area, as a result of plantlet differentiation, this did not occur (Table 23). The size of the explant, the donor plant from which the explant was obtained and the culture period, as well as the interaction of these three factors, significantly affected the rate of fresh mass accumulation by explants (Table 23).

Least significant differences were calculated between the means of the transformed data. The weighted means are shown (Table 24).

Table 24. Means (in grams) weighted on the basis of wound surface area for comparison purposes, showing the factors affecting the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants.

Donor Plant	Culture period (months)	Explant size (cm) 1,0x1,0	1,0x0,5	0,5x0,5	Mean fresh mass
1	1	0,130	0,119	0,128	0,126
	2	0,160	0,168	0,158	0,162
	3	0,166	0,201	0,170	0,178
	4	0,184	0,255	0,214	0,218
Mean fresh mass:		0,160	0,186	0,167	0,171
2	1	0,095	0,062	0,072	0,076
	2	0,126	0,070	0,063	0,086
	3	0,143	0,064	0,038	0,082
	4	0,187	0,070	0,059	0,106
Mean fresh mass:		0,138	0,066	0,058	0,088
3	1	0,070	0,088	0,081	0,079
	2	0,095	0,114	0,079	0,096
	3	0,096	0,110	0,080	0,095
	4	0,109	0,135	0,070	0,104
Mean fresh mass:		0,092	0,112	0,077	0,094
Mean mass of donor plants.	1	0,098	0,090	0,093	0,094
	2	0,127	0,117	0,100	0,115
	3	0,135	0,125	0,096	0,119
	4	0,160	0,153	0,114	0,143
Mean fresh mass of donor plants.		0,130	0,121	0,101	0,117

The rate of fresh mass accumulation by the explants differed according to the donor plant. Fresh mass data for plant 1 and plant 3 supported the hypothesis ($p = 0,05$), while fresh mass data for plant 2 did not (Table 25). In the case of plant 2, the 1,0 x 1,0 cm explants accumulated more fresh mass relative to the other explant sizes than expected under the hypothesis.

During the first month in culture, the accumulation of fresh mass attributable to plantlet initiation was similar for all explant sizes ($p > 0,05$). For 1,0 x 1,0 cm explants, the most significant increase in the mass of plantlets was observed after two months in culture ($p = 0,01$). On the 1,0 x 0,5 cm explants, the most significant increase in the mass of plantlets was observed at four months in culture ($p = 0,05$). No significant increase in the mass of plantlets on 0,5 x 0,5 cm explants was observed during the four month culture period ($p > 0,05$).

The mean mass of plantlets initiated on different sized explants was compared (Table 25). Initially it was found that plantlets initiated on 1,0 x 1,0 cm explants weighed more than those initiated on smaller explants (Table 25). However, after four months in culture, plantlet mass was the same, regardless of the initial size of the explant (Table 25).

Table 25. The mean mass (in grams) of plantlets on different sized explants at one month intervals during the culture period.

Culture Period (months)	Size of explant (cm) 1,0x1,0	1,0x0,5	0,5x0,5
1	0,093	0,051	0,087
2	0,034	0,016	0,023
3	0,027	0,015	0,013
4	0,020	0,020	0,020

3.5.3. Summary of Results.

- Donor plants differed in ability to differentiate plantlets and the rate at which they accumulated fresh mass.
- Plantlets differentiated on the cut (wound) surface on explants.
- The smaller the explant, the more economical its use for regenerating plantlets.
- The larger the explant, the larger the initial mass of the plantlets regenerated.
- After 16 weeks in culture, the mass of plantlets was unaffected by the size of the explant from which they were derived.

3.5.4. Discussion.

Changes in tissue metabolism in response to wounding are similar to those following the induction of organogenesis in tissues. Cytochemical investigations by Patel & Berlyn (1983) revealed various steps leading to adventitious shoot development *in vitro*:-

1. Increased DNA, RNA and basic nuclear protein synthesis.
2. Accumulation of lipid, protein and starch reserves.
3. Utilisation of reserves in organogenetic domains and their mobilisation in subadjacent tissue by lipase and amylase activity.
4. Organ formation concomitant with increased enzyme activity associated with energy production and utilisation, increased acid phosphatase, glucose - 6 - phosphatase and succinate dehydrogenase activity. Intense localisation of reducing substances, chiefly ascorbic acid and peroxidases.

In response to wounding, Macnicol (1976) observed similar metabolic changes. A rise in the respiration rate (induced respiration) occurred in response to wounding. The magnitude of this respiration was proportional to the rate of DNA, RNA and protein synthesis. The induced respiration was accompanied by increased glycolytic cycle, tricarboxylic acid cycle and pentose phosphate pathway activity, as well as synthesis of polyphenols, oxidative and hydrolytic enzymes and phenylalanine ammonium lyase.

van Aartrijk & Blom-Barnhoorn (1983) and Douglas (1985) report an interaction between explant size and productivity: the incidence of plantlets on explants rose

with decreasing explant size up to some minimum explant size, after which it decreased once again. This effect was attributed to the wound response metabolism of the tissues (van Aartrijk & Blom-Barnhoorn, 1983); smaller explants have relatively greater wound surfaces than larger explants.

The inhibitory effect of extensive wound areas on the ability of explants to initiate plantlets in vitro is probably the result of the low energy status of the tissues. Macnicol (1976) observed that the greater the relative wound surface on an explant, the higher the induced respiration rate. According to van Aartrijk & Blom-Barnhoorn (1983) the wound effect extends 2 - 5 mm from the cut surface of the explant. By dividing 1,0 cm leaf discs into an inner and an outer ring, Macnicol (1976) showed a similar rise in the level of hexose monophosphates in both areas of the disc. However, ATP in the inner ring was always higher than in the outer ring, suggesting that wounding lowered the available ATP level in the outer disc tissue.

In this experiment, plantlets differentiated in association with cut (wound) surfaces on explants. A constant number of plantlets differentiated per unit wound surface on the explant, regardless of the size of the explant. As smaller explants have a relatively larger cut surface area per unit area, 0,5 x 0,5 cm explants were the most economical explants to use for regenerating plantlets, in terms of the amount of tissue required.

Although the larger explants had plantlets that initially gained mass rapidly, after sixteen weeks in culture no difference could be observed between the mass of plantlets derived from different sized explants.

CHAPTER 4.

THE EFFECT OF PHYTOHORMONONES ON ORGANOGENESIS IN ORNITHOGALUM LEAF EXPLANTS IN VITRO.

4.1 Introduction.

Organogenesis comprises cytodifferentiation and morphogenesis. Cytodifferentiation refers to the de-differentiation of a cell and the formation, by means of cell division, of a cluster of cells in close contact, usually confined within the old cell wall, which are competent to be stimulated to organogenetic growth. Such a group of cells is referred to as a generalized primordium (Letham, Goodwin & Higgins, 1978).

During morphogenesis, organs re-differentiate. Differentiation depends on the activation, evocation or release of a wide group of genetic factors which produce proteins responsible for differentiation (Thimann, 1977).

Gene expression, or protein synthesis, has been shown to be regulated by plant growth regulators at both the transcriptional and translational levels (Moore, 1979).

It is the balanced interaction of hormones which is responsible for organogenesis (Thimann, 1977). However, while the commitment to an organ type depends on the hormonal environment, this in turn is governed by environmental factors (notably light, temperature, medium choice and other nutritional factors) and genetic factors (ploidy, age of the primordium and strain differences) of both the explant and the donor plant (Letham, Goodwin & Higgins, 1978).

The aim of the investigations reported in this chapter was

to assess the role of the phytohormones Napthaleneacetic acid (NAA) and Benzylaminopurine (BAP) and their interaction on *in vitro* plantlet initiation and the related rate of fresh mass accumulation by *Ornithogalum* 'Rollow' and *Ornithogalum maculatum* leaf explants *in vitro*.

Explants, 1,0 x 1,0 cm in size, derived from leaves of greenhouse grown donor plants were sterilized and innoculated onto Nel's (1981) medium. Four different auxin concentrations (0,0; 0,1; 1,0; 2,0 $mg.dm^{-3}$ NAA) were combined with four different cytokinin concentrations (0,0; 1,0; 2,0; 4,0 $mg.dm^{-3}$ BAP) to yield a total of 16 different hormone combinations. The explants were cultured for 16 weeks (*Ornithogalum* 'Rollow') and 12 weeks (*Ornithogalum maculatum*) and subcultured at 8 week intervals.

Initially, 20 replicates were used in each treatment, although some losses occurred as a result of contamination and test tube breakage. Data on the number of plantlets initiated by explants in response to the phytohormone concentration in the medium was obtained from 248 *Ornithogalum* 'Rollow' and 204 *Ornithogalum maculatum* explants. Fresh mass data was obtained from 264 *Ornithogalum* 'Rollow' explants and from 228 *Ornithogalum maculatum* explants.
Fresh mass determinations were made at 4 week intervals, and plantlets were counted at weekly intervals. Absolute growth rates were determined from the fresh mass data (Hunt, 1978).

Analyses of fresh mass data and plantlet counts were done using the BMDPAM programme to estimate missing values and BMDP2V for the analysis of variance of the data (Dixon, 1985). Time is regarded as a single variable with fourteen different levels. To assess the effects of various phytohormone combinations on the rate of plantlet initiation, regressions were calculated and the angle of inclination of the regression line was used as an indication

of the influence of phytohormones on the rate of plantlet initiation relative to the experimental mean.

4.2 RESULTS.

4.2.1 The influence of phytohormones on *in vitro* plantlet initiation by *Ornithogalum* 'Rollow' and *Ornithogalum maculatum* explants.

Of the two *Ornithogalums* tested, only *Ornithogalum* 'Rollow' explants initiated plantlets *in vitro*. The phytohormone levels in the medium were found to significantly affect plantlet initiation on *Ornithogalum* 'Rollow' explants (Table 26).

TABLE 26. Results of an analysis of variance showing the influence of phytohormones on plantlet initiation by *Ornithogalum* 'Rollow'.

Source of Variation.	Variance ratio.
Experimental mean	796,67 **
BAP	6,44 **
NAA	3,76 **
Interaction: BAP x NAA	5,01 **
Time[T]	475,75 **
Interaction: Time x BAP	5,83 **
: Time x NAA	2,72 **
: Time x BAP x NAA	5,01 **

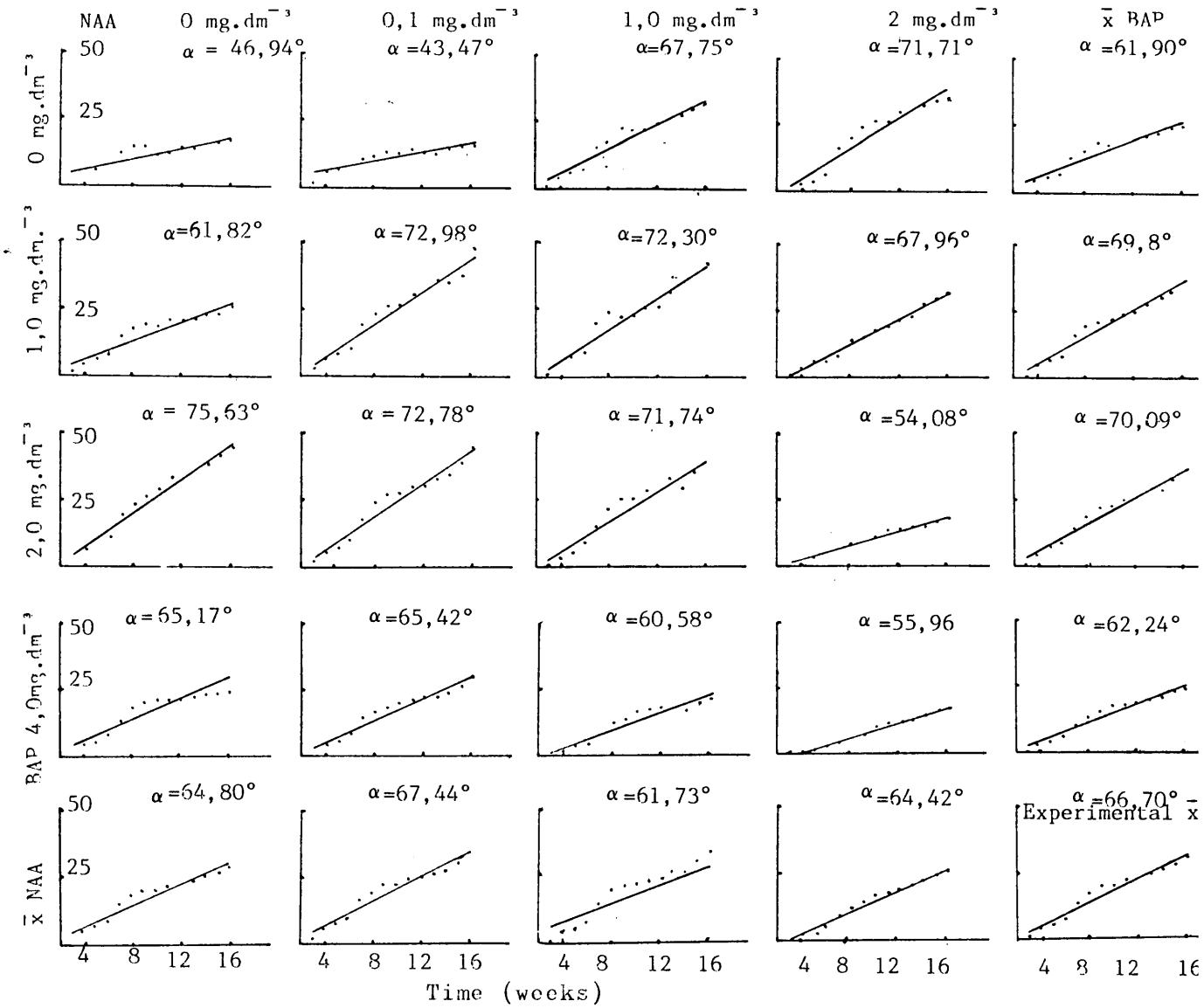
[T] Time is regarded as a single variable, with fourteen levels.

** : Highly significant result ; Reject H^{O} at $p = 0,01$.

Figure (8) shows linear regressions of the effect of a given phytohormone combination on the rate of plantlet initiation over time. For comparisons, the angles of incidence (α) of

the regressions were calculated. The larger the angle, the more successful the treatment. The performance of all treatments was assessed relative to the experimental mean (α = 66,70°).

The optimal NAA concentration for plantlet initiation appears to be 0,1 mg.dm^{-3} irrespective of the BAP concentration (as suggested by Nel, 1981). The optimal BAP concentration irrespective of the NAA concentration in the medium lay between 1,0 - 2,0 mg.dm^{-3}BAP.



α : The angle of incidence of the regression line (tan m)

Figure 8. Linear regression showing the effect of the phyrohormone concentration and the length of the culture period on plantlet initiation in vitro

BAP concentrations of 4,Ø mg.dm^{-3} appeared supra-optimal, inhibiting *in vitro* plantlet initiation. An interaction between the concentrations of NAA and BAP in the nutrient medium occurred (Figure 8). The following relationship between phytohormone concentrations was apparent:-

Cytokinin concentration.	Optimal auxin concentration.
Ø,Ø mg.dm^{-3} BAP	1,Ø-2,Ø mg.dm^{-3} NAA
1,Ø mg.dm^{-3} BAP	Ø,1-2,Ø mg.dm^{-3} NAA
2,Ø mg.dm^{-3} BAP	Ø,Ø-1,Ø mg.dm^{-3} NAA
4,Ø mg.dm^{-3} BAP	supra optimal cytokinin concentration.

The strong time dependence of plantlet initiation is stressed by the linear relationship which exists between the length of the culture period and the number of plantlets on the explant. Slight deviations from linearity occur when the explants are subcultured onto fresh medium after eight weeks.

Bulb development on the explants was inhibited by BAP, but multiple shoot formation by plantlets at concentrations of BAP below 4,Ø mg.dm^{-3} BAP was stimulated (Figure 9). Naphthaleneacetic acid added to the medium was involved in multiple shoot formation on explants, and, in combination with BAP promotes branching of adventitious shoots.

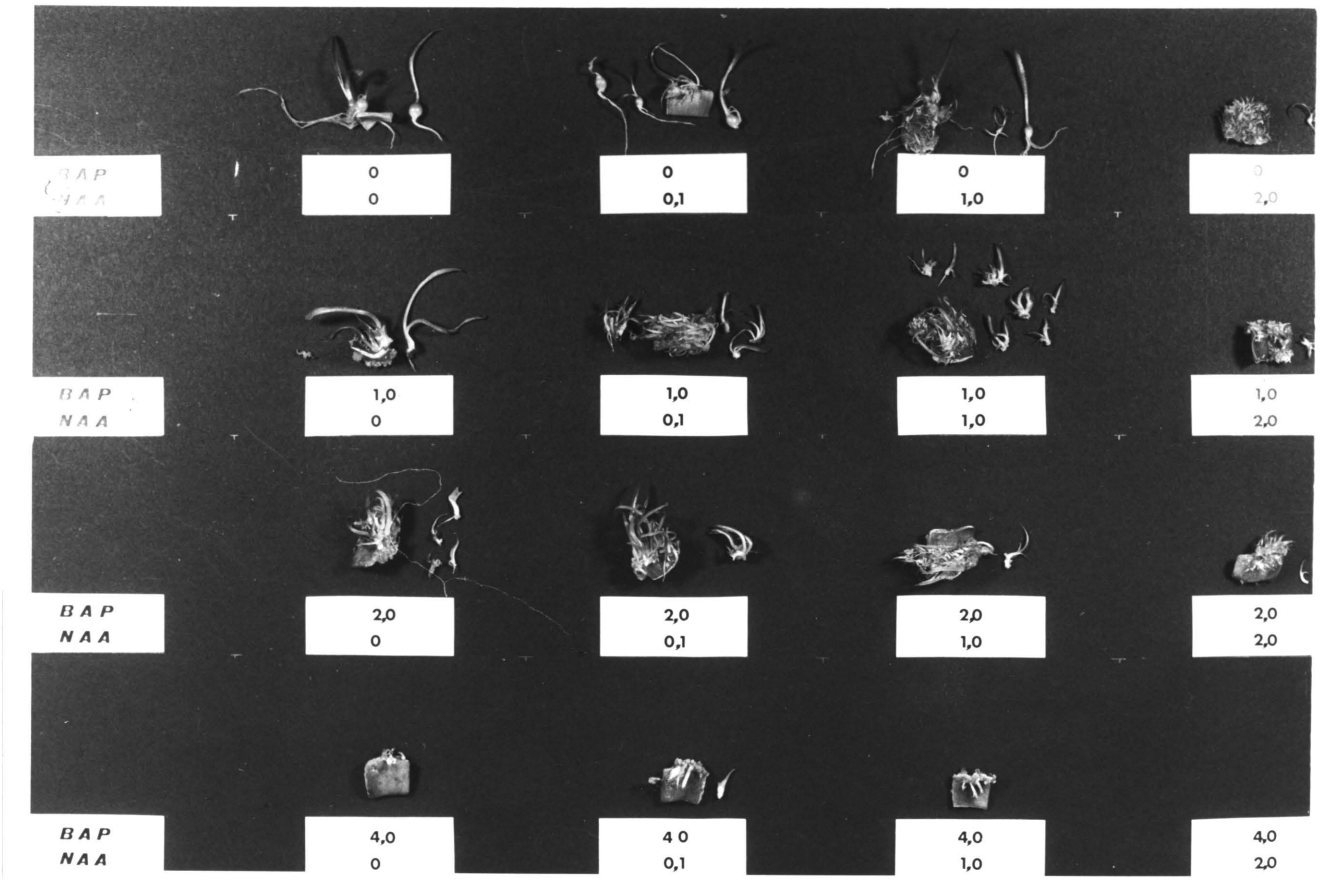


Fig. 9. The effect of phytohormones and their interaction on plantlet formation on Ornithogalum 'Rollow' explants in vitro. (Phytohormone concentration expressed in $mg.dm^{-3}$.)

4.2.2 The influence of phytohormones on the rate of fresh mass accumulation by Ornithogalum 'Rollow' and Ornithogalum maculatum in vitro.

The phytohormone concentrations, both alone (Ornithogalum 'Rollow' Table 27) and through their interaction (Ornithogalum 'Rollow' and Ornithogalum maculatum Table 27) determine the fresh mass of the explant. In Ornithogalum 'Rollow' where plantlet differentiation occurred on the explant, the rate of plantlet initiation and thus fresh mass accumulation was also influenced by the in vitro phytohormones.

Table 27. Analysis of variance showing the effect of phyto hormones and the culture period on fresh mass accumulation by Ornithogalum 'Rollow' and Ornithogalum maculatum explants in vitro.

Source of variation.	Variance ratio.	
	Ornithogalum 'Rollow'	Ornithogalum maculatum
Experimental mean	730,70 **	867,56 **
BAP	6,95 **	
NAA	5,28 **	
Interaction: BAP x NAA	1,57 **	2,45 **
Time	266,61 **	1,52
Interaction: BAP x Time	7,54 **	
NAA x Time	4,09 **	
BAP x NAA x Time	1,68 **	0,75

** : Highly significant result; reject H^{O} at p = 0,01

* : Significant result; reject H^{O} at p = 0,05.

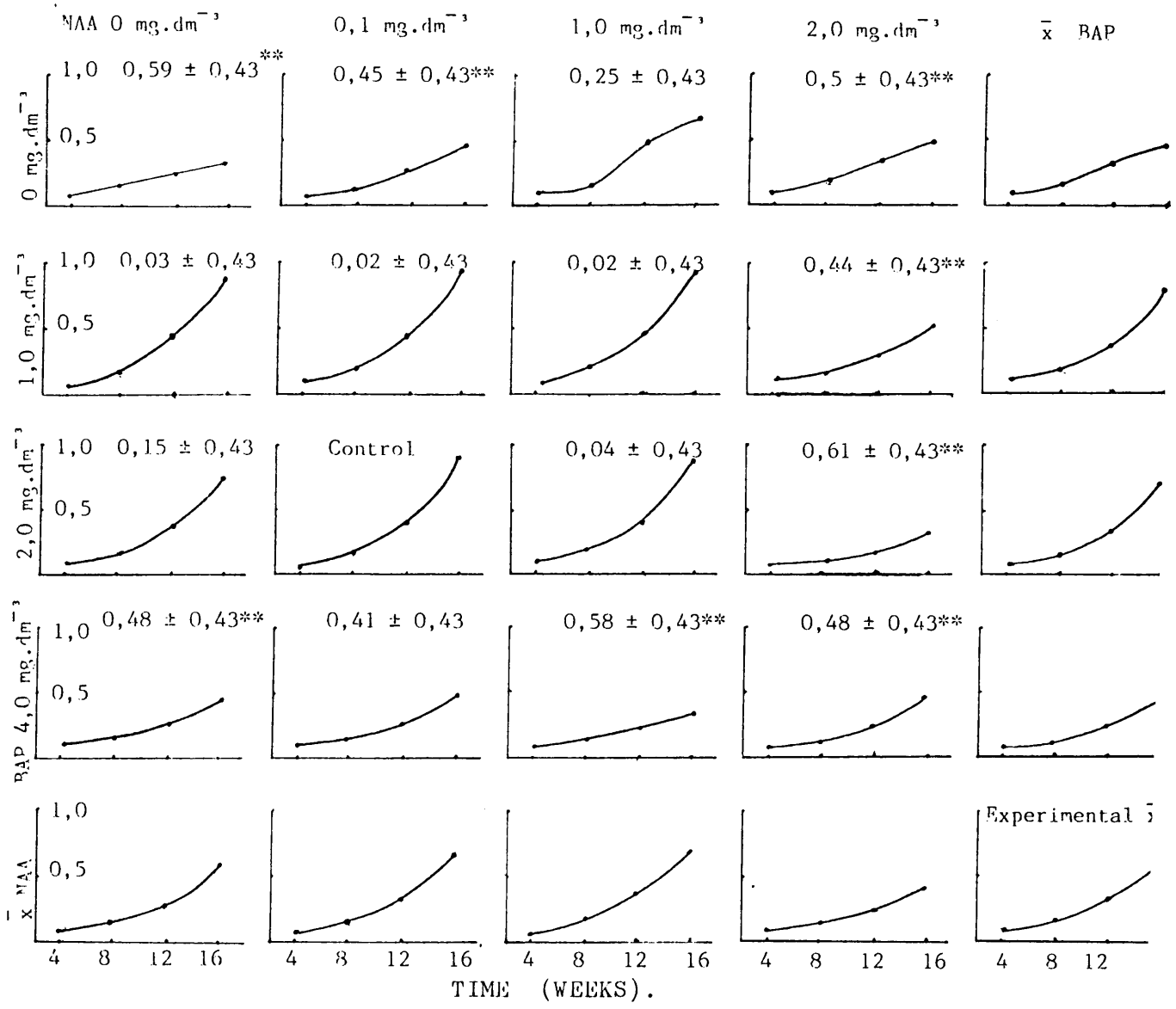


Figure 10. The effect of phytohormones on the fresh mass accumulation rate of Ornithogalum 'Rollow' explants in vitro.

4.2.2.1 Fresh mass accumulation by Ornithogalum 'Rollow' explants.

The relationship between the rate of fresh mass accumulation and time deviates from linearity (Figure 10). The resultant asymptotic growth curves which cannot be transformed to linear form, indicate that fresh mass accumulation by explants is a function of the linear dimensions of the explant and the initiated plantlets on the explant. The rate of fresh mass accumulation was also determined by the phytohormones in the culture medium (Figure 10). From the graph (Figure 10) it appears that the concentration of NAA was optimal between 0,1 and 1,0 mg.dm^{-3} while the optimal BAP concentration was between 1,0 and 2,0 mg.dm^{-3}. Confidence limits for contrasts to determine the influence of interactions between the phytohormones in the culture medium were calculated (Figure 10). A positive value indicated that the treatment performed less well than the control (a $\bar{X}$ = $\bar{x}$ control - $\bar{x}$ treatment). The following interaction between the phytohormones was apparent:-

Cytokinin concentration.	Optimal auxin concentration.
0,0 mg.dm^{-3} BAP	1,0 mg.dm^{-3} NAA
1,0 mg.dm^{-3} BAP	0,0-1,0 mg.dm^{-3} NAA
2,0 mg.dm^{-3} BAP	0,0-1,0 mg.dm^{-3} NAA
4,0 mg.dm^{-3} BAP	0,1 mg.dm^{-3} NAA

4.2.2.2 Fresh mass accumulation by Ornithogalum maculatum explants.

Ornithogalum maculatum explants failed to differentiate plantlets in vitro. Thus, after an initial response, no change in the response of explants to various combinations of phytohormones could be detected over time (Table 27). The response of the explants fresh mass to various

phytohormone combinations was compared (Figure 11).

Test statistics were calculated to test whether results differed significantly from the control (Table 28). As no plantlet initiation occurred, a significant value indicated that the phytohormone combination adversely affected the rate of fresh mass accumulation. The following interaction occurred between the fresh mass of the explants and the combination of phytohormones in the culture medium:-

Cytokinin concentration.	"Optimal" auxin concentration.
Ø,Ø $mg.dm^{-3}$ BAP	Ø,Ø-Ø,1 $mg.dm^{-3}$ NAA
1,Ø $mg.dm^{-3}$ BAP	Ø,Ø-1,Ø $mg.dm^{-3}$ NAA
2,Ø $mg.dm^{-3}$ BAP	Ø,Ø-1,Ø $mg.dm^{-3}$ NAA
4,Ø $mg.dm^{-3}$ BAP	Ø,Ø-1,Ø $mg.dm^{-3}$ NAA

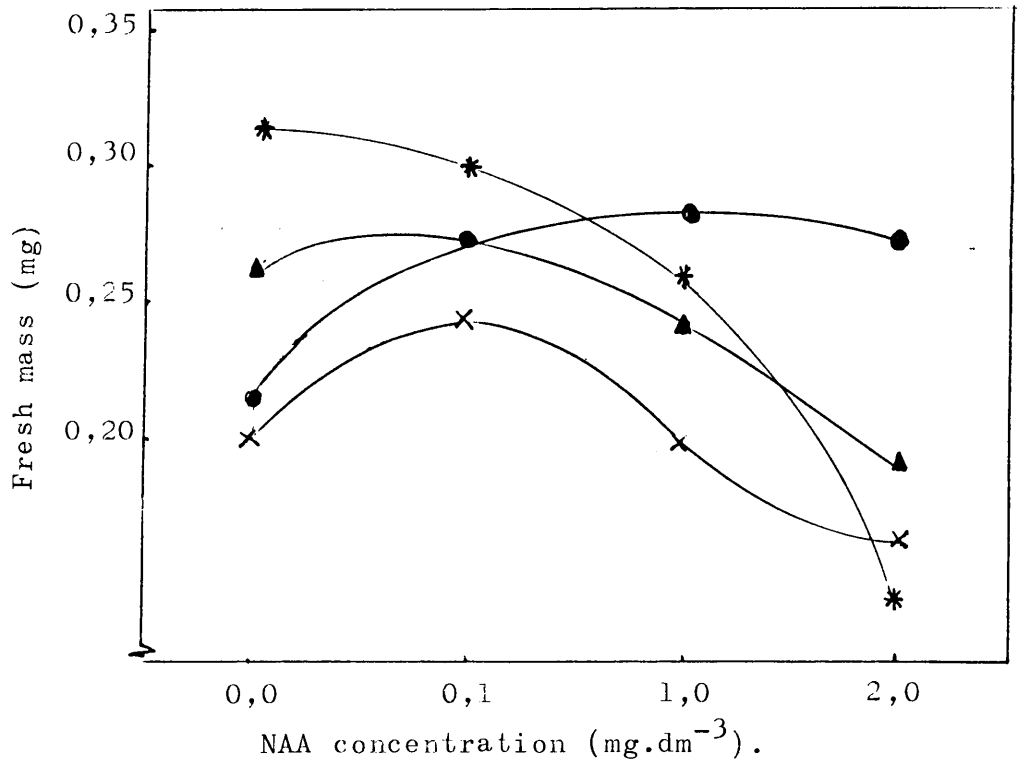


Figure 11. The interaction between napthaleneacetic acid (NAA) and benzylaminopurine (BAP) and its effect on fresh mass accumulation by Ornithogalum maculatum. Concentrations of BAP: 0 mg.dm^{-3} (x); 1 mg.dm^{-3} (●); 2 mg.dm^{-3} (*); 4 mg.dm^{-3} (▲).

Table 28. Test statistics to determine the effect of phytohormone combinations on fresh mass accumulation by Ornithogalum maculatum explants.

BAP concentration	NAA concentration. Ø mg.dm^{-3}	Ø,1 mg.dm^{-3}	1 mg.dm^{-3}	2 mg.dm^{-3}
Ø,Ø mg.dm^{-3}	1,15	1,82	6,81**	7,26**
1,Ø mg.dm^{-3}	Ø,91	Ø,91	Ø,91	5,22*
2,Ø mg.dm^{-3}	Ø,11	Control^{T}	3,86	6,35*
4,Ø mg.dm^{-3}	5,45*	Ø,Ø7	13,62**	21,79**

^{T} Nel's (1981) suggested phytohormone combination was taken as the control.

NOTE: To test the null hypothesis ($a\bar{x} = 0$) the test statistic

$$T = \frac{\text{ss for contrasts}}{\text{MSE}}$$

is referred to the F distribution; where T is the test statistic, and MSE is the mean square error.

$F_{1,213\ ;\ Ø,95} = 3,89$: * indicates a significant reduction in fresh mass as compared to the control.

$F_{1,213\ ;\ Ø,99} = 6,76$: ** indicates a highly significant reduction in fresh mass as compared to the control.

4.3 Summary of Results.

Exogenous NAA and BAP applications are not essential for *in vitro* plantlet initiation on Ornithogalum

'Rollow' explants, nor are they capable of inducing organogenesis in *Ornithogalum maculatum* explants which failed to differentiate plantlets *in vitro*.

The optimum phytohormone concentration for plantlet initiation and fresh mass accumulation by *Ornithogalum 'Rollow'* was determined by the interaction between the concentration of the phytohormones in the medium. Increasing BAP levels resulted in decreasing NAA requirements.

- The complexity of the explant resulted in non-homogenous responses to the exogenously applied phytohormones, as a result, dose-response relationships could not be established for the phytohormones tested.

4.4 Discussion.

Since the discovery that organogenesis can be manipulated by adjusting the auxin:cytokinin balance in the nutrient medium (Skoog & Miller, 1957), *in vitro* multiplication techniques have been successfully applied to many plant species.

Nel (1981) reported optimal shoot formation on explants of *Ornithogalum* hybrids in response to Ø,1 $mg.dm^{-3}$ NAA and 2,Ø $mg.dm^{-3}$ BAP. In this experiment this phytohormone combination was considered to be the control relative to which all other exogenous hormone combinations were judged.

Plantlet initiation occurred on *Ornithogalum 'Rollow'* explants, but *Ornithogalum maculatum* explants failed to respond to the same experimental conditions under which *Ornithogalum 'Rollow'* explants initiated plantlets *in vitro*.

The optimal exogenous concentration of a given plant hormone for plantlet initiation on *Ornithogalum 'Rollow'* explants and from fresh mass accumulation by *Ornithogalum 'Rollow'*

and Ornithogalum maculatum explants was found to be determined by the levels of other exogenous hormones in the explants' environment. With increasing exogenous cytokinin concentrations, the optimal exogenous auxin requirement for plantlet initiation decreased. At Ø,Ø mg.dm^{-3} BAP the optimal auxin concentration was between 1,Ø - 2,Ø mg.dm^{-3} NAA, while at 2,Ø mg.dm^{-3} BAP the optimal auxin concentration decreased to Ø,Ø - 1,Ø mg.dm^{-3} NAA. A similar trend in the phytohormone levels required for fresh mass accumulation by these Ornithogalum 'Rollow' explants was observed. At Ø,Ø mg.dm^{-3} BAP the optimal auxin concentration was 1,Ø mg.dm^{-3} NAA while at 4,Ø mg.dm^{-3}, Ø,1 mg.dm^{-3} NAA in the medium was optimal.

A clear interaction between auxin and cytokinin on fresh mass accumulation by Ornithogalum maculatum explants was not observed. The optimal auxin concentration at Ø,Ø and 4,Ø mg.dm^{-3} BAP was Ø,1 mg.dm^{-3} NAA. Between 1,Ø and 2,Ø mg.dm^{-3} BAP explants accumulated fresh mass over a range (Ø,Ø - 1,Ø mg.dm^{-3} NAA) of auxin concentrations.

The synthetic auxin, NAA, used in this experiment was chosen because:-

1). It is more stable than IAA, and media containing it may be stored for longer periods of time.

2). It is effective over a wider range of concentrations than most other auxins.

In the absence of BAP, auxin appeared to stimulate organogenesis (Figure 8); suppressing bulb development, shoot elongation and ultimately, root formation, with increasing concentrations (Figure 9).

Hussey (1976) reports that the cytokinins, kinetin or BAP (Ø,12 - 2,Ø mg.dm^{-3}) had no effect on plantlet initiation by Ornithogalum thyrsoides explants. Generally, monocotyledons differ from dicotyledons in that exogenous

cytokinins are not essential for *in vitro* shoot initiation (Letham, Goodwin & Higgins, 1978). In this experiment, although BAP was not essential, concentrations between 1,0 - 2,0 $mg.dm^{-3}$ were optimal for organogenesis (Figure 8) and fresh mass accumulation (Figure 10) by *Ornithogalum* 'Rollow'. Slightly higher concentrations (2,0 - 4,0 $mg.dm^{-3}$) stimulated fresh mass accumulation by *Ornithogalum maculatum* explants.

Explants from *Ornithogalum* 'Rollow', *Ornithogalum thyrsoides* (Hussey, 1976) and *Ornithogalum conicum* (Halaban, Galun & Halevy, 1965) have been shown capable of differentiating plantlets *in vitro* in the absence of exogenous phytohormones. Unlike *Ornithogalum thyrsoides*, *Ornithogalum* 'Rollow' failed to differentiate plantlets directly on the leaf bases *in vivo*.

The absence of a requirement for exogenous phytohormones is termed habituation, and implies that sufficient levels of phytohormones are produced by the explant organs of species of the genus *Ornithogalum* to allow organogenesis to occur independently from exogenous phytohormone levels.

Two treatments are generally used to induce rooting *in vitro*. These treatments stimulate rooting during different phases of organogenesis (Letham, Goodwin & Higgins, 1978). Firstly, roots are induced on media with equimolar concentrations of auxin and cytokinin. Reports on monocotyledonous cultures indicate that auxin alone may be rhizogenic - another indication of the cytokinin autonomy of these plants (Letham, Goodwin & Higgins, 1978). This treatment may stimulate cytodifferentiation (primordium development) in the presence of sufficient levels of auxin for subsequent root formation (Letham, Goodwin & Higgins, 1978). Withdrawal of cytokinin and/or auxin from the medium may counteract the effect of suppression of root development by exogenous hormones in the culture medium

(Letham, Goodwin & Higgins, 1978). Hussey (1976) reported a stimulatory effect of IAA and an inhibitory effect of NAA on rooting in Ornithogalum thyrsoides. The second method for inducing root formation, used by Nel (1981) is the omission of phytohormones from the nutrient medium (Letham, Goodwin & Higgins, 1978). No direct rooting of the explant occurred on Ornithogalum 'Rollow' explants as observed on Ornithogalum thyrsoides explants (Hussey, 1976). Roots occurred sporadically on the in vitro plantlets on all media.

The failure of an explant to undergo organogenesis in response to an exogenous phytohormone may result from the environmental conditions to which the explant is exposed. Environmental factors such as light, temperature and medium composition may all be involved, as well as genetic factors (ploidy, age of the tissue and generic variability).

Hussey (1975) found it impossible to predict the in vitro response of members of the monocotyledonous families Amaryllidaceae, Liliaceae and Iridaceae on the basis of taxonomic relationships.

The donor plant from which the explant is derived also determines the explants' ability to undergo organogenesis. The physiological state of the donor plant and its nutritional and environmental conditioning are important. In addition, when explant material is selected, the following characteristics of the donor material must be considered (Tran, Thanh Van, 1981) :-

i. The photosynthetic rate of the donor plant.
ii. Any host/parasite interrelation.
iii. Any symbioses which may exist.

When a plant is difficult to propagate in vitro the difficulties can sometimes be overcome by manipulating environ-

mental factors to which the explant or the donor plant is exposed, or by substituting one explant source for another. Manipulating these factors may achieve success. However, it does not promote the understanding of the processes controlling organogenesis.

The regulation of organogenesis by hormones is little understood. One of the problems involved in understanding the mechanism of organogenetic differentiation is the limitations which are imposed by the complexity of available explant material. Different tissues respond differently to the same plant hormone concentrations and exert feedback effects on each other. In order to establish dose-response relationships between hormones and organogenetic processes (which is necessary for controlling organogenesis) it is essential to simplify the experimental system or explant. The target cells involved in morphogenesis must be removed from the heterogeneous mass of cells which constitute the explant organ (Tran Thanh Van, 1981). Tran Thanh Van (1981) suggests the use of single homogenous layers of tissue on which uniform responses to various stimuli can be obtained. A drawback of this technique is the reduction in viability which often occurs in small explants. However, this can be overcome in many instances by culturing several small explants together (Seabrook, 1980).

CHAPTER 5.

THE EFFECT OF CARBOHYDRATES IN VITRO ON THE GROWTH YIELD OF ORNITHOGALUM LEAF BASE EXPLANTS.

5.1 Introduction.

This chapter comprises two experiments. In the first experiment, sucrose and mannose (a glucose stereoisomer encountered in cell walls but sparingly utilised for growth (Maretzki, Thom & Nickell, 1974) were added to Nel's (1981) medium containing 0,1 mg.dm^{-3} NAA and 2,0 mg.dm^{-3} BAP to give a total carbohydrate concentration not in excess of 80 g.dm^{-3} as follows:-

		Mannose (g.dm^{-3})				
		0	10	30	50	70
sucrose	0	x	x	x	x	x
(g.dm^{-3})	10	x	x	x	x	x
	30	x	x	x	x	
	50	x	x	x		
	70	x	x			

The purpose of the experiment was to determine to what extent the osmotic contribution of carbohydrates to the medium stimulated growth. The response of two Ornithogalums, the species maculatum, and the cultivar 'Rollow' were tested. Mannose was used in preference to mannitol, as Maene & Debergh (1985) showed that mannitol was toxic at concentrations greater than 20 g.dm^{-3}

Leaf base explants were supported on filter paper bridges to eliminate the osmotic effect of agar in the nutrient medium (Reinert & Yeoman, 1982). The osmotic potential of the nutrient medium was determined initially, together with the explant fresh mass. Fresh mass determinations and plantlet

counts were made after 3 and 6 weeks in culture. Three hundred and eighty-seven Ornithogalum 'Rollow' and fifty-eight Ornithogalum maculatum explants were used for this experiment.

Dry masses were determined according to the microwave drying technique of Ferreira (1982), and the water content of the explants was derived from the data according to Noggle & Fritz (1976).

In the second experiment, sucrose at:- 0; 10; 20; 30; 40; 50; 60; 70; 80; 90; 100 $g.dm^{-3}$ was added to Nel's (1981) medium solidified with 6 $g.dm^{-3}$ agar. Phytohormones, which are known to interact with carbohydrates in the nutrient medium were omitted from the medium (Tran Thann Van, 1977).

Leaf base explants, 1,0 x 1,0 cm in size were used. Explant fresh mass was determined initially and together with the incidence of plantlets at four week intervals for sixteen weeks. Two hundred explants were involved in this experiment.

The aim of this experiment was to determine the optimal sucrose concentration in Nel's (1981) medium for adventitious shoot initiation. As Ornithogalum maculatum failed to differentiate plantlets under the experimental conditions, only the response of the cultivar 'Rollow' was tested.

The effect of the various sucrose concentrations on the resultant plantlets was assessed in terms of shoot length and root formation.

The treatments in the different experiments comprised twenty replicates. The normality of the distribution was checked using BMDP5D (Dixon, 1985) and anovas were produced using

BMDP2V (Dixon, 1985). Where within-treatment variance was uniform, contrasts were used. To ensure the stated confidence limits on *ad hoc* contrasts (used to generate hypotheses), the Scheffe procedure (Brown & Hollander, 1977) was used. Where within-treatment variance differed significantly, pairwise t-tests (based on separate sample variances) were used to assess the significance of the results.

5.2 Results.

5.2.1 The effect of carbohydrates on the water potential of Nel's (1981) medium.

Considerable variability between thermocouple measurements of media water potential occurred. On the basis of the measurements made using liquid media, the following relationship between the initial mannose concentration (x) and the water potential (y) of the medium is proposed:-

$$y = -162 - 165{,}89\,x \quad : r = -0{,}99$$

Similarly, the relationship between the sucrose concentration (x) and the water potential (y) of the nutrient media including data obtained for media containing 80 $g.dm^{-3}$ rather than 70 $g.dm^{-3}$ sucrose, was:-

$$y = -180{,}53 - 6{,}51x \quad : r = -0{,}96$$

The ratio of initial to final water potential is given in Table 29. The decrease in water potential during the subculture period was more marked at higher initial carbohydrate concentrations.

Table 29 . Average water potentials of media (-kPa) on which Ornithogalum 'Rollow' was subcultured showing the ratio of final:initial water potential (as a percentage value).

Sucrose ($mg.dm^{-3}$)		Mannose ($mg.dm^{-3}$)	0		10		30		50		70	
0	Initial		193,7	1,2%	332,5	1,1%	253,3	1,7%	387	1,4%	340,7	1,3%
	Final		228,5		377,5		434,5		524		451	
10	Initial		266,7	1,3%	284,3	1,6%	241	1,5%	1086	1,2%	699	1,1%
	Final		353		460		357		1291		750	
30	Initial		262,7	1,0%	330	0,7%	698,7	1,0%	534,7	3,5%		
	Final		256,5		234,5		710,7		1860			
50	Initial		412,5	2,0%	329	2,2%	353,5	4,3%				
	Final		805		717,7		1532,7					
70	Initial		254,3	2,9%	343,3	2,8%						
	Final		748		969							

5.2.2 The effect of the carbohydrates in liquid media on the rate of callus differentiation by Ornithogalum maculatum explants and plantlet initiation on Ornithogalum 'Rollow' explants.

Ornithogalum maculatum explants failed to differentiate plantlets in vitro. The incidence of callus formation rose with increasing sucrose concentrations (S^{*} = 2,607**; p = 0,01) from 0,0 to 80,0 g.dm^{-3} sucrose.

The concentration of sucrose in liquid media did not affect the number of plantlets produced by Ornithogalum 'Rollow' explants in vitro. The X^2 value, although not significant (X^2 =9,433; p = 0,0511) occurs between the 90 and 95% confidence limits. Of all the explants tested, 32,6% produced plantlets, while 67,4% failed to respond in culture. The lack of an in vitro response indicates that additional factors to those controlled by the experimental conditions are involved in determining the in vitro response. The rate of plantlet formation on the explants which differentiated plantlets is presented in Table 30.

Table 30. The effect of sucrose concentration on in vitro plantlet initiation rates on Ornithogalum 'Rollow' explants.

Sucrose concentration.	Frequency of shoot production by explants.
0,0 g.dm^{-3}	24,5
10,0 g.dm^{-3}	24,4
30,0 g.dm^{-3}	20,7
50,0 g.dm^{-3}	15,2
70,0 g.dm^{-3}	15,2

The distribution of plantlets formed on the explants was also calculated (Table 31).

Table 31. The frequency of plantlet occurrence on Ornithogalum 'Rollow' explants.

Number of plantlets/explant.	Percentage of total explants.
0/ explant	67,4 %
1-5/ explant	14,8 %
6-10/ explant	14,2 %
>10/ explant	3,6 %

The concentration of mannose in the medium failed to significantly affect the rate of callus formation on Ornithogalum maculatum explants (X^2 = 8,09 NS ; p = 0,05) or plantlet initiation (X^2 = 8,25 NS ; p = 0,08) on Ornithogalum 'Rollow' explants. The interaction between mannose and sucrose in the medium failed to significantly affect the rate of plantlet initiation on explants (X^2 = 68,583 NS ; p = 0,14). However, the interaction between mannose and sucrose in the medium affected the incidence of callus formation on Ornithogalum maculatum explants, (X^2 = 33,584* ; p = 0,05). The incidence of callus increased with increasing carbohydrate concentrations in the nutrient medium.

Of all the explants initiating plantlets, 80,9% contained chlorophyll. The concentration of carbohydrate in the medium did not significantly affect the incidence of chlorophyll in explants (X^2 = 29,343 NS; p = 0,061) Neither the concentration of sucrose alone (X^2 = 1,193 NS; p = 0,879) nor mannose (X^2 = 3,738 NS; p = 0,443) affected the chlorophyll content of the explants.

5.2.3 The effect of sucrose in agar solidified media on organogenesis and differentiation of plantlets on Ornithogalum 'Rollow' explants.

Variance ratios derived from an analysis of variance (Table 32) indicate that the sucrose concentration in agar solidified media significantly affects the initiation of plantlets by Ornithogalum 'Rollow' explants. Similarly, the length of the culture period also determines the number of plantlets formed.

Table 32. Results of an analysis of variance showing the effect of sucrose on plantlet initiation by Ornithogalum 'Rollow' explants.

Source of Variation.	Variance ratio.
Sucrose Concentration.	2,80**
Culture Period.	78,15**
Interaction: Sucrose conc. x culture period	2,96**

** Highly significant Result; reject H^{O} at p = 0.01.

Contrasts to determine the significance of the treatment effect indicated that a minimum of 30 $g.dm^{-3}$ sucrose in the medium was required for optimal plantlet proliferation. Increasing the sucrose concentration to 100 $g.dm^{-3}$ failed to have any additional effect on plantlet initiation. At or below 10 $g.dm^{-3}$ sucrose in the medium, plantlets failed to develop (Table 33).

Table 33. Contrast results showing the effect of sucrose concentration on plantlet initiation on Ornithogalum 'Rollow' explants.

Sucrose concentration.	Test Statistic (F).
Ø $g.dm^{-3}$	No shoot initiation observed.
1Ø $g.dm^{-3}$	No shoot initiation observed.
2Ø $g.dm^{-3}$	-13.11**
3Ø $g.dm^{-3}$	Control^{T}
4Ø $g.dm^{-3}$	Ø,Ø2
5Ø $g.dm^{-3}$	Ø,76
6Ø $g.dm^{-3}$	Ø,Ø2
7Ø $g.dm^{-3}$	Ø,22
8Ø $g.dm^{-3}$	Ø,41
9Ø $g.dm^{-3}$	Ø,14
1ØØ $g.dm^{-3}$	1,63

** : Highly significant result, Reject H^{O} at at p = Ø.Ø1.

T : Control corresponds to sucrose concentration suggested by Nel (1981).

A contrast was used to determine the effect of the sucrose concentration on the rate of shoot elongation *in vitro* (Table 34). The optimal sucrose concentration for shoot elongation is 3Ø $g.dm^{-3}$ sucrose. Higher or lower sucrose concentrations adversely affect shoot elongation.

Table 34. Contrast results showing the effect of the sucrose concentration in the medium on shoot elongation by Ornithogalum 'Rollow' explants.

Sucrose concentration.	Test statistic.
0 $g.dm^{-3}$	No shoot initiation observed
10 $g.dm^{-3}$	No shoot initiation observed
20 $g.dm^{-3}$	$-5,41^{**}$
30 $g.dm^{-3}$	Control^{T}
40 $g.dm^{-3}$	$-5,16^{**}$
50 $g.dm^{-3}$	$-4,96^{**}$
60 $g.dm^{-3}$	$-5,13^{**}$
70 $g.dm^{-3}$	$-8,44^{**}$
80 $g.dm^{-3}$	$-8,73^{**}$
90 $g.dm^{-3}$	$-12,50^{**}$
100 $g.dm^{-3}$	$-10,51^{**}$

** : Highly significant result, Reject H^{O} at p = 0,01.

T Control corresponds to sucrose concentration suggested by Nel (1981).

The effect of sucrose on rooting was also noted. *In vitro* plantlets rooted on media containing 30 to 60 $g.dm^{-3}$ sucrose (Table 35). At higher or lower levels, rooting was inhibited.

Table 35. Contrast results showing the effect of the sucrose content of the nutrient medium on the number of roots formed on *in vitro* plantlets.

Sucrose concentration.	Test statistic.
0 $g.dm^{-3}$	No root initiation observed.
10 $g.dm^{-3}$	No root initiation observed.
20 $g.dm^{-3}$	-3,54**
30 $g.dm^{-3}$	Control[T]
40 $g.dm^{-3}$	-1,75
50 $g.dm^{-3}$	-2,44
60 $g.dm^{-3}$	-0,86
70 $g.dm^{-3}$	-5,67**
80 $g.dm^{-3}$	-4,68**
90 $g.dm^{-3}$	-8,34**
100 $g.dm^{-3}$	-8,91**

** : Highly significant result : Reject H^{O} at $p = 0,01$

[T] Control corresponds to sucrose concentration suggested by Nel (1981).

5.2.4 The effect of carbohydrates on fresh mass accumulation by *Ornithogalum maculatum* and *Ornithogalum* 'Rollow' explants on liquid media.

The anova (Table 36) showed a highly significant effect of carbohydrates in the medium on *Ornithogalum* fresh mass accumulation.

Table 36. Results of an analysis of variance showing the effect of the carbohydrate content of the nutrient medium on the rate of fresh mass accumulation by Ornithogalum 'Rollow' and Ornithogalum maculatum explants in vitro.

Source of variation.	Culture Period: 3 weeks O.M.	3 weeks 'Rollow'	6 weeks O.M.	6 weeks 'Rollow'	Change in fresh mass between 3-6 weeks in culture. O.M.	Change in fresh mass between 3-6 weeks in culture. 'Rollow'
Treatment	5,26**	4,92**	5,55**	4,12**	3,13**	1,84**
Equality of means test; variances not assumed equal.						
Welch	5,22**	4,51**	6,53**	4,63**	4,22**	1,94**
Brown-Forsythe	5,15**	4,95**	5,40**	4,16**	3,26**	1,87**
Equality of variance test	2,17**	1,53NS	3,96**	2,00**	3,71**	2,95**

** : Highly significant result; reject H^0 at $p = 0,01$.

The rate of fresh mass accumulation by shoot-forming Ornithogalum 'Rollow' tissues was lower than that of non-shoot forming Ornithogalum maculatum tissues (Table 37).

Table 37. Experimental means showing the rate of fresh mass accumulation in Ornithogalum maculatum and Ornithogalum 'Rollow' explants (in grams).

Explant source	Culture Period 3 weeks	6 weeks	Change in fresh mass between 3-6 weeks in culture.	SEM*
Ornithogalum maculatum	0,059	0,071	0,012	0,003
Ornithogalum 'Rollow'	0,018	0,024	0,006	0,002

*SEM : Standard error of the mean.

Contrasts were carried out where variances were equal and pairwise t-tests based on separate sample variances were used where the sample variances differed significantly, (Table 36), to determine the effect of carbohydrates in the medium on the rate of fresh mass accumulation. No significant effects of carbohydrates in the medium on fresh mass accumulation could be obtained from the Ornithogalum maculatum data based on the t-tests. Anovas are more robust, while t-tests have more stringent requirements for significance. This explains the apparent contradiction in results.

After three weeks in culture, the sucrose content in the medium affected the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants (Table 38).

Table 38. Results of a contrast in population means showing the influence of sucrose on the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants in vitro after 3 weeks in culture.

Sucrose concentration.	Test statistic.
Ø $g.dm^{-3}$	2
1Ø $g.dm^{-3}$	1
3Ø $g.dm^{-3}$	Control ^{T}
5Ø $g.dm^{-3}$	Ø,312
7Ø $g.dm^{-3}$	1,44
8Ø $g.dm^{-3}$	1,72

^{T} The control was taken to be 3Ø $g.dm^{-3}$ sucrose as proposed by Nel (1981). Test statistics did not differ significantly at $p = Ø,Ø1$, thus H^{O} cannot be rejected.

A significant interaction between the concentration of sucrose and mannose in the medium on the rate of fresh mass accumulation (Table 39) was observed after three weeks in culture. At certain sucrose concentrations in the medium, the mannose concentration had a significantly adverse effect on the rate of fresh mass accumulation (Table 39).

Table 39. The results of a contrast in population means showing the effect of mannose at various sucrose concentrations on the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants in vitro after three weeks in culture.

Sucrose concentration.	Mannose concentration.	Test statistic.
0 $g.dm^{-3}$	10; 30; 50; 70 $g.dm^{-3}$	1,65
10 $g.dm^{-3}$	10; 30, 50; 70 $g.dm^{-3}$	3,15*
30 $g.dm^{-3}$	10; 30; 50 " $g.dm^{-3}$	2,79*
50 $g.dm^{-3}$	10; 30 $g.dm^{-3}$	2,57
70 $g.dm^{-3}$	10 $g.dm^{-3}$	1

* Test statistic: Reject H^{O} at at the 95% confidence limit.

In instances where the combination of sucrose and mannose adversely affected the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants, the concentration of mannose involved was determined (Table 40).

Table 40. Results of contrasts in population means showing the effect of the mannose concentration on the fresh mass accumulation rate of Ornithogalum 'Rollow' explants on media containing 10 and 30 $g.dm^{-3}$ sucrose.

Mannose.	Test statistics. 10 $g.dm^{-3}$ sucrose.	30 $g.dm^{-3}$ sucrose.
10 $g.dm^{-3}$	0,205	2,67
30 $g.dm^{-3}$	2,84	24,00 **
50 $g.dm^{-3}$	2,74	16,67 **
70 $g.dm^{-3}$	4,60*	Not tested

** Highly significant result, Reject H^o at p = 0,01.

* Significant result, Reject H^o at p = 0,05.

The inhibitory effect of mannose in the medium was only notable in the presence of sucrose, where the concentration of sucrose in the medium was relatively low (Table 40).

After six weeks in culture, pairwise t-tests revealed no significant effects of sucrose or mannose on the rate of fresh mass accumulation *in vitro*. The apparent contradiction between these results and those of the variance analysis (Table 36) may be ascribed to the more robust nature of the anova and the more stringent requirements of the t-test for significance of results to be proved.

5.2.5 The effect of sucrose on fresh mass accumulation by Ornithogalum 'Rollow' explants on agar solidified media.

Both the sucrose concentration and the length of the culture period both significantly affected fresh mass accumulation by these explants (Table 41).

Table 41. Results of an analysis of variance showing the effect of sucrose, culture period and the interaction of these variables on Ornithogalum 'Rollow' explant fresh mass.

Source of variation.	Variance ratio.
Sucrose concentration	5,18**
Culture period	82,78**
Interaction: Sucrose x Culture period.	4,25**

** : Highly significant result; Reject H^{O} at p = Ø,Ø1.

From the contrast (Table 42), it was evident that optimal fresh mass accumulation occurred at sucrose concentrations greater than 1Ø g.dm^{-3}. Increasing the sucrose concentration in the medium to 1ØØ g.dm^{-3} failed to additionally stimulate fresh mass accumulation (Table 42).

Table 42. Results of a contrast showing the effect of the sucrose concentration on the rate of fresh mass accumulation by Ornithogalum `Rollow' explants in vitro.

Sucrose concentration.	Variance ratio.
0 $g.dm^{-3}$	$-7,58^{**}$
10 $g.dm^{-3}$	$-4,01^{*}$
20 $g.dm^{-3}$	0,05
30 $g.dm^{-3}$	Control ^{T}
40 $g.dm^{-3}$	$4,39 \times 10^{-5}$
50 $g.dm^{-3}$	1,57
60 $g.dm^{-3}$	1,06
70 $g.dm^{-3}$	0,31
80 $g.dm^{-3}$	2,04
90 $g.dm^{-3}$	2,58
100 $g.dm^{-3}$	1,01

* : Significant result; Reject H^{O} at p = 0,05

** : Highly significant result; Reject H^{O} at at p=0,01.

T : Control corresponds to sucrose concentration suggested by Nel (1981)

5.2.6 Percentage moisture contents of Ornithogalum maculatum and Ornithogalum `Rollow' explants in vitro.

The percentage moisture contents of Ornithogalum maculatum and Ornithogalum `Rollow' explant tissues were determined after six weeks in culture. The percentage moisture contents of these tissues were found to be similar. The average moisture content of Ornithogalum maculatum tissues was 98,74% and of Ornithogalum `Rollow' tissues the moisture content was 98,71%. The moisture content of the tissues remained unaffected by the changing carbohydrate content of the medium.

5.3 Summary of Results.

- The ability of explants to differentiate plantlets under the experimental conditions depended on the genome.

- The optimal carbohydrate content in the medium depended on phytohormone levels in the medium and the type of differentiation occurring.

- After a six week subculture period, the osmotic effect of carbohydrates in the nutrient medium on explants was negligible.

5.4 Discussion.

Generally, the efficiency with which explants photosynthesize *in vitro* is limited. As a result, autonomous growth of explants *in vitro* without an added carbon and energy source is not feasible (Reinert & Yeoman, 1982).

In addition to their nutritional role, carbohydrates also exert an osmotic effect on culture media. Under conditions of constant temperature and pressure, the chemical potential of water in any system decreases with the log of the solute concentration (Noggle & Fritz, 1976). The water potentials of nutrient media at the end of the subculture period, are a function of the nutrients removed from the medium during growth (which raises the water potential), water loss from the medium as a result of evaporation and transpiration Yoshida *et al.* (1973) and Dunwell & Thurling (1985), (which lowers the water potential) and as a result of the interaction between components of the media and the degree of dissociation of ions in solution. Because of the many components involved, these water potentials can only be determined by direct measurement (Table 33).

The water potentials of the culture media declined over the

culture period. This drop in water potential has been reported in the literature as constituting a stress factor in callus, cell (Yoshida, Kobayashi & Yoshida, 1973) and organ cultures (Dunwell & Thurling, 1985) which, while initially stimulatory, could ultimately cause the onset of dormancy.

During this experiment, the explants remained unaffected by the reduction in water potentials of the media which occurred during the six week culture period. Reinert & Yeoman (1982) report the water potential of Murashige & Skoog's (1962) medium as - 415 kPa (or - 508 kPa where agar is present in the medium). The water potentials of molal solutions of sucrose are reported by Michel (1972). From the data, the maximum solute potential at a sucrose concentration of 100 $g.dm^{-3}$ would be expected to be approximately -1000 kPa. This value lies within the range (0 to -1500 kPa) of water potentials of leaves of plants rooted in well watered soils (Noggle & Fritz, 1976). Under the experimental conditions, most observations lay well within this range. At no stage during the six week culture period did the level of solutes in the medium constitute a stress factor.

The genotype of the explant determined whether differentiation would occur; the rate (Ball & Soma, 1965) and type of differentiation (Tran Thanh Van, 1977) is determined by the interaction between the phytohormone and carbohydrate balance in the tissue.

In this experiment, Ornithogalum maculatum explants failed to differentiate callus in response to the same conditions under which Ornithogalum 'Rollow' differentiated plantlets.

According to Brown, Leung & Thorpe (1979), the essential difference between shoot and non-shoot forming tissues is the accumulation of starch in the former - essential in or-

der to meet the high energy requirements of shoot formation. Accumulated starch and free sugars in the tissues supply the substrates for energy production via glycolysis and oxidative phosphorylation. In addition, NADPH supplied reducing power via the pentose phosphate pathway (Brown & Thorpe, 1979). Factors which delay (Thorpe & Meier, 1972) or inhibit (Thorpe & Murashige, 1969) starch accumulation have a similar effect on organogenesis.

Brown, Leung & Thorpe (1979) found that the accumulated starch in shoot forming tissues raised their water potential, at least prior to the onset of differentiation. In this experiment, the water contents of shoot forming Ornithogalum 'Rollow' tissues and Ornithogalum maculatum tissues producing callus were similar after six weeks in culture. The higher fresh mass of Ornithogalum maculatum tissues was attributable to the increased rate of cell division during callus formation.

Maretzki, Thom & Nickell (1974) found that the ability of explants to utilise carbohydrates in the nutrient medium depended on the species and clone. The form in which carbohydrates are utilised also depended on environmental factors (Tran Thanh Van, 1981), the stage of development of the explant and the concentration in the medium (Jeffs & Northcote, 1967). In this experiment, the phytohormone content of the nutrient medium was also found to be an important determinant of the effect of carbohydrates on growth.

Jeffs & Northcote (1967) noted that only disaccharides with an alpha glycosyl radical at the non-reducing end of the molecule are capable of initiating organogenesis. They propose that these molecules combine with other molecules in the presence of phytohormones to exert their effect on differentiation. At the molecular level, this forms an inducer / repressor system (Shoemaker, Conche & Galbraith,

1986) which controls organogenesis (Jeffs & Northcote, 1967). According to Maretzki, Thom & Nickell (1974), glucose and sucrose, the forms in which carbohydrates are transported within the plant, are the two forms most likely to promote optimum growth.

Sucrose was found to stimulate plantlet initiation on Ornithogalum 'Rollow' explants only where phytohormones were omitted from the culture media. More than 10 $g.dm^{-3}$ was required for plantlet initiation, 30 $g.dm^{-3}$ sucrose being optimal for initiation and subsequent shoot development. To maintain optimal rates of fresh mass accumulation in the absence of exogenous phytohormones, 10 $g.dm^{-3}$ sucrose was required, higher sucrose concentrations were without effect. Rooting occurred on media containing 30 - 60 $g.dm^{-3}$ sucrose. In the absence of phytohormones higher sucrose concentrations inhibited rooting. Callus formation on Ornithogalum maculatum was stimulated by increasing the sucrose content of liquid media containing phytohormones.

An interaction between carbohydrates in the medium was noted. Mannose in combination with sucrose had an inhibitory effect on fresh mass accumulation by Ornithogalum 'Rollow' explants during the first three weeks of the culture period. This effect occurred when mannose was present in media at concentrations greater than 30 $g.dm^{-3}$ and provided the level of sucrose in the medium was equivalent to, or lower than, the mannose concentration. The effect was no longer apparent after six weeks in culture.

The carbohydrate content of the medium did not significantly affect the rate of fresh mass accumulation of Ornithogalum maculatum or Ornithogalum 'Rollow' in liquid media in the presence of phytohormones. On solid media, in the absence of phytohormones, a minimum of 10 $g.dm^{-3}$ sucrose was required for optimal fresh mass accumulation, further increases being without effect.

CHAPTER 6.

THE EFFECT OF THE AGAR AND SUCROSE CONTENT OF THE NUTRIENT MEDIUM ON GROWTH OF ORNITHOGALUM 'ROLLOW' EXPLANTS IN VITRO.

6.1 Introduction.

The agar used in Nel's (1981) medium was the most expensive media constituent. The use of agar as support matrices in culture vessels reduces the labour which would otherwise be involved in assembling alternative support systems (Conner & Meredith, 1984). In addition, in some instances, it prevents the development of glassiness, waterlogging and translucency (syn. hyperhydric transformation) (Debergh, 1982). However, addition of agar to media complicates cleaning of culture vessels, reduces the number of explants which may be added to the culture vessel and may add statistically significant amounts of calcium, magnesium, potassium and sodium to the medium (Chung et al., 1980; Debergh, 1982). In addition, the availability of cytokinins is reduced, decreasing the propagation rate in some instances, an effect which cannot be compensated for by raising the cytokin level in the medium (Debergh, 1982).

As both sucrose (Reinert & Yeoman, 1982) and agar (Brown, Leung & Thorpe, 1979) are reportedly osmotically active in culture media, the effects of the agar concentration on the growth of explants from the leaf bases of Ornithogalum 'Rollow' were tested over a range of sucrose concentrations in this experiment. Initially, each experimental treatment comprised twenty replicates. Data from a total of 257 explants was used in this experiment.

Special grade agar (Lot HD 004) supplied by Biolab Chemicals (Pty.) Ltd., was used at 0,0; 5,0; 6,0; 7,0; 10,0; 15,0 and 20,0 $g.dm^{-3}$, in Nel's (1981) medium containing 0,1 $mg.dm^{-3}$

NAA and 2,0 mg.dm^{-3} BAP and either 30,0 or 45,0 g.dm^{-3} sucrose. The pH values of the media were adjusted to pH 6,2 prior to autoclaving.

The experimental results were analyzed using BMDP - P series programmes. The effect of the agar and sucrose concentrations in the medium on plantlet initiation and the chlorophyll content of explants was assessed after 6 weeks using Chi square tests (Dixon, 1985). The fresh mass accumulation of explants was measured initially and at 3 and 6 week intervals, while the rate of dry mass accumulation, the water content of the explants and the water potential of the nutrient media were determined after 6 weeks in culture. Fresh and dry mass data and data on the water potentials of the nutrient media were analyzed using BMDP 2V analysis of variance (Dixon, 1985). The significance of the treatment effects where they were observed was assessed using contrasts (Brown & Hollander, 1977).

6.2 Results.

6.2.1. The effect of the agar and sucrose content of the medium on the incidence of plantlets on *Ornithogalum* 'Kollow' explants in vitro.

Increasing the sucrose content of the medium from 30,0 to 45,0 g.dm^{-3} was found to significantly reduce the number of plantlets formed by the explants (X^2 = 16,081 **; p = 0,0029) (Table 43).

Table 43. The percentage frequency with which plantlets occurred on all media under the experimental conditions.

Number of Plantlets	Sucrose concentration. 30,0 g.dm^{-3}	45,0 g.dm^{-3}	Total.
0	17,9	29,6	47,5
1 - 5	8,6	7,0	15,6
6 - 10	12,1	7,0	19,1
11 - 15	7,0	2,7	9,7
> 15	3,9	4,3	8,2
Total	49,4	50,6	100,0

The agar content of the medium was not found to have a significant effect on the incidence of plantlets on explants (X^2 = 26,142NS; p = 0,35).

A significant interaction between the levels of sucrose and agar in the media on the incidence of plantlets on explants was observed (X^2 = 35,174**; p = 0,0008).

The highest incidence of plantlets was attained on media containing 30,0 g.dm^{-3} sucrose at agar concentrations greater than 15,0 g.dm^{-3} and at agar concentrations greater than 10,0 g.dm^{-3} in media containing 45 g.dm^{-3} sucrose (Table 44).

Table 44. The percentage of explants in each treatment which formed plantlets on the different media.

Agar concentration	Sucrose concentration. 30 g.dm^{-3}	45 g.dm^{-3}
0,0 g.dm^{-3}	44,4 %	55 %
5,0 g.dm^{-3}	66,7 %	18,8 %
6,0 g.dm^{-3}	50,0 %	31,6 %
7,0 g.dm^{-3}	61,1 %	26,3 %
10,0 g.dm^{-3}	63,2 %	50,0 %
15,0 g.dm^{-3}	65,0 %	50,0 %
20,0 g.dm^{-3}	100,0 %	55,6 %

6.2.2 The effect of the agar and sucrose content of the medium on the fresh mass of Ornithogalum 'Rollow' explants.

Agar and sucrose significantly affected the fresh mass of explants *in vitro* (Table 45). However, a significant interaction between these two factors on fresh mass was only evident after three weeks in culture, when plantlets began forming on the explants. A significant change in the influence of agar on fresh mass accumulation was noted during the culture period.

Table 45. Results of an analysis of variance showing the effect of agar, sucrose and their interaction on explant fresh mass accumulation.

Source of variation. between 3 and 6 weeks in culture.	Variance ratio. 3 weeks	6 weeks	Changes in fresh mass
Mean	550,59**	578,32**	1,10
Agar	39,50**	32,20**	2,85*
Sucrose	14,76**	6,00*	3,39
Interaction Agar x sucrose	1,54	2,91**	0,36

** : Highly significant Result; reject H^O at at p = 0,01
* : Significant Result ; reject H^O at at p = 0,05.

The fresh mass of the explants was significantly higher on media containing 45,0 $g.dm^{-3}$ as opposed to 30,0 $g.dm^{-3}$ sucrose (Table 46).

Table 46. Results of a contrast in population means showing the influence of the sucrose concentration in the medium on the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants.

Culture period.	Test value.
3 weeks	-3,85**
6 weeks	-2,63**

Control : 30,0 $g.dm^{-3}$ sucrose (Nel, 1981).
Negative sign : Implies concentration mean > Control.

The presence of agar in the medium led to a significant in-

crease in explant fresh mass (Table 47). Decreasing the agar content of the medium to 5,0 g.dm^{-3} increased the fresh mass of the explants significantly, while increasing the agar content of the medium to 15,0 or 20,0 g.dm^{-3} led to a significant decrease in explant fresh mass (Table 47).

Table 47. Results of a contrast in population means showing the effect of the agar content of the nutrient medium on explant fresh mass.

Contrast.	Test statistic.	
	3 weeks.	6 weeks.
7,0 - 0,0 g.dm^{-3}	+9,29**	+7,40**
7,0 - 5,0 g.dm^{-3}	-2,56*	-3,89**
7,0 - 6,0 g.dm^{-3}	+1,07	-0,57
7,0 - 10,0 g.dm^{-3}	+2,73*	+0,44
7,0 - 15,0 g.dm^{-3}	+6,59**	+3,86**
7,0 - 20,0 g.dm^{-3}	+7,81**	+6,15**

Control : 7,0 g.dm^{-3} agar (Nel,1981).
Positive sign : Implies concentration mean > control.
Negative sign : Implies concentration mean < control.
** : Highly significant result; Reject H^{O} at p = 0,01.
* : Significant result; Reject H^{O} at p = 0,05.

A contrast in population means revealed that after six weeks in culture, optimal fresh mass accumulation occurred on media containing 5,0 g.dm^{-3} agar and 45,0 g.dm^{-3} sucrose (Test statistic = 8,46**; p > 0,01), when compared to all other media tested in this experiment.

6.2.3 The effect of the agar and sucrose content of the medium on the dry mass of *Ornithogalum* 'Rollow' explants.

The sucrose and agar content of the nutrient medium both

significantly affected explant dry mass (Table 48). No significant interaction between the agar and sucrose concentrations in the medium and explant dry mass were evident after six weeks in culture (Table 48).

Table 48. Results of an analysis of variance to determine the effect of the agar and sucrose content of the medium on *Ornithogalum* 'Rollow' explant dry mass after six weeks in culture.

Source of variation.	Variance ratio.
Mean	505,04**
Agar	3,22*
Sucrose	15,34**
Interaction: Agar x Sucrose	1,37

** : Highly significant result : Reject H^{O} at at $p = 0,01$.
* : Significant result : Reject H^{O} at $p = 0,05$.

Explants on media containing 45,0 $g.dm^{-3}$ sucrose had significantly greater dry masses than those on media containing 30 $g.dm^{-3}$ sucrose (Test statistic = -3,902** ; $p < 0,01$).

Explants on media lacking agar and those on media containing 20,0 $g.dm^{-3}$ agar had significantly lower dry masses than those on media containing 7,0 $g.dm^{-3}$ (Control suggested by Nel, 1981). The dry masses of explants on all other media failed to differ significantly from those on the control (Table 49).

Table 49. Results of a contrast in population means showing the effect of the agar content of the nutrient medium on Ornithogalum 'Rollow' explant dry mass after six weeks in culture.

Contrast.	Test statistic
7,0 - 0,0 $g.dm^{-3}$	3,60**
7,0 - 5,0 $g.dm^{-3}$	2,09
7,0 - 6,0 $g.dm^{-3}$	0,05
7,0 -10,0 $g.dm^{-3}$	0,75
7,0 -15,0 $g.dm^{-3}$	2,27
7,0 -20,0 $g.dm^{-3}$	2,89**

Control : 7,0 $g.dm^{-3}$ agar (Nel,1981).
Positive sign : Implies concentration mean < control.
** : Highly significant result : Reject H^{O} at p = 0,01.

6.2.4 The effect of agar and sucrose content of the medium on the percentage moisture content of explants.

Varying the agar and sucrose content of the nutrient medium failed to have a significant effect on the percentage moisture content of the explants (Table 50).

Table 50. Percentage moisture contents for Ornithogalum 'Rollow' explants on the different media used in this experiment.

Agar concentration.	Sucrose concentration. 30 g.dm^{-3}	45 g.dm^{-3}
0,0 g.dm^{-3}	98,88	99,03
5,0 g.dm^{-3}	98,82	99,08
6,0 g.dm^{-3}	98,57	98,50
7,0 g.dm^{-3}	98,54	98,55
10,0 g.dm^{-3}	98,40	98,46
15,0 g.dm^{-3}	98,82	98,85
20,0 g.dm^{-3}	98,97	99,07
Average	98,71	98,79

6.2.5 The influence of explant growth on the water potentials of media containing different agar and sucrose concentrations.

Both the agar and sucrose content of the medium were found to have a significant effect on its water potential after 6 weeks of explant subculture (Table 51).

Table 51. Results of an analysis of variance to determine the effect of explant growth on the water potential of the nutrient medium.

Source of variation.	Variance Ratio.
Mean	2754,69**
Agar	10,43**
Sucrose	100,12**
Interaction : Agar x Sucrose	1,40

** : Highly significant result ; Reject H^{o} at p = 0,01.

Media containing 45,0 g.dm^{-3} sucrose were found to have sig-

nificantly lower water potentials than media containing 30,0 $g.dm^{-3}$ sucrose at the end of a six week culture period (Test statistic = $-11,51^{**}$; $p < 0,01$).

The water potential of media containing agar were significantly lower than those of media without agar (Table 52).

Table 52. Results of contrasts to determine the effect of the agar content of the medium on medium water potential.

Contrast.	Variance ratio.
7,0 - 0,0 $g.dm^{-3}$	$8,64^{**}$
7,0 - 5,0 $g.dm^{-3}$	0,02
7,0 - 6,0 $g.dm^{-3}$	0,28
7,0 -10,0 $g.dm^{-3}$	-0,89
7,0 -15,0 $g.dm^{-3}$	1,84
7,0 -20,0 $g.dm^{-3}$	1,85

Control: 7,0 $g.dm^{-3}$ (Nel 1981).
Negative sign : Implies concentration mean > control.
Positive sign : Implies concentration mean < control.
** : Highly significant result; Reject H^{o} at $p = 0,01$.

6.2.6 The influence of sucrose and agar in the medium on the chlorophyll content of the explants.

The sucrose concentration of the medium significantly affected the chlorophyll content of the explants ($X^2 = 4,379^{*}$; $p = 0,04$). Of explants grown on media containing 30,0 $g.dm^{-3}$ sucrose, 54,2% possessed chlorophyll, while 45,8% of explants on media containing 45,0 $g.dm^{-3}$ sucrose had chlorophyll.

The agar content of the medium was without significant effect on the chlorophyll content of explants (X^2 = 8,941 NS ; p = 0,18).

No significant interaction between the sucrose and agar content of the medium on the chlorophyll content of the explants was noted (X^2 = 21,978 NS; p = 0,06).

6.3 Summary of Results.

- Agar did not significantly affect plantlet initiation or explant chlorophyll content. Optimum fresh and dry mass occurred on media containing agar, but 15,0 and 20,0 $g.dm^{-3}$ respectively, depending on the sucrose content of the medium, proved inhibitory to growth.

- Increasing sucrose levels in the medium reduced the incidence of chlorophyll and plantlets, but led to increasing fresh and dry explant masses.

- Increasing the sucrose and agar concentrations significantly reduced media water potentials, but failed to affect explant percentage moisture contents after a six week culture period.

6.4 Discussion.

Organogenesis occurred more frequently on media containing 30,0 as opposed to 45,0 $g.dm^{-3}$ sucrose (Table 43). The inhibitory effects of supra-optimal sucrose concentrations have previously been reported (Galiba & Erdei, 1986; Brown & Thorpe, 1980a; and Takayama & Misawa, 1980).

Associated with a higher frequency of organogenesis was a higher concentration of chlorophyll. Tran Thanh Van (1977) found that the presence of chloroplasts is essential for bud formation, even though CO_2 measurements indicate that the

rate of photosynthesis *in vitro* is low. The concentration of sucrose, but not agar, in the nutrient medium affected the chlorophyll content of the explants.

Carbohydrates *in vitro* function as carbon and energy sources, but also have an osmotic role in the medium (Brown Leung & Thorpe, 1979). Although a minimum osmotic requirement must be satisfied in order for organogenesis to proceed, osmotic effects alone were insufficient to induce organogenesis (Brown, Leung & Thorpe, 1979).

In this experiment, the sugar content of the media did not affect the water content of the tissues significantly (Table 50). Osmotic adjustment or the accumulation of organic compounds and salts in the cells did not occur, as the ratio of fresh to dry mass in the tissues remained constant. The adverse effect of supra-optimal sucrose concentrations on growth cannot be interpreted as the result of osmotic stress placed on the explant by the medium. A similar conclusion was reached by Takayama and Misawa (1980) who were able to alleviate the adverse effect of supra-optimal sucrose concentrations on growth by raising the salt content of the nutrient medium.

In addition to their osmotic role, carbohydrates affect cellular metabolism directly (Brown & Thorpe, 1980 b). Although hormones are generally recognised as controlling organogenesis, the combination, type and concentration of sugars in the medium may reverse the effect of the hormone balance in the medium (Tran Thanh Van, 1977). The physiological state of the donor plant at the time of excision and the inter-tissue correlations also affect organogenesis *in vitro* (Tran Thanh Van, 1981).

Although explants grown on media containing 30,0 $g.dm^{-3}$ sucrose produced more plantlets, explants on media containing 45,0 $g.dm^{-3}$ sucrose had greater fresh and dry masses.

On average, 98,75% of the explant is composed of water (Table 50), yet the water content of the explants was unaffected by the various media. As a result, these mass increases can only be ascribed to the increased rate of undifferentiated growth on media containing 45,0 $g.dm^{-3}$ sucrose. Brown & Thorpe (1980 b) found that, prior to primordium formation, shoot-forming tissues grow at a relatively slower rate than non shoot-forming tissues. This is a direct consequence of the altered metabolism of shoot-forming cells and the high energy requirements of the organogenic processes (Brown & Thorpe, 1980 b).

The presence of agar in the medium had no effect on the incidence of plantlets on explants. In the absence of agar in the medium, a significant decrease in medium water potential occurred (Table 52), as well as a reduction in explant fresh and dry mass (Tables 47 and 49 respectively). As the moisture content of the explants was unaffected by the agar content of the medium (Table 50), a reduced rate of undifferentiated growth on media without agar was responsible for the reduced rates of fresh and dry mass accumulation. The high pH of the nutrient medium (pH 6,2 was used in this experiment) appears responsible. Murashige (1977) found it necessary to reduce the pH of liquid media to pH 5,0 to prevent the precipitation of media components essential to growth.

It is not apparent why organogenesis was unaffected by the absence of agar in the nutrient medium. It has been shown in this experiment that conditions which were optimal for fresh and dry mass accumulation (45,0 $g.dm^{-3}$ sucrose and 5,0 $g.dm^{-3}$ agar) were not necessarily optimal for organogenesis. It may be that the altered metabolism of tissues which undergo organogenesis (Brown & Thorpe, 1980 b) change the <u>in vitro</u> requirements of these tissues.

No significant differences in the water potentials of the

agar media over the concentrations used were observed (Table 52). The agar effect appeared to result from the reduced contact between the explant and the medium (Debergh, 1982).

As the concentration of sucrose in the nutrient medium increased from 30,0 to 45,0 g.dm-3, so the optimum agar concentration range for plantlet initiation increased from 10,0 to 15,0 $g.dm^{-3}$. Yosnida et al.(1982) observed that factors, for example, medium hardness, or differentiation of the plant body which reduce the contact between the medium and the explant also increase transpiration rates of the explant. This in turn reduces the tolerance of explants to high osmotic pressures in the medium.

The linear relationsnip which exists between medium hardness and the agar content of the medium is determined by the agar brand, which for this reason, should always be specified in tissue culture experiments.

CHAPTER 7.

THE EFFECT OF THE TISSUE CULTURE MEDIUM pH ON THE GROWTH OF ORNITHOGALUM 'ROLLOW' TISSUE CULTURES ON NEL'S (1981) MEDIUM.

7.1 Introduction.

Plants in tissue culture are able to withstand pH values between pH 4,0 and pH 7,2 (Reinert & Yeoman, 1982). The detrimental effects of pH are generally related to ion availability and nutrient uptake, rather than cell damage (Murashige & Skoog, 1962; Behrend & Mateles, 1975; Reinert & Yeoman, 1982 and Skirvin et al., 1986). Substrate utilization (Martin & Rose, 1976) and the rate of auxin (Macdowall & Sirois, 1977), for example, 2,4-D (Kurkdjian et al., 1981) and abscisic acid uptake into cells (Reinert & Yeoman, 1982), is affected by pH.

Skirvin et al.(1986), suggest that as the pH-values of culture media vary under the conditions existing in different laboratories "all tissue culture researchers should take care to measure the pH of the nutrient medium both before and after autoclaving as well as at various times during culture". The extent to which autoclaving and phytohormone additions affected the buffering capacity of Nel's (1981) modified Murashige and Skoog (1962) medium was determined in this experiment.

In addition to physical factors which influence pH in vitro, the presence of plant tissues are also known to significantly affect the medium's ultimate pH (Skirvin et al., 1986). These changes are related to the utilization of nitrate and ammonium within culture media. The removal of NO_3^- results in a drift towards neutral pH, while NH_4^+ utilization results in rapid media acidification (Behrend & Mateles, 1975). As pH is dependent on the relative con-

centration of nitrate and ammonia, its influence on growth cannot readily be assessed in unbuffered culture media. For this reason, experiments to determine the influence of pH on the growth of Ornithogalum 'Rollow' were done at controlled pH using a Citrate - K_2HPO_4 buffering system, because of the wide buffering range of this combination. The concentration of buffer per dm^3 (modified from Dawson et al., 1979) was as follows:-

pH	Concentration of citric acid ($g.dm^{-3}$) (1)	Concentration of K_2HPO_4 ($g.dm^{-3}$) (2)	Molarity of buffer soln. $mol.dm^{-3}$
5,0	1,02	1,79	$1,51 \times 10^{-3}$
5,5	0,90	2,00	$1,58 \times 10^{-3}$
5,7	0,86	2,07	$1,60 \times 10^{-3}$
6,0	0,78	2,20	$1,63 \times 10^{-3}$
6,2	0,72	2,30	$1,66 \times 10^{-3}$
6,5	0,60	2,48	$1,71 \times 10^{-3}$
7,0	0,38	2,56	$1,65 \times 10^{-3}$

(1) 0,02 moles citric acid per dm^3
(2) 0,04 moles K_2HPO_4 per dm^3

Media were autoclaved at 1,2 $kg.cm^{-2}$ and 121° C for 15 minutes in an autoclave manufactured by Hirayama Manufacturing Co., Tokyo (Model # HA3D).

As poor agar gelation occurs with decreasing medium pH (Murashige, 1977), agar was not used in these experiments. Leaf base explants of Ornithogalum 'Rollow' were supported on Whatman's number 1 filter paper bridges (Dodds & Roberts, 1982).

In this experiment data was collected from 175 explants. All parameters used in this experiment (except where otherwise stated) were measured after 6 weeks in culture. Ex-

plant dry mass was determined according to the microwave drying technique of Ferreira (1982). The percentage moisture content of the tissues was calculated according to Noggle & Fritz (1976).

Water potentials of the nutrient medium at the pH-values used in the experiment were determined in a screen cage thermocouple psychrometer manufactured by Merrill, Utah.

The effect of autoclaving and hormones on the buffering capacity of Nel's (1981) medium was determined on unbuffered media.

The stock solutions used for phytohormones consisted of 29,7% (v/v) ethanol and phytohormones at concentrations of Ø,2 $mg.cm^{-3}$.

The medium was acidified using Ø,1 N HCl and made alkaline with Ø,1 N NaOH. The resultant changes in pH were checked using a Knick 646 digital pH meter using an Ingold U 455 electrode. The pH meter was standardised using 2 buffers (pH 7,ØØ and pH 4,Ø1).

Initially, each treatment comprised twenty replicates. The experimental data was analyzed using BMDP - P series programmes (Dixon, 1985). The effect of pH on the number of plants formed on explants, their chlorophyll content and the contamination rate was assessed using Chi-square tests (Dixon, 1985). Where necessary, Yates' correction for continuity was used where the samples were small in size to guard against the possibility of erroneously rejecting the null hypotheses (Brown & Hollander, 1977).

The effect of pH on fresh and dry mass data was assessed using BMDP 2V (Dixon, 1985), an analysis of variance. The distribution of the data was first checked for normality using BMDP 5D, histograms and univariate plots (Dixon,

1985). To determine the significance of treatment effects, where these were observed, the multiple range tests of Student-Newman-Keuls were used at the appropriate confidence limits.

The curve establishing the quadratic relationship between medium water potential and pH was fitted using Genstat V (Copyright Lawes Agricultural Trust, 1984).

7.2 Results.

7.2.1 The buffering capacity of Nel's (1981) medium.

The buffering capacity of Nel's (1981) medium and the effect of autoclaving hormones at the concentrations suggested by Nel (1981) (0,1 $mg.dm^{-3}$ NAA and 2,0 $mg.dm^{-3}$ BAP) on medium pH was assessed (Figure 12). Addition of phytohormones to Nel's (1981) medium over the pH range studied (pH 2,16 - 9,02) resulted in more alkaline or acid medium depending on the initial pH-value. Autoclaving the medium led to an acidification particularly in the range pH 6,7 to pH 9,02 (Figure 12). The graph shows that the minimum buffering capacity of Nel's (1981) medium falls between pH 3,0 and pH 8,00. However, neither the addition of hormones to the medium, nor autoclaving appeared to significantly affect the buffering capacity of Nel's (1981) medium.

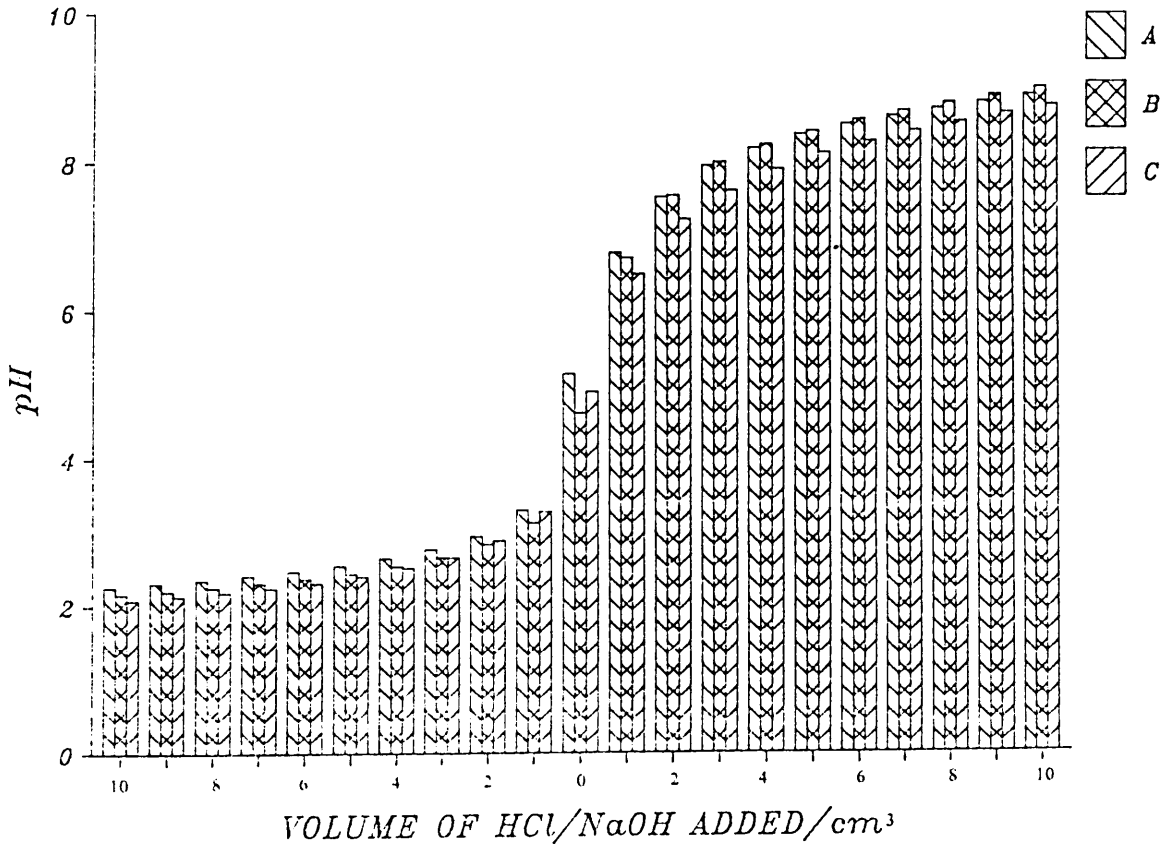


Figure 12. The buffering capacity of Nel's (1981) medium. Before phytohormones were added (A); after the addition of 0,1 mg.dm^{-3} napthaleneacetic acid (NAA) and 2,0 mg.dm^{-3} benzylaminopurine (BAP) (B), and after medium (B) was sterilized by autoclaving (C).

7.2.2 The effect of culture medium pH on the incidence of plantlets on Ornithogalum 'Rollow' explants.

The pH of the medium did not significantly affect the frequency with which plantlets occurred on explants (X^2 = 19,438 NS; p = Ø,Ø8). Of the explants, 72% failed to produce plantlets; 11,4% produced between 1 and 5 plantlets and 16,6% produced more than 5 plantlets after six weeks in culture. The relatively low regenerative ability of the explants indicates an adverse effect of the buffer on regeneration.

7.2.3 The effect of medium pH on explant fresh mass.

The distribution of fresh mass data was checked using BMDP - 5D histograms and univariate plots (Dixon, 1985) and found to be normally distributed.

The anova revealed a significant effect of pH on the rate of fresh mass accumulation by Ornithogalum 'Rollow' explants (Table 53).

Table 53. Results of an analysis of variance to determine the effect of pH on Ornithogalum 'Rollow' explant fresh mass.

Source of Variation.	Variance ratio.		
	3 weeks	6 weeks	(3-6 weeks)
Treatments	4,66**	3,45**	1,44
Equality of means test; variances are not assumed equal.			
Welch	3,23**	4,59**	1,36
Brown-Forsythe	4,57**	3,32**	1,44
Levene's test for equality of variance	Ø,62	1,98	2,7Ø*

** : Highly significant result; Reject H^O at p = Ø,Ø1

* : Significant result; Reject H^O at p = Ø,Ø5.

According to the multiple range test of Student-Newman-Keuls (Dixon, 1985), at the 99% confidence limit the optimum pH range for fresh mass accumulation lay between pH 5,0 and 6,0 after three weeks and pH 5,0 and 6,5 after six weeks (Fig. 13). Given sufficient time, Ornithogalum `Rollow' explants were capable of accumulating fresh mass over a wide range of pH values.

Figure 13. Results of the Student-Newman-Keuls multiple range test at the 99% confidence limit to determine the optimum pH for fresh mass accumulation (in grams) by Ornithogalum `Rollow' explants.

pH 5,0	pH 5,5	pH 5,7	pH 6,0	pH 6,2	pH 6,5	pH 7,0
Mean ($\bar{x}$) change in fresh mass between 0 and 3 weeks in culture:						
Suboptimal						
Optimal						
($\bar{x}$)0,03	0,02	0,02	0,05	0,02	0,01	0,01
Mean change in fresh mass between 0 and 6 weeks in culture:						
Suboptimal						
Optimal						
($\bar{x}$)0,03	0,02	0,02	0,04	0,02	0,01	0,00
n 26	30	21	24	29	22	23

7.2.4 The effect of medium pH on explant dry mass.

The data was checked using BMDP - 5D (Dixon, 1985), and found not to have a normal distribution. A non-parametric anova revealed a significant effect of medium pH on dry mass accumulation by Ornithogalum `Rollow' explants in vitro (Table 54).

Table 54. Results of an analysis of variance to determine the effect of medium pH-value on dry mass accumulation by Ornithogalum 'Rollow' explants in vitro.

Source of variation	Variance ratio.
Treatment	7,Ø8*
Equality of means test; variances are not assumed to be equal.	
Welch	4,99
Brown-Forsythe	7,Ø8*
Levene's test for equality of variance	Ø,ØØ

* : Significant result; Reject H^{O} at p = Ø,Ø5.

The optimal pH for dry mass accumulation lay between pH 5,5 and pH 7,Ø (Figure 14).

Figure 14. Results of a Student-Newman-Keuls multiple range test used at the 95% confidence limit to determine the optimum pH for dry mass accumulation (in grams).

	pH 5,Ø	pH 5,5	pH 5,7	pH6,Ø	pH 6,2	pH 6,5	pH 7,Ø
	Mean ($\bar{x}$) explant dry mass after 6 weeks in culture:						
Suboptimal							
Optimal							
(x)	Ø,Ø15	Ø,Ø22	Ø,Ø17	Ø,Ø23	Ø,Ø27	Ø,Ø27	Ø,Ø17
n	12	12	12	12	12	12	12

7.2.5 The effect of medium pH-value on the percentage water content of explants.

Varying the pH of the nutrient medium failed to significantly affect the moisture content of Ornithogalum `Rollow' explants after 6 weeks in culture (Table 55).

Table 55. Percentage moisture contents of Ornithogalum `Rollow' explants from media with different pH values after six weeks in culture.

pH	% moisture content.
5,0	99,29 %
5,5	99,95 %
5,7	99,19 %
6,0	99,99 %
6,2	98,84 %
6,5	98,80 %
7,0	99,38 %

7.2.6 The influence of medium pH on the water potential of the nutrient medium before and after explant subculture.

A quadratic relationship was shown to exist between pH and the water potential of Nel's (1981) medium buffered with citrate and K_2HPO_4 (Table 56). The water potentials of nutrient media give an indication of the solute content of the medium and thus the nutrients available to the plant for growth.

Table 56. Results of an analysis of variance to determine the relationship between pH and solute potential before and after explant subculture.

Source of Variation		Variance ratio. Initial value	value after 6 weeks
pH		10,183**	7,225**
relationship:	linear	8,779*	4,669
	quadratic	25,908**	21,121**
	cubic	1,661	8,371*
Deviations		8,249**	3,063
Coefficient of variation		24,6%	54,0%

** : Highly significant result; Reject H^{O} at p = 0,01

* : Significant result; Reject H^{O} at p = 0,05.

The relationship (Figure 15), determined using a thermocouple psychrometer showed that before explant subculture, most nutrients were available in culture media between pH values of 5,6 and 6,5. After 6 weeks of explant subculture, the situation was reversed. Few nutrients were available for growth between these pH limits.

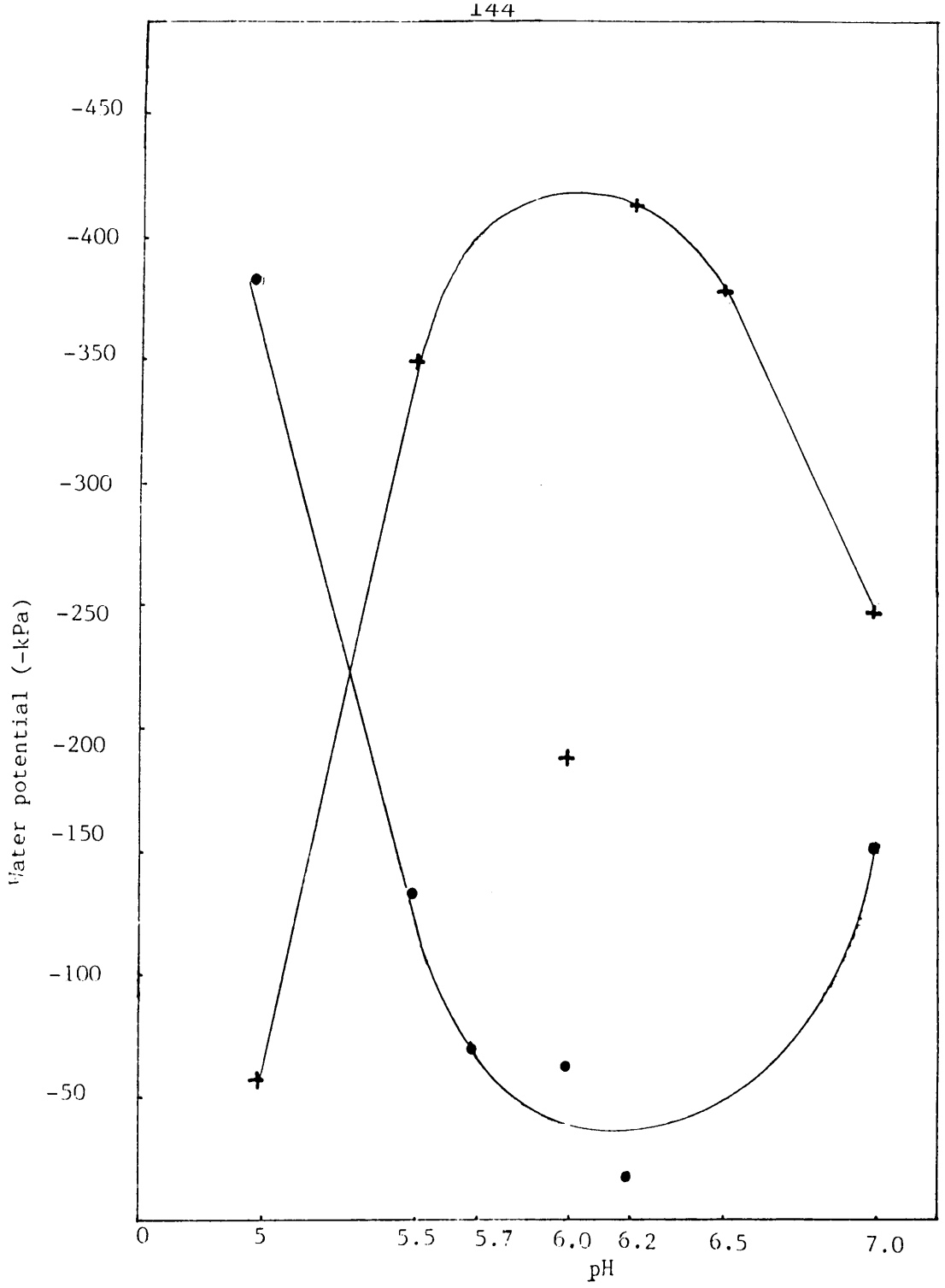


Figure 15. The effect of citrate - K_2HPO_4 buffering system on the water potential of Linsmaer & Skoog's (1965) medium initially (×) and after Ornithogalum 'Rollow' explants were subcultured for six weeks (•).

7.2.7 The effect of medium pH on the chlorophyll content of Ornithogalum 'Rollow' explants after six weeks in culture.

The pH of the medium significantly affected the chlorophyll content of explants (X^2 = 19,800*; p=0,05) 61 % of explants on media at pH 5,0 possessed chlorophyll, while 93,1 % of explants at pH 6,2 and 100 % of explants at pH 6,5 had chlorophyll (Table 57). These values correspond to the minimum and maximum concentration of buffer used in the experiment (See Materials & Methods).

Table 57. Results of Chi square (X^2) tests to determine whether significant differences between chlorophyll contents of Ornithogalum 'Rollow' explants cultured at pH values other than pH 6,2 (Nel, 1981) occurred.

pH values contrasted.	X^2
5,0 - 6,2	8,006*
5,5 - 6,2	0,574 (C)
5,7 - 6,2	0,146 (C)
6,0 - 6,2	0,106 (C)
6,5 - 6,2	10,415*(C)
7,0 - 6,2	3,457 (C)

(C) Refers to Yates' correction according to Brown & Hollander (1977).

* : Significant Result; $X^2 > X^2_{1;0,008}$ at p = 0,05).

7.2.8 The effect of culture medium pH on the visible contamination rates in Ornithogalum 'Rollow' explants.

The pH of the culture medium significantly affected the visible contamination rate (X^2 = 21,499** ; p = 0,002) (Table 58). At pH 5,0, 53,8 % of the explants were con-

taminated as compared to 13,8 % at pH 6,2. At pH 7,0, 65,2 % of the explants were contaminated.

Table 58. Results of Chi square tests to determine whether contamination rates (X^2) at pH 6,2 (Nel, 1981) differed significantly from those at other pHs used in the experiment.

pH	X^2
5,0 - 6,2	9,989*
5,5 - 6,2	4,069
5,7 - 6,2	2,710
6,0 - 6,2	2,863
6,5 - 6,2	0,665 (C)
7,0 - 6,2	14,628*

(C) : Refers to Yates' correction according to Brown & Hollander (1977).

* : Significant Result; $X^2 > X^2_{1;0,008}$ at $p = 0,05$.

7.3 Summary of Results.

- The buffering capacity of Nel's (1981) medium was minimal between pH 3,0 and pH 8,0.

- The incidence of plantlets on Ornithogalum 'Rollow' explants was unaffected by pH values between 5,0 and 7,0.

- The optimum rate of fresh mass accumulation occurred over a pH range of 5,0 - 6,0 (after 3 weeks) and 5,0 - 6,5 (after 6 weeks).

- Optimal dry mass accumulation occurred in the pH range between pH 5,5 and 7,0.

- The percentage moisture content of the explants was un-

affected by the pH value.

- Water potential data show that during culture, most nutrients are available and utilized between pH 5,6 and 6,5.

- Chlorophyll content of explants was lowest at pH 5,00 and highest at pH 6,5. The concentration of K_2HPO_4 - citric acid buffer was lowest at pH 5,0 and highest at pH 6,5. A relationship between the chlorophyll content of explants and the amount of buffer in the medium occurred.

- Contamination rates were highest at pH extremes (5,0 and 7,0) used in the experiment. This corresponded with media having the highest concentrations of solutes at the end of the culture period.

7.4 Discussion.

The nutrient media used in tissue culture are generally poorly buffered at the pH values most commonly utilized for growth (Reinert & Yeoman, 1982). In this experiment the buffering capacity of Nel's (1981) medium was lowest between pH 3,0 and pH 8,0 (Figure 12).

Neither autoclaving, nor the addition of the phytohormone concentrations suggested by Nel (1981) altered the buffering capacity of the medium. Autoclaving the medium resulted in significant media acidification between pH 6,7 and pH 9,02. Skirvin et al. (1976) found a similar effect of autoclaving on pH. He attributed this effect to chemical reactions within the medium. According to Skirvin et al. (1976) factors which could influence autoclaving include autoclave temperature, type of autoclave, position within the autoclave, quality of mineral nutrients, water quality and duration of autoclaving.

Singha (1982) showed that increasing the concentration of agar in the nutrient medium tended to counteract the effect of reduction in pH with autoclaving. However, he points out that the effect of both agar and autoclaving on the pH of the medium is so slight as to be almost negligible.

Martin & Rose (1976) found evidence that medium pH exerts it's effect on growth in culture media through controlling the balance between ammonium and nitrate utilisation in culture media. In their investigations on Ipomea, immediate post inoculation utilisation of ammonia and not nitrate occurred between pH 6,2 and pH 7,1. Nitrate, but not ammonium utilisation occurred at pH 4,8 and both compounds are utilised at pH 5,6.

Skirvin et al. (1976) found that, regardless of the initial pH of the medium, plant tissues placed on the culture media tend to modify the pH of these media within a few days. The ultimate pH lay within a very narrow range, which appeared independent of the initial pH of the medium.

As a result in the alteration of medium pH with explant growth (Skirvin et al., 1986; Rose & Martin, 1975) it is difficult to assess the effect of external pH on the metabolism of cells. To prevent pH variation during the culture period, Nel's (1981) medium was buffered over a range of pH values using Citric acid - K_2HPO_4 buffer solutions (Dawson et al.,1979). To reduce the sodium content of the medium, K_2HPO_4 was substituted for Na_2HPO_4. This buffer system was chosen for its effectiveness over a wide pH range (2,6 - 7,6). At and above pH 7,Ø a white precipitate was formed. As no precipitate formed when the pH of unbuffered media was adjusted, the precipitate is probably calcium orthophosphate, which has low solubility at high pH.

No effect of pH on the ability of explants to produce

plantlets was observed, however, buffering the medium appeared to adversely affect plantlet initiation as only 28 % of the explants produced plantlets *in vitro*. Other buffering systems used in culture media have been shown to have similar adverse effects on growth. Street & Henshaw (1966) and Reinert & Yeoman (1982) found that significant buffering by KH_2PO_4 and K_2HPO_4 is only achieved at soluble phosphate levels inhibitory to growth. Sheat *et al.*(1959), quoted by Reinert & Yeoman (1982) found that, while buffering could be achieved with sparingly soluble calcium phosphates, these reduce the availability of micronutrients, making them unavailable and exerting an inhibitory effect on plant growth. The most effective buffering systems are those devised by Martin & Rose (1976) and Martin, Rose & Hui (1977). Cells were grown in batch cultures in fermentor vessels, the pH of the medium was adjusted automatically using HCl and NaOH or ammonium hydroxide.

The percentage moisture content of the tissues was unaffected by the culture medium. The optimum dry mass accumulation rates occurred on media in the pH ranges from 5,5 to 7,Ø. Martin, Rose & Hui (1977) attributed the low dry masses at pH 5,Ø to the low rate of sugar conversion to biomass, as a result of the metabolic energy required for maintaining tissues cultured at low pH. The optimum fresh mass accumulation occurred between pH 5,Ø and pH 6,Ø after 3 weeks, and pH 5,Ø and 6,5 after 6 weeks. The optimal pH of culture media for fresh and dry mass accumulation lies between pH 5,5 and pH 6,5.

The adverse effects of pH on growth are generally attributed to reduced ion availability, rather than direct cellular damage (Reinert & Yeoman, 1982). The solutes in solution lower the chemical potential of water in the system (Noggle & Fritz, 1976). Thus, the water potential of a system provides a measure of the nutrients available for growth in the system.

At pH values between 5,5 and 7,0 most of the solutes in the nutrient medium were utilized for growth during the six week subculture period. At pH 5,0 a rise in the concentration of solutes occurred over the culture period. The cause of this rise in solutes is not known.

The chlorophyll content of the tissues correlated well with the concentration of buffer used in the medium. The chlorophyll content was minimal where the buffer concentration was lowest and vice versa.

The highest visible contamination rates in this experiment coincided with the pH extremes (5,0 and 7,0). At these pH-values the solutes available in the medium after 6 weeks of explant subculture were at a maximum. The contamination rates observed in this experiment probably reflect the ability of the explant and its contaminating micro-organisms to compete for resources under a given set of environmental conditions.

CHAPTER 8.

THE EFFECT OF CULTURE TEMPERATURE ON THE GROWTH YIELD OF ORNITHOGALUM 'ROLLOW' EXPLANTS IN VITRO.

8.1 Introduction.

Literature concerning the effect of temperature in relation to the requirements for commercial flower production is available for the genus Ornithogalum (von Maatsch et al., 1964 ; Shaub et al., 1971; Shaub & Halevy, 1971, Halevy, Mor & Valershtein, 1971 and Jansen van Vuuren,1983). Some work has also been done on the effect of temperature and other factors, on the control of bulb dormancy in vitro (Halaban, Galun & Halevy, 1983). No information is available on the temperature requirements for bulb formation. This study was aimed at determining the effect of culture temperature on the growth of Ornithogalum 'Rollow' explants in vitro.

Leaf base explant tissue from Ornithogalum 'Rollow' plants was grown axenically on Nel's (1981) medium. This medium contained 0,1 $mg.dm^{-3}$ NAA and 2,0 $mg.dm^{-3}$ BAP. The cultures were grown in controlled environment cabinets at constant temperatures of 15; 22; 25 and 32°C +/- 1°C.

Explants were inspected initially, and after six weeks in culture, to determine the incidence of plantlets, callus, chlorophyll and contamination. Fresh mass was determined initially, and after six weeks in culture.

Data was analyzed using X^2 tests and BMDP 2V's analysis of variance. The significance of treatment effects where observed using the anova, were assessed using the multiple range test of Student-Newman-Keuls. One hundred and twenty-four explants were involved in the study.

Explants were photographed using a stereo dissecting microscope at 10x magnification (Scale : 10 mm = 1 mm).

8.2 Results.

8.2.1 The effect of culture temperature on plantlet initiation by Ornithogalum 'Rollow' explants in vitro.

Temperature was found to significantly affect the incidence of plantlets on Ornithogalum 'Rollow' explants after six weeks in culture ($X^2 = 47,321^{**}$; $p = 0,0000$). The percentage frequency with which plantlets occurred on explants at various culture temperatures is given in Table 59.

Table 59. Percentage frequency of plantlet occurrence at various culture temperatures in vitro.

Number of plants per explant.	Culture temperature. 15° C	22° C	25° C	32° C	Total.
0	55,3	6,4	27,7	10,6	100 %
1 - 5	13,0	43,5	34,8	8,7	100 %
6 - 10	3,4	17,2	51,7	27,6	100 %
> 10	4,5	18,2	54,5	22,7	100 %

Significantly more plantlets formed on explants cultured at 22 and 25° C than on explants cultured at 15° C ($X^2 = 32,93^{**}$; $p = 0,001$). The number of plants formed on explants cultured at 32° C did not differ significantly from the number occurring on explants cultured at 22 and 25° C ($X^2 = 3,26$ NS; $p = 0,05$).

8.2.2 The effect of culture temperature on fresh mass accumulation by Ornithogalum 'Rollow' explants in vitro.

The data was checked for normality using BMDP 5D histograms

and univariate plots (Dixon, 1985). The data was not normally distributed, and as a result of its distribution, it was decided to transform the data using a log normal transformation. The use of this transformation did not alter the outcome of the variance analysis significantly. The culture temperature significantly affected the fresh mass accumulated by explants after six weeks in culture (Table 60).

Table 60. Analysis of variance to determine the effect of culture temperature on fresh mass accumulation by Ornithogalum 'Rollow' explants in vitro after six weeks in culture.

Source of variation	Variance ratio.	
	Untransformed Data	Log-Normal Transformation
Culture Temperature	4,76**	3,49*
Equality of means test; variances are not assumed to be equal.		
Welch	5,79**	3,93*
Brown-Forsythe	4,72**	3,28*
Levene's test for equality of variance	6,51**	1,80

** : Highly significant result; Reject H^{O} at p = 0,01

* : Significant result; Reject H^{O} at p = 0,05.

The significance of treatment effects were assessed using the multiple range test of Student-Newman-Keuls. The optimum culture temperature for fresh mass accumulation lay between 22 - 32^{O} C (Table 61).

Table 61. The effect of culture temperature on Ornithogalum `Rollow' explant fresh mass accumulation shown by the multiple range test of Student-Newman-Keuls at the 99 % confidence limit [T].

	15° C.	22° C.	25° C.	32° C.
suboptimal				
optimal				
mean ($\bar{x}$)	0,05g	0,06g	0,08g	0,07g
n	31	22	20	48

[T] For transformed data, the same result was obtained at the 95 % confidence level.

8.2.3 The effect of culture temperature on the incidence of callus on Ornithogalum `Rollow' explants.

The culture temperature significantly affected the incidence of callus on explants of Ornithogalum `Rollow' after six weeks in vitro ($X^2 = 7,811^*$; $p = 0,05$). The incidence of callus was reduced by increased culture temperatures (Table 62).

Table 62. The effect of culture temperature on the occurrence of callus on Ornithogalum `Rollow' explants in vitro.

Culture temperature.	Incidence of callus.
15° C.	58,1 %
22° C.	36,4 %
25° C.	37,5 %
32° C.	20,0 %

8.2.4 The effect of culture temperature on the incidence of chlorophyll in Ornithogalum 'Rollow' explants.

Culture temperature significantly affected the incidence of chlorophyll in Ornithogalum 'Rollow' explants in vitro (X^2 = 51,136**; p = 0,0000). Increased culture temperatures reduced the incidence of chlorophyll in Ornithogalum 'Rollow' explants in vitro (Table 63).

Table 63. The effect of culture temperature on the occurrence of chlorophyll on Ornithogalum 'Rollow' explants in vitro.

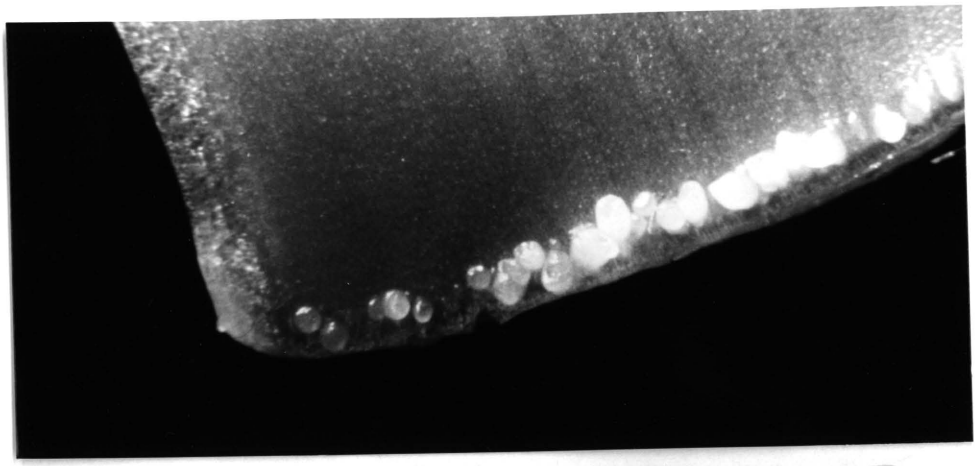
Culture temperature.	Incidence of chlorophyll.
15° C.	100,0 %
22° C.	45,5 %
25° C.	58,3 %
32° C.	0,0 %

8.2.5 The effect of culture temperature on the in vitro contamination rate.

Culture temperature was not found to significantly affect the contamination rate in vitro (X^2 = 3,96 NS ; p = 0,05).

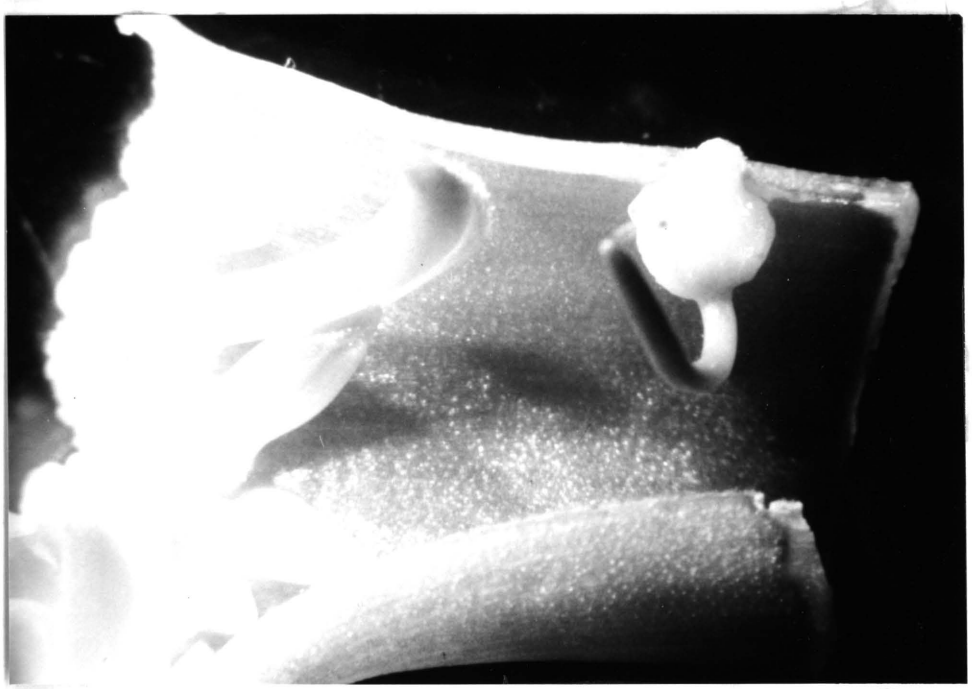
8.2.6 The effect of culture temperature on plantlet morphology.

Photographs were taken of explants cultivated at the temperatures used for this experiment to provide an indication of the effect of culture temperature on plantlet morphology (Figure 16).

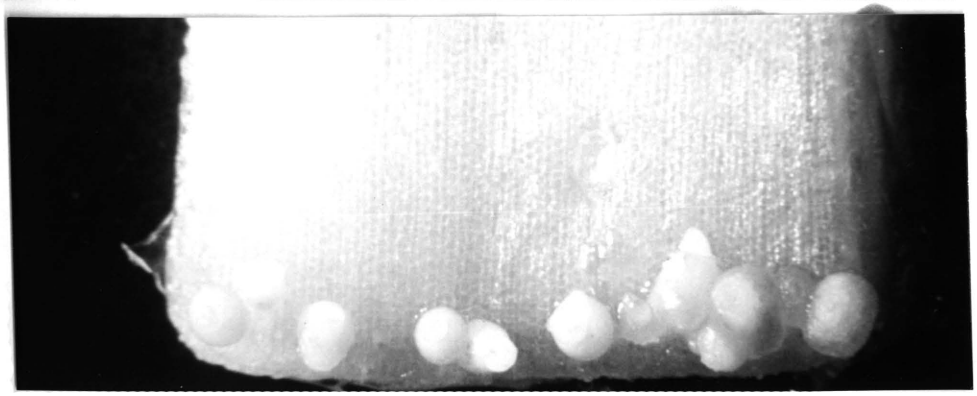


15°C

22°C



25°C



32°C

Figure 16. The effect of culture temperature on plantlet morphology.

8.3 Summary of Results.

- Explants cultured between 22° and 32° C initiated more plantlets and had greater fresh masses than explants cultured at 15° C.

- The incidence of callus and the chlorophyll contents of explants were highest at 15° C and decreased with increasing culture temperature.

- Contamination rates were unaffected by culture temperature.

8.4 Discussion.

According to Towill & Mazur (1976), plants respond to temperature stress in the same way as to osmotic stress. The cells lose water until equilibrium between the chemical potential of water inside and outside the cell is obtained. This physiological response is known to affect biochemical and physiological processes within the cell. Photosynthesis, respiration, solute permeability DNA and protein synthesis (Bernstam, 1978) are all affected. Molecular changes causing these responses could include alterations in the solubility and concentrations of solutes, compaction of macromolecules, changes in pH and membrane alterations.

Dormancy in bulb plants is under the control of environmental factors. Of these factors tested on Ornithogalum arabicum, temperature alone affected dormancy, light intensity and day length being without effect (Shoub & Halevy, 1971). Ornithogalum bulbs do not require vernalization to break dormancy. Tompsett (1985) for Ornithogalum thyrsoides and Shoub & Halevy (1971) for Ornithogalum arabicum both report a high temperature pre-storage requirement in these bulbs to break dormancy. Although initiation and

development of the inflorescence are favoured by a succession of temperatures which reflect the morphological and physiological processes occurring (Went,1953), Shoun & Halevy (1971) and Jansen van Vuuren (1983) found that in Ornithogalum arabicum and Ornithogalum thyrsoides respectively, inflorescence development is possible over a wide range of temperatures (5^{o} - 30^{o} C).

In this experiment, Ornithogalum 'Rollow' explants cultured between 22^{o} and 32^{o} C produced plantlets and accumulated fresh mass at comparable rates. At 15^{o} C growth was retarded. Hussey (1976) reported culturing Ornithogalum thyrsoides explants at 20^{o} C, while Nel (1981) cultured Ornithogalum leaf explants in vitro at 23^{o} C, and reported that, unlike mature bulbs or normal bulblets in vivo, these plants did not become dormant during December, but continued growing, producing flowers within four months in vivo.

The incidence of callus and chlorophyll in explants decreased with increasing culture temperature. Similar results obtained by Pence & Caruso (1986) working on crown gall tumours in Nicotiana tabacum were attributed to reduced cytokinin levels in tissues cultured at high temperatures (Amasino & Miller, 1983). At 33^{o} C, tumour tissues in vitro were green and leafy, while at 21^{o} C, tumour tissue was white and callus like.

The effect of temperature on growth is largely attributed in tissue culture studies to its effect on plant hormone activity. Most evidence is based on growth responses to exogenous applications of these hormones (Halaban, Galun & Halevy, 1965; Stimart & Ascher, 1981 a & b; Seabrook & Cummings, 1982; van Aartrijk & Blom-Barnhoorn, 1983), although some direct measurements of hormone levels in tissues have been made (Amasino & Miller, 1983; Radin & Hendrix, 1986; Pence & Caruso, 1986).

Although many attempts to break dormancy using plant hormones have been made, few of these attempts have been successful (van Staden, 1978). Halevy, Mor & Valershtein (1971), found that only certain stages in the development of Ornithogalum arabicum bulbs could be related to the gibberellic acid concentration in the bulbs. Depending on the stage of development, however, dormancy is probably under the control of more than one plant hormone.

The response of tissues in vitro to different temperature regimes is influenced by the physiological preconditioning of the tissues at the time of explanting (Stimart & Ascher, 1981 b), the age of the tissue (Julien, 1983) and the morphological and physiological characteristics of the explant (Seabrook & Cumming, 1982).

In addition to evidence for the effect of culture temperature on plantlet initiation and fresh mass accumulation in vitro, some evidence that bulb morphology was affected was obtained, but not quantified statistically. Leaf development was largely limited to explants cultured between 22^{o} and 25^{o} C, while bulb diameter increased with increasing culture temperatures to a maximum at 32^{o} C. Callus formation was stimulated by low culture temperature. Further information on the in vivo performance and survival of in vitro bulbs produced under different culture temperatures is required before the commercial significance of their morphologies can be assessed.

CHAPTER 9.

GENERAL DISCUSSION.

The optimum environmental conditions for the *in vitro* propagation of a species and a cultivar of *Ornithogalum* were determined. The cultivar chosen for its broad genetic background was *Ornithogalum* 'Rollow' and the species *Ornithogalum maculatum*. The genetic isolation of the species *Ornithogalum maculatum* in the breeding programme was suggested by its lack of response to conditions under which *Ornithogalum* 'Rollow' initiated plantlets.

The work was undertaken as considerable scope for the commercial exploitation of the genus as pot plants and cut flowers exists both on the local market and overseas.

Multiplication *in vitro* was effected by the stimulation of adventitious shoot formation directly on the explant. A possible disadvantage of the technique is the loss of viability which is associated with repeated subculture (Hussey, 1976). However, it is rapid and shows low variability.

A factorial experimental design was used in all experiments. Parameters studied were the main effects of the variables (called factors) being investigated and the interactions between them (Stuart, 1967). This experimental design is appropriate to exploratory studies where a number of factors are involved in determining the observed response and where results must lead to recommendations that have application over a wide range of conditions. Generally it is necessary to use more than one parameter when assessing the effect of an experimental variable as the influence of the variable need not be the same on all parameters.

The most important determinant of the rate of *in vitro* increase and the quality of the regenerated plants is the initial explant. The impact of explant source, site on the donor organ, orientation relative to the basal medium, additional wounding to the explant and size of the explant on growth yield were studied.

The *in vitro* regeneration of *Ornithogalums* from a wide range of organs; leaves (Nel, 1981); bulb scales and inflorescence stalks (Hussey, 1975, 1976); stigma, style and ovary tissues (Chung *et al.*, 1980) is reported in the literature. So as to avoid the loss of a season between flowering and clonal propagation, the viability of leaf, inflorescence bract and inflorescence stalk explants were compared after one third of the flowers on the inflorescence opened, and selections could be made. Bulb material was avoided as this cannot be used where single plant selections are made in a breeding programme. The most viable source of explants at anthesis was the bract in the axils from which branches of the raceme arise.

The explant fresh mass was a function of the initial explant size, while the explants most efficient at biomass accumulation were those which differentiated plantlets.

Prior to anthesis, leaves are used as explants. They are abundant, obtained with minimal damage to the donor plant, and relatively free of micro-organisms. The donor plant and the site on the donor leaf from which the explant was removed, significantly affected the viability of the explant in terms of their ability to regenerate plantlets *in vitro*. The lower half of the leaf provided the most viable explants, with less satisfactory explants being derived from the upper half of the leaf.

The rate of biomass accumulation by explants depended on their ability to differentiate plantlets. It appears that,

during the first twelve weeks of the culture period, when explants accumulated fresh mass as a linear function of the culture period, fresh mass is determined by the number of plantlets on the explant. After twelve weeks, the rate of fresh mass accumulation increased. Despite the variations to which they are subject, both plantlet callus and fresh masses provide information concerning explant viability.

Variable effects of explant orientation on regenerative ability are reported in the literature (Paek, 1982; Wietsma, 1984). Four orientations of Ornithogalum 'Rollow' explants were compared. Leaf explants were placed on the medium horizontally (with their abaxial or adaxial surface in contact with the medium), and vertically (with polar or apolar orientation). The effect of explant orientation on growth varied depending on the donor plant. Adverse effects were associated with vertical apolar explant orientation, implicating auxin involvement which is transported in a polar direction in plant tissue. As a consequence of this orientation, plantlet initiation was delayed and fresh mass accumulation by these explants was slower in consequence. By the eighth week in culture plantlet initiation was similar, regardless of explant orientation. However, the fresh masses of the explants with vertical, apolar orientations were significantly lower.

Presumably variations in the phytohormone levels in the donor plants during development (Rudnicki & Nowak, 1976) would affect explant response to a given orientation . In addition, the redistribution of auxin would affect levels of other phytohormones in the tissues. Transport of auxin via the phloem is likely to be negligible due to callose deposition in sieve tubes in response to wounding (Esau, 1960). The existence of gradients in regenerative ability in explants (J.G. Janse van Rensburg, pers. comm.) as well as between sites of vascular tissue (Yanagawa & Sakanishi, 1980) implicate the involvement of the epidermis, cortex, xylem

and pith (Goldsmith, 1977) in secondary auxin transport.

Stimulation of plantlet initiation in response to wounding is extensively reported in the literature (Hussey, 1976; van Aartrijk & Blom-Barnhoorn, 1983; Paek, 1982 and Douglas, 1985). A grid of small squares pattern cut into the surface of Ornithogalum 'Rollow' explants was found to inhibit plantlet initiation on explants although the subsequent differentiation of these plantlets on explants was unaffected.

Wounding results in a biphasic response (Macnicol, 1976). The initial response or "wound" respiration lasts 15 - 40 minutes and is unrelated to plantlet initiation (Macnicol, 1976). The "induced" or "developed" respiration occurs after 1 - 2 hours and may last several days (Macnicol, 1976). The magnitude of the latter respiration depends on the extent of DNA and protein synthesis (Macnicol, 1976), but is independent of the age of the explant. Where wounding has a stimulatory effect, this probably results from the increased citric acid cycle, pentose phosphate pathway and glycolytic activity (Macnicol, 1976). The effect of wounding depends on the explant's ability to recover from membrane damage (van Aartrijk, Blom-Barnhoorn & Bruinsma, 1985 b). Macnicol (1976) observed an inverse relationship between energy charge and the distance from the wound surface. The wound effect extends 2 - 5 mm from the cut surface (van Aartrijk & Blom-Barnhoorn, 1983). The decreased ability of Ornithogalum 'Rollow' explants to initiate plantlets in response to wounding was probably a consequence of the energy required to restore membrane integrity.

Metabolic changes in response to wounding are similar to those which occur in response to plantlet initiation (Patel & Berlyn, 1983; Macnicol, 1976). To determine to what extent plantlets arise in association with wound surfaces on the leaf explants, the regenerative ability of (1,0 x 1,0

cm; 0,5 x 1,0 cm; 0,5 x 0,5 cm) explants were compared. Plantlets were found to arise in association with wound surfaces on the explant, rather than randomly distributed on the explant. The regenerative ability per unit wound surface area remained constant for the explant sizes tested, irrespective of the wound surface area of the explant.

Growth yield is determined by the chemical composition and the physical qualities of the medium. Generally, extant media are adequate for plantlet initiation *in vitro* (Gamborg *et al.*, 1976). The medium chosen for experimental work was that of Nel (1981) - a modified Linsmaer & Skoog (1965) medium proposed for mass propagating *Ornithogalum* cultivars *in vitro*. The influence of phytohormones, carbohydrates, agar and pH-value on growth yield are discussed. Since the discovery by Skoog & Miller (1957) that organogenesis could be induced by adjusting the auxin : cytokinin balance in the medium, *in vitro* techniques have been applied to many plant species. Manipulating the benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) contents of Nel's (1981) medium proved insufficient to induce plantlet initiation on *Ornithogalum maculatum* explants. Plantlet initiation on *Ornithogalum* '*Rollow*' explants occurred in the absence of exogenous phytohormones, indicating that sufficient levels of phytohormone were present in these tissues to satisfy the organogenic requirement.

Unlike *Ornithogalum thyrsoides*, which was unaffected by the cytokinin content of the nutrient medium (Hussey, 1976), BAP stimulated organogenesis (at concentrations between 1,0 and 2,0 $mg.dm^{-3}$) and fresh mass accumulation by *Ornithogalum* '*Rollow*' explants (at concentrations between 2,0 and 4,0 $mg.dm^{-3}$). The increasing genetic instability of *Ornithogalum thyrsoides* on repeated subculture, is attributed by Hussey (1976) to the decreasing ability for autonomous cytokinin synthesis by *Ornithogalum thyrsoides* tissues with repeated subcultures.

A significant interaction between phytohormones in the medium occurred, which affected plantlet initiation and fresh mass accumulation by Ornithogalum explants. As the level of BAP in the medium increased, so the optimal level of NAA in the medium for both plantlet initiation and fresh mass accumulation decreased. The concentration of phytohormones of 0,1 $mg.dm^{-3}$ NAA and 2,0 $mg.dm^{-3}$ BAP suggested by Nel (1981) occurred within the concentration range of phytohormones giving optimal yields.

The interaction between phytohormones and the carbohydrate balance in the medium determined the rate and type of differentiation (Tran Thanh Van, 1977). The ability of the explant to differentiate plantlets was determined by the genome with Ornithogalum maculatum explants failing to differentiate plantlets under conditions suitable for plantlet differentiation by Ornithogalum 'Rollow' explants.

Only polysaccharides with an α - glycosyl radical at the non-reducing end of the molecule are capable of inducing organogenesis (Jeffs & Northcote, 1967). These molecules combine with other molecules in the presence of phytohormones to exert their effect on differentiation. At the molecular level, this forms an inducer / repressor system (Shoemaker, Conche & Galbraith, 1986) which controls organogenesis (Jeffs & Northcote, 1967).

The form in which explants utilise carbohydrates is determined by the species or clone (Maretzki, Thom & Nickell, 1974), the concentration of carbohydrates in the medium, the stage of development of the explant (Jeffs & Northcote, 1967) and environmental conditions (Tran Thanh Van, 1981). According to Maretzki, Thom & Nickell (1974) glucose and sucrose, the major transport forms of carbohydrates in the plant, are the two forms most likely to promote optimum growth.

Sucrose was found to stimulate plantlet initiation and biomass accumulation by Ornithogalum 'Rollow' explants, but only in the absence of phytohormones in the medium. Nel (1981) suggested that 30 $g.dm^{-3}$ sucrose was optimal for plantlet initiation on agar solidified media. Callus formation on Ornithogalum maculatum explants was stimulated by increasing the sucrose content of the culture medium. An interaction occurred between carbohydrates in the culture medium - mannose at concentrations greater than 30 $g.dm^{-3}$ in the presence of equivalent or lower sucrose concentrations inhibited fresh mass accumulation by Ornithogalum 'Rollow' explants. In the absence of phytohormones, 10 $g.dm^{-3}$ sucrose was required in tissue culture media of Ornithogalum 'Rollow' before fresh mass accumulation occurred.

In addition to their nutritional role in culture media, carbohydrates exert an osmotic effect. The soluble concentration determines the water potential of the culture medium (Noggle & Fritz, 1976), which initially, or after six weeks of explant subculture, was not found to constitute a stress factor. While a drop in water potential during culture may initially stimulate organogenesis (Yoshida, Kobayashi & Yoshida, 1973) later growth inhibition is often a consequence (Dunwell & Thurling, 1985).

That the adverse effects of supraoptimal sucrose concentrations on growth cannot be interpreted as the result of osmotic stress was demonstrated by Takayama & Misawa (1980) who were able to alleviate the adverse effects of supraoptimal sucrose concentrations on growth by raising the salt content of the nutrient medium.

The optimum agar concentration in Nel's (1981) medium depended on the factors being investigated and the carbohydrate content of the nutrient medium. A significant interaction occurred between the agar and sucrose in culture

media with the highest incidence of plantlets on media containing more than 15,0 $g.dm^{-3}$ agar at 30,0 $g.dm^{-3}$ sucrose. Fresh and dry mass accumulation was stimulated by the agar, however, 15,0 and 20,0 $g.dm^{-3}$ agar respectively proved inhibitory. The water content of the tissue was unaffected by the agar content of the medium, although media containing agar showed significantly higher water potentials than those lacking agar.

The effect of agar on growth is believed to result from the reduced contact between the explant and the medium, which raises the transpiration rate of the explant (Yoshida, Kobayashi & Yoshida, 1973), reducing the tolerance of the explant for low medium water potentials.

According to Reinert & Yeoman (1982) media are generally poorly buffered at the pH-values most commonly utilised for growth. The buffering capacity of Nel's (1981) medium was minimal between pH 3,0 and pH 8,0. Neither the addition of phytohormones to the medium, nor autoclaving, significantly affected the buffering capacity of Nel's (1981) medium. Although medium acidification which was attributed to media interactions (Skirvin et al., 1986) occurred between pH 6,7 and pH 9,02, the effect was so slight as to be almost negligible, and was removed by the addition of agar to culture media (Singha, 1982).

Martin & Rose (1976) observed that medium pH affected the form in which nitrogen was removed from the medium by the explant. From pH 6,2 to pH7,1 ammonium, but not nitrate, was utilised. At pH 5,6 both ammonium and nitrate were utilised, while at pH 4,8, nitrate was used in preference to ammonium. As nitrogen and ammonium are the major buffering components of the medium, their utilisation affects medium pH.

Skirvin et al. (1986) noted that explants modify the pH of

the culture media so that, regardless of initial pH, the final pH of the medium lies within a narrow band.

To study the effect of a given pH on growth, a $K_2 HPO_4$ - citric acid buffer combination was included in the nutrient medium. The buffer was chosen because of its wide buffering capability. A toxic effect of this buffer on growth was observed. Few plantlets were initiated on explants under the experimental conditions. However, maximum chlorophyll retention by explant tissues occurred on media containing the highest buffer concentrations.

The adverse effect of pH on growth has been attributed to its effect on nutrient and phytohormone availability. A logarithmic relationship was established between the pH-value and the water potential of Nel's (1981) medium. The water potential gives an indication of the solute concentrations in media and thus the nutrient availability.

In Nel's (1981) medium most nutrients were available for growth in the pH range pH 5,6 to pH 6,5 at the beginning of the culture period. At pH-values higher than pH 7,0 a white precipitate, probably calcium orthophosphate, formed when buffer was added to the nutrient medium. After six weeks in culture the situation was reversed and solute availability was minimal between pH 5,6 and pH 6,5.

Although plantlet initiation was unaffected by pH-values between pH 5,0 and pH 7,0, optimum fresh mass accumulation occurred between pH 5,0 and pH 6,0 (after three weeks), and pH 5,0 and pH 6,5 (after six weeks), in culture. Optimum dry mass accumulation occurred between pH 5,5 and pH 7,0. Martin, Rose & Hui (1977) attributed the low dry masses at pH 5,0 to the low rate of carbohydrate conversion to biomass, as a result of the metabolic energy required for maintaining tissues cultured at low pH. The percentage moisture content of explants was unaffected by pH.

The contamination visible on explants was highest at pH extremes (pH 5,6 and pH 7,6). These correspond to media having the highest solute concentrations at the end of the culture period and probably indicate the ability of microorganisms and plant tissues to compete for resources under the environmental conditions existing *in vitro*.

In practice it has been observed that the optimal cultural environment is the one that most closely simulates the plant's natural habitat.

The effect of culture temperature on organogenesis *in vitro* is largely attributed to its effect on phytohormone concentrations. The incidence of plantlets and the fresh mass of explants was optimal at culture temperatures from 22^{o} to 32^{o} C. At a temperature of 15^{o} C, plantlet initiation and fresh mass accumulation were inhibited. The chlorophyll content of these tissues was high, as was the incidence of callus. Similar results obtained by Amasino & Miller (1983) were attributed to the reduced cytokinin levels in tissues cultured at high temperatures.

In analysing the relationship between the *in vitro* explant and its environment in terms of growth yield, little information was gained about the fundamental physiological processes that control the response of plants to their environment. However, valuable clues are obtained (Hunt, 1978).

SUMMARY.

Rapid multiplication of chincherinchee (*Ornithogalum* species) by tissue culture.

by
Penelope Anne Landby

Supervisor : Prof. H.A. van de Venter.
Department : Botany.
Degree : M.Sc.

A study of factors affecting morphogenesis by *Ornithogalum* 'Rollow' and *Ornithogalum maculatum* explants was made to facilitate the rapid multiplication of products of the breeding programme of the Vegetable and Ornamental Plant Research Institute in South Africa.

The water potential of Nel's (1981) medium declined insufficiently over the culture period to constitute a physical stress on explants. The inclusion of agar and a citrate - K_2HPO_4 buffer system lowered the water potential of the culture medium significantly. Where the medium was buffered, a quadratic relationship existed between medium water potential and pH. During culture, most nutrients were utilised between pH 5.6 and pH 6.5.

The buffering capacity of the culture medium was minimal between pH 3.0 and pH 8.0. Neither the addition of phytohormones to the medium, nor autoclaving significantly affected the buffering capacity of the medium.

Ornithogalum maculatum explants failed to differentiate plantlets under the culture conditions in this study.

The number of plantlets differentiated on *Ornithogalum* 'Rollow' explants was linearly related to the culture period.

Prior to anthesis, leaf explants were used. A gradient in regenerative ability favouring the leaf base was observed. Apolar vertical orientation of these explants from some donor plants delayed plantlet initiation, implicating auxin involvement in the process.

Plantlets arose in association with wound surfaces on the explant. The number of plantlets initiated was proportional to the wound surface on the explant. Additional wounding to explants inhibited morphogenesis, probably as a result of the diversion of energy away from organogenic processes in order to restore membrane integrity.

After anthesis, the viability of leaf explants decreased. Bracts in the axils of raceme branches were the best explants at this stage in the development of the donor plant.

Napthaleneacetic acid (NAA) and benzylaminopurine (BAP) while not essential for morphogenesis, stimulated plantlet initiation on explants. Imbalances in the phytohormone concentrations affected plantlet incidence and morphology. The higher the BAP concentration, the lower the optimal NAA concentration.

Plantlet initiation was optimal at sucrose concentrations of 30 $g.dm^{-3}$ and was suspended at concentrations lower than 10 $g.dm^{-3}$. Supra-optimal sucrose concentrations inhibited shoot elongation, stimulating plantlet initiation only where phytohormones were not added to the culture medium. Root initiation only occurred at sucrose concentrations between 30 $g.dm^{-3}$ and 60 $g.dm^{-3}$.

The pH-value and agar content of the culture medium was

without effect on plantlet initiation, although the use of the buffer pair, citrate and K_2HPO_4 inhibited plantlet initiation.

Significantly more plantlets were initiated on explants cultured between 22 and 32°C than at 15°C. Leaf development was stimulated at temperatures between 22 and 25°C, while the diameter of bulbs increased to a maximum at 32°C.

Callus formation was stimulated by increasing sucrose concentrations in culture media and inhibited by increasing culture temperature.

For the first twelve weeks in culture, the rate of fresh mass accumulation by explants was dependent on the rate of plantlet and callus formation.

The phytohormone concentration affected fresh mass accumulation by influencing differentiation. Increasing the cytokinin concentration in the culture medium necessitated a reduction in the auxin content of the medium in order to acnieve optimal fresh mass accumulation.

The effect of the interaction between carbohydrates and other medium constituents determined the rate of fresh mass accumulation. Mannose at 30 $g.dm^{-3}$ interacted with sucrose at equivalent or lower concentrations delaying the rate of fresh mass accumulation by explants.

Fresh mass accumulation was optimal at agar concentrations between 5 and 15 $g.dm^{-3}$, at pH-values between pH 5,0 and pH 6,5 and at culture temperatures between 22 and 32°C.

The ratio of fresh to dry mass remained constant for all treatments. Dry mass accumulation was stimulated by increased carbohydrate concentrations, pH-values in the range pH 5,5 to pH 7,0 and agar concentrations below 20,0 $g.dm^{-3}$.

The average moisture content of Ornithogalum 'Rollow' and Ornithogalum maculatum explants was 98,7%. The moisture content of explants was unaffected by altering the medium carbohydrate content, pH-value or agar concentration as described in this study.

The chlorophyll content of explants decreased in response to increasing sucrose concentrations. Decreasing pH-values and buffer concentrations in culture media and increasing culture temperature also reduced the chlorophyll content of explants. The agar content of the culture medium did not affect the chlorophyll content of explants.

The contamination visible on explants varied depending on the source, age and stage of development of the explant material. Highest contamination rates occurred at pH extremes and probably indicated that contaminating micro-organisms had a competitive advantage over explant material under these conditions. Culture temperature was without effect on contamination rates.

SAMEVATTING.

Vinnige vermeerdering van Chincherinchee (Ornithogalum spesies) deur weefselkultuur.

deur
Penelope Anne Landby.

Promotor : Prof. H. A. van de Venter.
Departement : Plantkunde.
Graad : M.Sc.

Die faktore wat morfogenese by Ornithogalum 'Rollow' en Ornithogalum maculatum eksplante beïnvloed is bestudeer ten einde die vinnige vermeerdering tydens die teelprogram van die Navorsingsinstituut vir Groente en Sierplante te vergemaklik.

Die waterpotensiaal van Nel (1981) se medium verander minimaal tydens die groeiperiode en die eksplante was dus nie aan enige fisiese vogspanning onderwerp nie. Indien agar en 'n sitraat - K_2HPO_4 buffersisteem by die medium ingesluit was, net die waterpotensiaal betekenisvol verlaag. 'n Kwadratiese verband tussen die waterpotensiaal van die medium en die pH is waargeneem wanneer 'n bufferisisteem in die medium ingesluit is. Optimale verbruik van voedingselemente in kultuur is tussen 'n pH van 5,6 en 6,5 waargeneem.

Die bufferkapasateit van die medium was minimaal tussen pH 3,0 en 8,0. Byvoeging van groeistowwe en autoklavering het nie die bufferkapasiteit van die medium betekenisvol beïnvloed nie.

Geen differensiasie van plantjies het voorgekom by Ornithogalum maculatum eksplante nie. Die aantal plantjies wat vanaf Ornithogalum 'Rollow' eksplante gedifferensieer het, was verwant aan die tydperk in kultuur.

Blaareksplante is voor antese gebruik. Die regenerasievermoë van die blare het 'n gradiënt gevorm met die optimale gebied aan die blaarbasis. Indien eksplante vanat sommige donorplante op 'n apolêre, vertikale manier georiënteer is, was inisiasie van plantjies vertraag. Hierdie waarneming dui op die betrokkenheid van ouksiene in die proses.

Plantjies is in die omgewing van die wondingsgebied op die eksplante gevorm. Die aantal plantjies wat gevorm het was eweredig aan die omvang van die wondingsoppervlak. Morfogenese is egter geïnhibeer indien die eksplante verder gewond is; moontlik word die energie gebruik om membraanintegriteit te herstel in plaas van organogenese.

Die vermoë om plantjies vanaf blaareksplante te regenereer neem af as die donorplant reeds geblom het. In hierdie geval was die skutblaartjies in die oksels van die bloeistele die beste om te gebruik as eksplante.

Naftaleenasynsuur (NAA) en bensielaminopurien (BAP) was nie noodsaklik vir morfogenese nie maar het wel die inisiasie van plantjies op die eksplante gestimuleer. Ongeballanseerde groeistof konsentrasies het die hoeveelheid plantjies wat gevorm het, sowel as hul morfologie, beïnvloed. 'n Hoër BAP-konsentrasie het 'n laer optimale NAA-konsentrasie tot gevolg gehad.

Die optimale sukrosekonsentrasie vir plantinisiasie was 30 $g.dm^{-3}$ en by konsentrasies laer as 10 $g.dm^{-3}$ is plantjies nie gevorm nie. Supra-optimale sukrosekonsentrasies het die verlenging van spruite geïnhibeer en plantinisiasie in

media sonder groeistowwe gestimuleer. Wortelvorming het voorgekom by sukrosekonsentrasies tussen 30 g.dm^{-3} en 60 g.dm^{-3}.

Die pH-waarde en die agarkonsentrasie van die medium het nie plantinisiasie beïnvloed nie terwyl die bufferpaar, sitraat en K_2HPO_4, plantinisiasie geïnhibeer het.

Kulture geweek tussen 22°C en 32°C het betekenisvol meer plantjies as die by 15°C gelewer. Temperature tussen 22°C en 25°C het blaarontwikkeling gestimuleer terwyl die bolddeursnit maksimaal was by 32°C.

Kallusvorming is gestimuleer deur toenemende sukrosekonsentrasies in die medium en geïnhibeer deur verhoogde temperatuur.

Die toename in varsmassa van kulture is gedurende die eerste twaalf weke deur die mate van plant- en kallusproduksie bepaal.

Die konsentrasie groeistowwe in die medium speel ook 'n rol tydens varsmassatoename aangesien dit differensiasie beïnvloed het. Verhoogde sitokinienkonsentrasies in die medium gaan gepaard met 'n verlaagde ouksienkonsentrasie ten einde 'n optimale toename in varsmassa te verkry.

Varsmassatoename is deur die interaksie tussen koolhidrate en ander bestandele in die medium bepaal. Die interaksie tussen mannose (30 g.dm^{-3}) en sukrose van 'n gelyke of laer konsentrasie het die toename in varsmassa van eksplante vertraag.

Varsmassatoename was optimaal by agarkonsentrasies tussen 5 g.dm^{-3} en 15 g.dm^{-3}, pH waardes tussen pH 5 en 6,5 en temperature tussen 22°C en 32°C.

Die verhouding tussen vars- en droë massa het konstant gebly oor al die behandelings. 'n Toename in droë massa is gestimuleer deur 'n toename in koolhidraatkonsentrasies, pH-waardes tussen pH 5,5 tot 7,0 asook agarkonsentrasies laer as 20 $g.dm^{-3}$.

Die gemiddelde voginhoud van Ornithogalum 'Rollow' en Ornithogalum maculatum eksplante was 98.7%. Die voginhoud van eksplante is nie deur die koolhidraatkonsentrasie, pH-waarde of agarkonsentrasie, soos beskryf, beïnvloed nie.

Met verhoogde sukrosekonsentrasies het die chlorofilinhoud van die eksplante verlaag. Chlorofilinhoud het ook verminder as gevolg van verlaagde pH-waardes en bufferkonsentrasies in die media asook 'n toename in temperatuur. Agarkonsentrasies het geen invloed op die chlorofilinhoud gehad nie.

Variasie in visuele kontaminasie van eksplante is deur die bron van eksplante, ouderdom asook die stadium van ontwikkeling van die donorweefsel beïnvloed. Die hoogste frekwensie kontaminasie het voorgekom by pH uiterstes. Mikroorganismes is waarskynlik tydens kompetitisie onder hierdie toestande bevoordeel. Temperatuur het nie die mate van kontaminasie beïnvloed nie.

ACKNOWLEDGMENTS.

I should like to thank the following people and organizations:-

The University of Pretoria for use of their facilities.

My promoter, Prof. H.A van de Venter.

The staff of Biometric Services, especially Dr. B. Isenberg, Mr. D. Beukes, Miss A. Bosman, Miss E. Campher, Miss S. Griesel, Miss M. Odendaal.

The staff of the Vegetable and Ornamental Plant Research Institute, Roodeplaat, especially Miss J. G. J. van Rensburg and Mrs. A. van der Mescht.

My family, and Drs. W. and G. Hudson for their help and support at all times.

APPENDIX.

The general background of the Ornithogalum 'Rollow'.

O. alticolum (Bainskloofvlei) x *O. flavissimum* (Robberg) | -------- 8374
O. fergussoniae (Kruisfontein) x *O. thyrsoides* (Porterville) |

O. alticolum (Bainskloof) x *O. flavissimum* (Robberg) | x *O. thyrsoides* (Porterville) - 8388
O. flavissimum (Robberg) x *O. miniatum* (Caledon) |

8374 x 8388
|
8395 x (*O. alticolum* (Bainskloof) x *O. brownleii* (Grahamstown))
|
8397 x *O. conicum*[T]
|
75/103/33 cultivar 'Rollow'.

[T] *O. conicum* was derived from crosses of this species out of the following areas:-
Elandsbaai, Snorkfontein, Aurora and Duikerfontein.

REFERENCES.

ABELES, F.B. 1973. Ethylene in plant biology. Academic Press Inc. New York.

ABELES, F.B. & ROBINSTEIN, B. 1964. Regulation of ethylene evolution and leaf abscission by auxin. Plant Physiol. 39: 963-969.

AMASINO, R.M. & MILLER, C.O. 1983. Effect of temperature on the morphology and cytokinin levels of tobacco crown gall tetroma tissues. Plant Sci. Lett. 28: 245-253.

BALL, E. & SOMA, K. 1965. Effect of sugar concentration on growth of the shoot apex of Vicia faba. In proceedings of an international conference on plant tissue culture. Ed. P.R. White & A.R. Grove. McCutchan Pub. Co. California.

BEHREND, J. & MATELES, R.I. 1975. Nitrogen metabolism in plant cell suspension cultures 1. Effect of amino acids on growth. Plant Physiol. 56: 584-589.

BERNSTAM, V.A. 1978. Heat effects on protein biosynthesis. Annu. Rev. Plant Physiol. 29: 25-46.

BROWN, B.W. & HOLLANDER, M. 1977. Statistics. A bio medical introduction. John Wiley & Sons. New York.

BROWN, D.C.W., LEUNG, D.W.M. & THORPE, T.A. 1979. Osmotic requirements for shoot formation in tobacco callus. Physiol. Plant. 46: 36-41.

BROWN, D.C.W. & THORPE, T.A. 1980 (a). Changes in water potential and its components during shoot formation in tobacco callus. Physiol. Plant. 49: 83-87.

BROWN, D.C.W. & THORPE, T.A. 1980 (b). Adenosine phosphate and nicotinamide adenine dinucleatide pool sizes during shoot initiation in tobacco callus. Plant Physiol. 65: 578-590.

CHUNG, J.D., CHUN, C.K., SUH, Y.K., LEE, D.W., BYUN, S.K., PARK, J.K. 1980. Studies on tissue culture of Ornithogalum thyrsoides in vitro. 1. Effect of plant growth regulators on callus formation and organogenesis from tissues of female organ (stigma, style and ovary). J. Korean Soc. Hortic. Sci. 21 (2): 198-203.

COCHRAN, W.G. & COX, G.M. 1957. Experimental designs. 2nd Edition. John Wiley & Sons. New York.

CONNER, A.J. & MEREDITH, C.P. 1984. An improved polyurethane support system for monitoring growth in plant cell cultures. Plant Cell Tissue Organ Cult. 3 (1): 59-68.

DAWSON, R.M.C., ELLIOTT, D.C., ELLIOTT, W.H. & JONES, K.M. 1979. Data for biochemical research. Oxford University Press. 2nd Ed.: 484-485.

DEBERGH, P. 1982. Physical properties of culture media in plant tissue culture. Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. Pub. Japan. Assn. for plant tissue culture. Tokyo.: 135-136.

de FOSSARD, R.A. 1981. Tissue culture for plant propagators. University of New England Printery. Armidale.

DENNE, M.P. 1966. Leaf development in Narcissus pseudonarcissus L.11. The comparative development of scale and foliage leaves. Ann. Bot. 24 (93): 32-47.

DIXON, W.J. 1985. B.M.D.P. Statistical Software Manual. University of California Press. California.

DODDS, J.H. & ROBERTS, L.W. 1982. Experiments in plant tissue culture. Cambridge University Press. New York.

DOUGLAS, G.C. 1985. Formation of adventitious buds in stem internodes of Populus hybrid TT 32 cultured in vitro : Effects of sucrose, zeatin, IAA and ABA. J. Plant Physiol. 121: 225-231.

DOWNS, R.J. & HELLMERS, H. 1975. Environment and the experimental control of plant growth. Academic Press, London.2.

DUNWELL, J.M. & THURLING, K. 1985. Role of sucrose in microspore embryo production in Brassica napus ssp. Olifera. J. Exp. Bot. 36 (170): 1478-1491.

DYER, D.A. 1976. The genera of South African flowering plants. Vol. 2. Gymnosperms and monocotyledons. Dept. Agric. Tech. Serv. P/Bag X 144, Pretoria.

ELIOVSON, S. 1957. South African flowers for the garden. Howard Timmins, Cape Town.

ESAU, K. 1960. Anatomy of seed plants. John Wiley & Sons, U.S.A.

FERREIRA, D.I. 1982. A rapid method for the determination of the moisture percentage in leaves of some Protea species. Agroplantae: 23-24.

GALIBA, G. & ERDEI, L. 1986. Dependence of wheat callus growth, differentiation and mineral content on carbohydrate supply. Plant Sci. 45: 65-70.

GAMBORG, O.L., MURASHIGE, T., THORPE, T.A. & VASIL, I.K. 1976. Plant tissue culture media. In vitro. 12 (7): 473-478.

GOLDSMITH, M.H.M. (1977). The polar transport of auxin. Annu. Rev. Plant Physiol. 28: 439-478.

HALABAN, R., GALUN, E. & HALEVY, A.H. 1965. Experimental morphogenesis of stem tips of Ornithogalum arabicum L. cultured in vitro. Phytomorphology 15: 379-387.

HALEVY, A.H., MOR, J. & VALERSHTEIN, J. 1971. Endogenous gibberellin level in O. arabicum and its relationship to storage temperature of bulbs and flower development. Acta Hortic. 23 (1): 82-87.

HUNT, R. 1978. Plant growth analysis. Edward Arnold. Pub. Camelot Press. Great Britain.

HUSSEY, G. 1975. Totipotency in tissue, explants and callus of some members of the Liliaceae, Iridaceae and Amaryllidaceae. J. Exp. Bot. 26 (91): 253-262.

HUSSEY, G. 1976. Plantlet regeneration from callus and parent tissues in Ornithogalum thyrsoides. J. Exp. Bot. 27 (97): 375-382.

JANSEN van VUUREN, P.J. 1983. Die invloed van temperatuur op bloeiwyse-inisiasie in differensiasie van Ornithogalum thyrsoides. Jacq. Msc. Agriculture. University of Pretoria.

JEFFS, R.A. & NORTHCOTE, D.H. 1976. The influence of indol-3yl acetic acid and sugar on the pattern of induced differentiation in plant tissue culture. J. Cell Sci. 2: 77-88.

JULIEN, M. 1983. Medium term preservation of mesophyll cells isolated from A. officianalis L. Development of a simple method of storage at reduced temperature. Plant Cell Tissue Organ Cult. 2: 305.

KAMP, M. & deHERTOGH, A.A. 1986. Anatomical and growth effects of ethephon on hyacinths and Narcissus during greenhouse forcing. Sci. Hortic. 29(3): 263-272.

KLESSER, P.J. & NEL, D.D. 1976. Virus diseases and tissue culture of some South African flower bulbs. Acta Hortic. 59: 71.

KURKDJIAN, A., MOROT-GUADRY, J.F., WUILLEME, Si LAMANT, A., JOLIVET, E. & GUERN, J. 1981. Evidence for an action of fusicoccin on the vacuolar pH of Acer Pseudo-platanus cells in suspension culture. Plant Sci. Lett. 23: 233-243.

LETHAM, D.S., GOODWIN, P.B. & HIGGINS, T.J.V. 1978. Phytohormones and related compounds - a comprehensive treatise Vol II. Phytohormones and the development of higher plants. Pub. Elsevier/North Holland Biomedical Press. Netherlands.

LINSMAER, E.M. & SKOOG, F. 1965. Organic growth factor requirement of tobacco tissue cultures. Physiol. Plant. 18: 100-127.

MACDOWALL, F.D.H. & SIROIS, J.C. 1977. Importance of time after excision and pH on kinetics of respiration of wheat coleoptile segments to added IAA. Plant Physiol. 59 (3): 405-410.

MACNICOL, P.K. 1976. Rapid metabolic changes in the wounding response of leaf discs following excision. Plant. Physiol. 57: 80-84.

MAENE, L. & DEBERGH, P. 1985. Liquid medium additions to established tissue cultures to improve elongation and rooting in vivo. Plant Cell Tissue Organ Cult. 5: 23-33.

MARETZKI, A., THOM, M. & NICKELL, L.G. 1974. Utilization and metabolism of carbohydrates in cell and callus cultures . Tissue culture and plant science proc. of 3rd international congress of plant tissue and cell culture. Ed. H. E. Street. Academic Press. London: 329-362.

MARTIN, S.M. & ROSE, D. 1976. Growth of plant cell (Ipomea) suspension cultures at controlled pH levels. Can. J. Bot. 54: 2838-2843. 1264-1270.

MARTIN, S.M., ROSE, D. & HUI, V. 1977. Growth of plant cell suspension cultures with ammonium as sole source of nitrogen. Can. J. Bot. 55: 2838-2843.

MICHEL, B.E. 1972. Solute potentials of sucrose solutions. Plant Physiol. 50: 196-198.

MOORE, T.C. 1979. Biochemistry and physiology of plant hormones. Springer-Verlag. New York.

MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-479.

MURASHIGE, T. 1977. Clonal crops through tissue culture. Plant tissue culture and its biotechnological applications. Ed. Barz, W.; Reinhard, E. & Zenk, M. H. Springer-Verlag. Heidelberg. : 392-403.

NEL, D.D. 1981. Rapid propagation of Ornithogalum hybrid in vitro. Agroplantae 13: 83-84.

NIIMI, Y. & ONOZAWA, T. 1979. In vitro bulblet formation from leaf segments of lilies, especially Lilium rubellum. Baker. Sci. Hort. (11) 4: 379-389.

NOGGLE, G.R. & FRITZ, G.J. 1976. Introductory plant physiology. Prentice-Hall Inc. New Jersey. U.S.A.

OBERMEYER, A.A. 1978. Ornithogalum: a revision of the Southern African species. Bothalia. 12 (3): 323-376.

PAEK, K.Y. 1982. Studies on the clonal propagation by the culture of bulb scale segments, inflorescence stems, and flower bulbs in hyacinth. In: A. Fujiwara (Editor). Plant Tissue Culture. 5th International Congress of plant tissue and cell culture. The Japanese Association for Plant Tissue Culture: 713-714.

PATEL, K.R. & BERLYN, G.P. 1983. Cytochemical investigations on multiple bud formation in tissue cultures of Pinus coulteri. Can. J. Bot. 61 (2): 575-585.

PENCE, V.C. & CARUSO, J.L. 1986. Auxin and cytokinin levels in selected and temperature induced morphologically distinct tissue lines of tobacco crown gall tumours. Plant Sci. 46: 233-237.

PERRY, P. 1985. The restructuring of the family Liliaceae. Veld and Flora : 66-68.

PILET, P.E. 1968. In vitro and in vivo auxin and cytokinin translocation. Biochemistry and physiology of plant growth substances. Ed. Wightman, F. & Setterfield, G. The Runge Press Ltd. Ottowa. Canada. : 993-1004.

RADIN, J.W. & HENDRIX, D.L. 1986. Accumulation and turnover of ABA in osmotically stressed cotton leaf tissue in relation to temperature. Plant Sci. 45: 37-42.

RAWAL, S.K. & MEHTA, A.R. 1982. Changes in enzyme activity and isopeoxidases in haploid tobacco callus during organogenesis. Plant Sci. Lett. 24: 67-77.

REINERT, J. & YEOMAN, M.M. 1982. Plant cell and tissue culture. A laboratory manual. Springer-Verlag. Berlin.

ROBB, S.M. 1957. The culture of excised tissues from bulb scales of Lilium speciosum. J. Exp. Bot. 8: 348-352.

ROSE, D. & MARTIN, S.M. 1975. Growth of suspension cultures of plant cells (Ipomea. sp.) at various temperatures. Can. J. Bot. 53 (4): 315-320.

RUDNICKI, R.M. & NOWAK, J. 1976. Studies on the physiology of Hyacinth bulbs (Hyacinthus orientalis L.). VI. Hormonal activities in Hyacinth bulbs during flower formation and dormancy release. J. Exp. Bot. 27 (97): 303-313.

SALISBURY, F.B. & ROSS, A.W. (eds) 1985. Plant physiology, 3rd edn. Wadsworth Publishing Co., Belmont.

SALTVEIT, M.E. & DILLEY, D.R. 1978. Rapid induction of wound ethylene from excised segments of etiolated Pisum sativum L., cv. Alaska. 1. Characterization of response. Plant Physiol. 61: 447-450.

SANIEWSKI, M., RUDNICKI, R.M. & NOWAK, J. 1978. Studies of the physiology of hyacinth bulbs (Hyacinthus orientalis L.). XII. The distribution of hormones in the hyacinth bulb in relation to its growth phase and elongation of the inflorescence. Physiol. Plant. 1 (1): 15-22.

SCOTT, N.S., MUNS, R. & BARLOW, E.W.R. 1979. Polyribosome content in young and aged wheat leaves subjected to drought. J. Exp. Bot. 30 (118): 905-911.

SEABROOK, J.E.A. 1980. Laboratory culture. In; Plant tissue culture as a source of biochemicals. Ed. J.E. Staba. Pub. C.R.C. Press Inc. Florida.

SEABROOK, J.E.A. & CUMMINGS, B.G. 1982. In vitro morphogenesis and growth of Narcissus in response to temperature. Sci. Hortic. 16: 185-190.

SHOEMAKER, R.C., CONCHE, L.J. & GALBRAITH, D.W. 1986. Characterization of somatic embryogenesis and plant regeneration in cotton (Gossypium hirsutum L.). Plant Cell Rep. 3: 178-181.

SHOUB, J. & HALEVY, A.H. 1971. Studies on the developmental morphology and thermoperiodic requirements for flower development in Ornithogalum arabicum L. Hortic. Res. II: 29-39.

SHOUB, J., HALEVY, A.H., MAATSCH, R., HERKLOTZ, A., BAKKER, T. & PAPENDRACHT, G. 1971. Control of flowering in Ornithogalum arabicum L. Hortic. Res. II: 40-51.

SINGHA, S. 1982. Influence of agar concentration on in vitro shoot proliferation of Malus sp. "Almey" and Pyrus communis "Seckel". J. Am. Soc. Hortic. Sci. 107 (4): 657-660.

SKIRVIN, R.M., CHU, M.C., MANN, M.L., YOUNG, H., SULIVAN, J. & FERMANIAN, T. 1986. Stability of tissue culture medium pH as a function of autoclaving, time and cultured plant material. Plant Cell Rep. 5: 292-294.

SKOOG, F. & MILLER, C.O. 1957. Chemical regulation of growth and organ formation in plant tissues grown in vitro. Symp. Soc. Exp. Biol. 11: 118.

STIMART, D.P. & ASCHER, P.D. 1978. Tissue culture of bulb scale sections for asexual propagation of Lilium longiflorum Thunb. J. Am. Soc. Hortic. Sci. 103 (2): 182-184.

STIMART, D.P. & ASCHER, P.D. 1981a. Foliar emergence from bulblets of L. longiflorum Thunb. as related to in vitro regeneration temperatures. J. Am. Soc. Hortic. Sci. 106 (4): 446-450.

STIMART, D.P. & ASCHER, P.D. 1981b. Developmental responses of L. longiflorum bulblets to constant or alternating temperature in vitro. J. Am. Soc. Hortic. Sci. 106 (4): 450-454.

STUART, A. 1967. Statistics. Chambers Encyclopedia Pergamon Press 13: 145-159.

TAKAYAMA, S. & MISAWA, M. 1980. Differentiation in Lilium bulbscales grown in vitro. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation in vitro. Physiol. Plant. 48: 121-125.

THIMANN, K.V. 1977. Hormone action in the whole life of plants. The University of Massachusetts Press. Amherst.

THORPE, T.A. & MEIER, D.D. 1972. Starch metabolism, respiration, and shoot formation in tobacco callus cultures. Physiol. Plant. 27: 363.

THORPE, T.A. & MURASHIGE, T. 1969. Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot. 48: 277-285.

TOMPSETT, A.A. 1985. Dormancy breaking in bulbs by burning over. Plantsman 7 (1): 40-52.

TOWILL & MAZUR, P. 1976. Osmotic shrinkage as a factor in freezing injury in plant tissue cultures. Plant Physiol. 57: 290-296.

TRAN THANH VAN, K. 1977. Regulation of morphogenesis. Plant tissue culture and its biotechnological applications. Ed. Barz, W.; Reinhard, E. & Zenk, M.H. Springer-Verlag. Heidelberg.: 367-385.

TRAN THANH VAN, K.M. 1981. Control of morphogenesis in in vitro cultures. Annu. Rev. Plant Physiol. 32: 291-311.

van AARTRIJK, J. & BLOM-BARNHOORN, G.J. 1983. Adventitious bud formation from bulb scale explants of Lilium speciosum. Thunb. in vitro. Effects of wouonding, TIBA and temperature. Z. Pflanzenphysiol. 110: 355-363.

van AARTRIJK, J., BLOM-BARNHOORN, G.J. & BRUINSMA, J. 1985a. Adventitious bud formation from bulb-scale explants of Lilium speciosum. Thunb. in vitro. Effects of aminoethoxyvinyl-glycine, 1-aminoacyclopropane-1-carboxylic acid and ethylene. Plant Physiol. 117: 401-410.

van AARTRIJK, J., BLOM-BARNHOORN, G.J. & BRUINSMA, J. 1985b. Adventitious bud formation from bulb-scale explants of Lilium speciosum. Thunb. in vitro. Production of ethane and ethylene. J. Plant Physiol. 117: 411-422.

van NIEKERK, H.A. 1965. Cytogenetic study of some Ornithogalum L. hybrids. Research project W-Eb76. Msc. Agriculture. University of Stellenbosch. Stellenbosch.

van NIEKERK, H.A. & de VILLIERS PIENAAR, R. 1968. 'n sitogenetiese ondersoek van 'n aantal hibriede in die genus Ornithogalum L. Proc. Third Congress, S.Afr. Gen. Soc. 1966.: 45-51. Pretoria.

van STADEN, J. 1978. The levels of endogenous cytokinins in daffodil bulbs stored under different environmental conditions. J. Plant Physiol. 86: 323-330.

von MAATSCH, R., HERKLOTZ, A., SHOUB, J. & HALEVY, A.H. 1964. Uber den Einflub der Lager temperatur auf dem Entwicklung von Ornithogalum arabicum L. Der Gartenbauwissenschaft 29 (11): 419-424.

WENT, F.W. 1953. The effect of temperature on plant growth. Annu. Rev. Plant Physiol. 4: 347-362.

WIETSMA, W.A. 1984. Vermeerdering van Fritillaria persica in kweekbuizen. Bloembollenkultuur : 1148-1149.

YAMADA, Y. 1978. Photoautotrophism in green cultured cells. In; Frontiers of plant tissue culture. Ed. T.A. Thorpe. International Association for Plant Tissue Culture. Canada : 481-496.

YAMAGAWA, T. & SAKANISHI, Y. 1980a. Studies on the regeneration of excised bulb tissues of various ornamentals and tunicate bulbs. I. Regenerative capacity of the segments from different parts of bulb scales. J. Jpn. Soc. Hortic. Sci. 48 (4): 495-502.

YANAGAWA, T. & SAKANISHI, Y. 1980b. Studies on the regeneration of excised bulb tissues of ornamentals and tunicate bulbs II. Morphological observations of bulblet formation from bulb scale segments. J. Jpn. Soc. Hortic. Sci. 49 (1): 119-126.

YOSHIDA, F., KOBAYASHI, T. & YOSHIDA, T. 1973. The mineral nutrition of cultured chlorophyllous cells of tobacco. 1. Effect of salts, sucrose, Ca, Cl and B in the medium on the yield, friability, chlorophyll contents and mineral absorption of cells.. Plant Cell Physiol. 14: 329-339.

YU, Y.B. & YANG, S.F. 1980. Biosynthesis of wound ethylene. Plant Physiol. 66: 281-285.