"The Initiation, Growth and Morphogenesis of Wheat Tissue Cultures"

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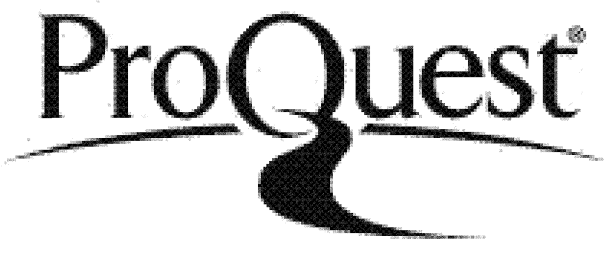

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CONTENTS

INTRODUCTION

The present study of growth and morphogenesis of wheat tissues *in vitro*, was undertaken in the hope of developing a technique whereby large numbers of identical plants could be regenerated from callus cultures. In recent years much attention has been focussed on the possibility of applying plant tissue culture techniques to plant breeding. With wheat, as with most other crop plants, plant breeding programmes are apt to stretch over many years of selection procedures and field trials before plants, homozygous for the desired traits, are available in sufficient quantity to be grown on a meaningful scale. Any techniques which could reduce the time factor involved must be of enormous benefit. It is during the early stages of obtaining large numbers of genetically identical plants that plant tissue culture could be invaluable. Ideally, it would be possible to select a plant with desirable characteristics, and use the microspores to obtain plants, either directly from the pollen or via callus. Such plants would contain only half the chromosome complement, and would yield homozygous 'diploid' plants upon application of suitable techniques to double the chromosome number.

Some progress has already been made towards this aim: several workers have reported callus formation and plantlet production from the cultured anthers of many Grasses, including wheat (Fujii, 1969; Kimata & Sakamoto, 1972; Wang, Chu, Sun, Wu, Yin & Hsü, 1973), rice (Nishi & Mitsouka, 1969) and *Lolium* and *Hordeum* (Clapham, 1971). However, as yet there is no reliable method for obtaining 'haploid' plantlets or callus in acceptable yield from Monocotyledons. This is in contrast with the case in some Dicotyledons, such as tobacco and other

Solanaceous species, where haploid plants can be obtained reproducibly and in high yield (Sunderland, 1971). In the absence of readily available haploid tissue, it was decided to study the growth and morphogenetic capacity of somatic wheat tissue (hereafter referred to as 'diploid' tissue, although most of the wheat varieties used were in fact hexaploid). Since the first reported successes in obtaining plant regeneration from somatic callus tissue of oats (Carter, Yamada & Takahasi, 1967) and rice (Nishi, Yamada & Takahasi, 1968), further progress has been made and several other cereals have been maintained in culture, including wheat. Clearly plantlet formation from wheat callus was in need of further study, since in the reported regenerations (Adachi & Katayama, 1969; Shimada, Sasakuma & Tsunewaki, 1969; Caramiello, 1970; Caramiello & Montacchini, 1970; Caramiello & Montacchini, 1973) a whole range of cultural conditions and different media had been used, and in no case had more than a very low proportion of cultures proved capable of producing shoots. Growth of the callus tissue was apparently slow and both growth and morphogenetic response depended upon the variety tested. Thus, one of the first requirements was to test a range of varieties of wheat and select those showing the best callus initiation and growth for further investigations.

Callus had been reported to form from germinating embryos, and from the coleoptile or roots of young seedlings. For convenience, it was decided to use newly germinating embryos of imbibed grain for routine callus initiation, but to test callus formation from many other parts of the wheat plant. Publications reported wheat callus to have been maintained on several different media, suggesting that it had no exacting mineral requirements. Murashige & Skoog (1962) medium was therefore chosen as the basic medium with which to study the

effects of the organic additives on growth and morphogenesis. It was hoped to culture wheat tissue both on semi-solid and in liquid media, and to observe morphogenetic changes under different conditions, with the object of obtaining plant regeneration.

Since the co-operating Industrial Laboratory was undertaking studies on wheat anther culture, it was decided to link up with this work by conducting an experiment, of limited scope, on anther culture. The particular experiment involved a study of pollen viability during the early stages of anther culture. It was hoped to use haploid callus, if this became readily available from the Industrial Laboratory, for further studies of growth and morphogenesis.

SECTION ONE : Materials and Methods

A. Culture media

Cultures were initiated and maintained on defined media. All glassware, whether used in the preparation of stock solutions or as culture vessels, was kept scrupulously clean, using methods described by Street (1973). Only deionised, double-distilled water was used in the preparation of medium, and all chemicals were, where possible, of A.R. grade.

(i) Constituents of the media and stock solutions

Several different media were used, including those of Gamborg*, 1968 ("B5"), Green & Phillips, 1975 ("G"), Smith**, 1967 ("S"), and Caramiello, 1970 ("C"), but the medium most regularly used was based on that of Murashige & Skoog, 1962 ("M").

Dispensed medium was stored for no longer than a week before use, so for convenience, the components of "M" were stored as concentrated stock solutions. Less frequently used media were made up fresh as required.

Detailed formulae of all media used may be found in the Appendix.

Each medium had the following components:

Mineral Salts

When making up concentrated stocks or dilute medium, each chemical was dissolved separately, and added carefully to the mixed solution, to avoid precipitation. For "M", a x10 concentrated stock

*see Gamborg, Miller & Ojima (1968)

** as modified by Mascarenhas (1971)

solution was stored at 4°C, and renewed every few weeks.

Vitamins

Most of the media contained the vitamin supplement of White (1963). The solution of the vitamins was stored frozen as a x100 concentration stock solution.

Iron source

A stock solution of the Ferric Sodium salt of Ethylene-diaminetetraacetic acid (FeEDTA) was stored at 4°C and diluted to give the appropriate level of iron required.

Carbon source

In most experiments 3% w/v of 'Analar' grade Sucrose was used as carbon course.

Supplements

Meso-inositol was stored as a x100 concentrated stock at 4°C, and diluted as required.

Casein Hydrolysate was freshly weighed out and added to the medium, when required.

2,4-dichlorophenoxyacetic acid (2,4-D), which was used in most media, was kept as a stock solution stored at 4°C.

The type and concentration of other growth hormones varied according to the object of the experiment, so solutions of these were not stored but made up as required. A full list of those used, plus suitable solvents, may be found in the Appendix.

Agar

10 g/l of 'lab m' type agar, added after correction of the pH, was used to solidify media for callus culture.

(ii) Preparation of the medium

Solutions of the chemical components were thoroughly mixed and made up to volume. The sucrose was added last of all, and once dissolved, the medium could be brought to the required pH by adding small amounts of 1N-NaOH or 1N-HCl (both solutions Analar grade).

Medium for liquid cultures was dispensed straight away into 250 ml Erlenmeyer flasks (60 ml per flask). For semi-solid medium, agar was added and dissolved by steaming the solution. To ensure uniform dispersion of agar, the medium was mixed with a magnetic stirrer whilst being dispensed, using an automatic dispenser* which accurately dispensed 25 ml aliquots to 100 ml Erlenmeyer flasks.

Muslin-covered non-absorbant cotton wool bungs were used to close the flasks and these were protected from moisture and dust by aluminium caps or foils. The medium was sterilised by autoclaving at 15 lb. sq. in.$^{-1}$ (121°C) for 15 min and allowed to cool slowly.

When plastic 'Sterilin' tubes or petri dishes were being used, the semi-solid medium was autoclaved as above, in volumes less than 2 litres, and then dispensed by hand-pouring under aseptic conditions. Sterilin tubes were tilted while cooling to obtain a slope in the agar.

Dispensed sterilised medium was stored at 15°C.

B. Conditions of culture

The cultures were placed in any one of three separate environments, differing in temperature and light intensity and spectrum:

*Filamatic Vial Filler, National Instrument Co., Baltimore 15, M.D., U.S.A.

Controlled Environment Room I

The light intensity here was much higher than in Controlled Environment Room II, since the cultures were placed on a shelf 2 feet below a series of fluorescent lights. Thus, all the cultures had access to the same type of light, although the intensity varied from 430 - 750 lux depending on position on the shelf. The lighting was arranged as an alternating series of "Daylight" (5) and "White" (4) fluorescent tubes.

Temperature was maintained at 25°C.

Controlled Environment Room II

This room had facilities for cultures in liquid medium. A horizontal rotary shaker* was arranged in the middle of the room, and the lighting (3 "Warm White" fluorescent tubes alternating with 2 "Grolux") was suspended overhead, giving an intensity in the range of 155 - 225 lux immediately below.

Cultures on solid medium were placed on shelves to one side of the room and therefore only received oblique light of low intensity (2.15 - 8.60 lux). A partitioned-off area allowed cultures to be kept completely in the dark.

The whole room had a controlled temperature of 25°C.

Growth cabinet

The Fisons Growth Cabinet was used mainly for experiments involving Immature Wheat Embryos. 9 Phillips 'Reflectalite-29' fluorescent tubes were directly above the top shelf, set on a 18 hr light, 6 h dark cycle. Because of the large number of cultures, 3 shelves

*L. & H. Engineering Co.Ltd., Bells Hill, Stoke Poges, Bucks, England.

were in use, and these had different temperatures and light intensities:

Top Shelf - light intensity 1180 lux; temperature 28°C

Middle Shelf - " " 80 - 183 lux; " 26 - 27°C

Bottom Shelf - " " 30 - 54 lux; " 25°C

Cultures were randomised and moved from shelf to shelf every few days, to ensure equal exposure to light of high intensity.

Details of the spectral composition of the different types of fluorescent tubes used may be found in the Appendix.

C. Source of material for culture

Grain

Grain of a number of varieties of wheat was obtained from the Plant Breeding Institute, Cambridge, and the Lord Rank Research Centre, High Wycombe. In both cases, the grain had generally been harvested at least one year previously.

When immature grain was required, this was obtained directly from plants grown under greenhouse conditions at the Lord Rank Research Centre. The embryos were excised aseptically onto agar, using facilities at the Research Centre, and the cultures transported straight away to a Growth Cabinet at Leicester University.

Initiating callus on the embryos of mature grain proved to be a convenient and reliable source of tissue for culture. A full list of all the varieties tested appears in the Appendix. Of these, 4 varieties which gave consistently good callus production were selected for use in further experiments. They were all varieties of Triticum

aestivum: Maris Ranger, Maris Ensign and the American dwarf varieties 6 wuk 3 and 6 wuk 4.

Plants

Where plants, at different stages of maturity, were required, these were grown from grain, under greenhouse conditions, either at the Lord Rank Research Centre, High Wycombe (temperature 16°C night, 29°C day) or at Leicester University (temperature 23°C, 16 h day).

Haploid plants, regenerated from wheat pollen callus, were donated by Dr. L. Ayre (Lord Rank Research Centre).

D. Initiation of cultures

All of the following procedures took place under aseptic conditions, either using a bench Laminar Airflow Unit, or a Sterile Cabinet Room isolated from the rest of the laboratory, as described by Street (1973).

All instruments used had been previously autoclaved, and between operations they were flamed in alcohol and cooled in sterile distilled water.

After inoculation, culture flasks were flamed and sealed firmly with sterile aluminium foil squares. Petri dishes were sealed with Nescofilm*. Sterilin tubes hadscrew-down caps and these were made finger-tight.

(i) Embryos from mature grain

Stored mature grain was imbibed in tap water for at least 16 h, by which time the endosperm had swollen and the embryos had

*Distributors: Nippon Shoji Kaisha Ltd., Osaka, Japan.

usually begun germination (were beginning to break through the pericarp). After imbibition, the grain was surface-sterilised by immersing in 25% v/v Domestos* for at least 10 min. The grain was then rinsed 5 times with sterile distilled water.

Embryos were excised with the aid of a binocular microscope. Fig. 1.1 shows the embryo in relation to the scutellum. The area of attachment of the embryo to the scutellum was cut through to free the embryo. Thus, the excised embryos were likely to be damaged, or even broken in two, but the degree of damage did not seem to influence the amount of callus production.

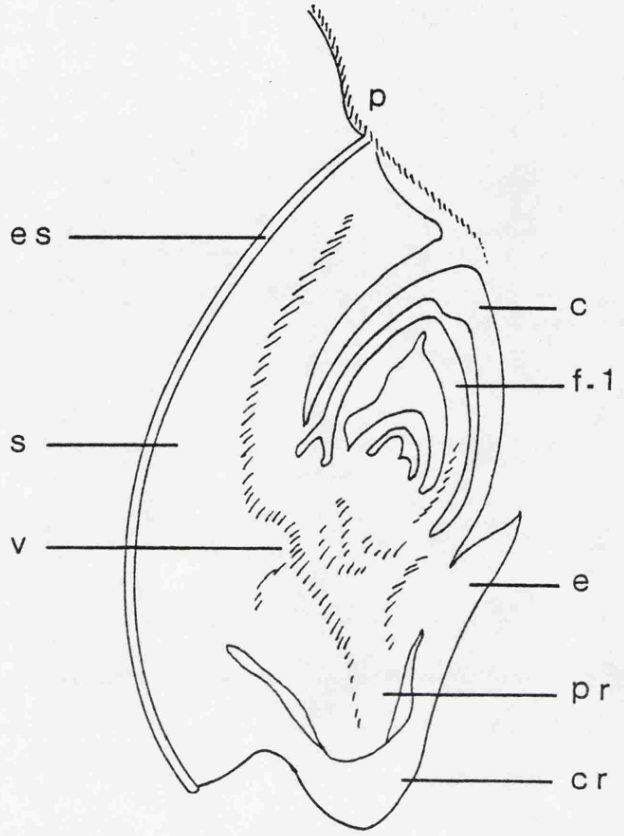
Excised embryos were placed on solid medium (one per flask) so that the cut surface was in contact with the agar.

Fig. 1.1 shows that the embryo of a mature grain already has a well-developed plumule, and this often continued growing after the embryo had been put into culture. It will be shown later (P.37) that enhanced concentrations of 2,4-dichlorophenoxyacetic acid and p-chlorophenoxyacetic acid could suppress this development of the embryo axis. Callus formation occurred at the base of the plumule and usually enclosed the rest of the embryo so that roots were more rarely formed during the first passage. A more detailed description of callus formation on the embryo will be found later (P.101).

Callus formation began a few days after excision of the embryo, but did not reach a suitable size for subculture until about 10 weeks. The first subculture involved using fine forceps to carefully take away as much of the callus as possible, leaving behind visibly organised structures such as the plumule and roots. The callus

*A commercial disinfectant, containing 10% available Chlorine.

Fig.1.1 : Diagram showing L.S. of the Wheat Embryo before excision from the Mature Grain.



The diagram shows the embryo still attached to the endosperm by the scutellum, s.

c - coleoptile, f-1 - first foliage leaf,

cr - coleorhiza, p - pericarp,

e - epiblast, pr - primary root,

es - epithelium of scutellum, v - vascular tissue.

was placed on fresh medium and could be subcultured every 5 weeks thereafter. It was considered important, especially where morphogenetic studies were concerned, to ensure that the callus subcultured was undifferentiated, but this could only be done visually so there was always the possibility that endogenous primordia or very tiny buds had been missed.

The appearance of the callus over succeeding passages did not depend entirely on the level of hormones used in the medium. Some pieces of callus on "M", with 1 mg/l 2,4-D, would grow slowly and remain completely undifferentiated, while others would increase their size more rapidly and start producing roots after 3 to 4 weeks. In any batch of grain used for an experiment, a fair amount of variation in response to culture would be found and this often made interpretation of results difficult.

Although at each subculture undifferentiated callus was being selected, the cultures did not lose their capacity for producing roots if placed on medium lacking auxin.

(ii) Embryos from immature grain

Plants were grown under greenhouse conditions at the Lord Rank Research Centre, and the date of anthesis of each ear recorded so that grain at different stages of development was available.

Unripe grain was carefully removed from the ear and soaked for less than 10 min in 25% v/v Sodium Hypochlorite solution, to which a small amount of Pyroneg detergent had been added as a wetting agent. They were rinsed by passing through a series of 4 petri dishes of sterile distilled water.

Immature grain from 9 to 25 days after anthesis was used, the embryo of the youngest stage being roughly 0.3 mm long. The endo-

sperm was fluid in all but the most advanced grain, so that the embryo and attached scutellum could be removed without damage, with the aid of a binocular microscope. Explants were placed, one per 'Sterilin' tube, usually with the scutellum nearest the agar, although, due to their small size, it proved difficult to ensure that this was always so.

Callus formed in much the same way as described for mature embryos. The scutellum did not always proliferate, but when it did, the callus was compact and more yellow in colour than callus from the embryo. Often, the callus from the embryo completely covered the explant so that it was difficult to see whether the scutellum had increased in size and initiated callus.

Callus was subcultured as for mature embryos, but the cultures were smaller because they were grown in smaller tubes and subcultured more often (every 4 weeks).

Immature embryo cultures were placed in the Growth Cabinet, with high light intensity and 18 h day 6 hr night cycle. They were not propagated for more than 4 passages.

(iii) Segments of the plant stem

Mature tillering plants were used, which had shoots at different stages of development. All of the nodes along the stem were found to be capable of producing callus, but the most active callus initiation occurred from the first node below the ear or from very newly-formed nodes.

9 cm lengths of stem, each including a node, were cut from the plant and immersed in 0.01% w/v Mercuric Chloride solution, to which a small amount of detergent had been added as a wetting

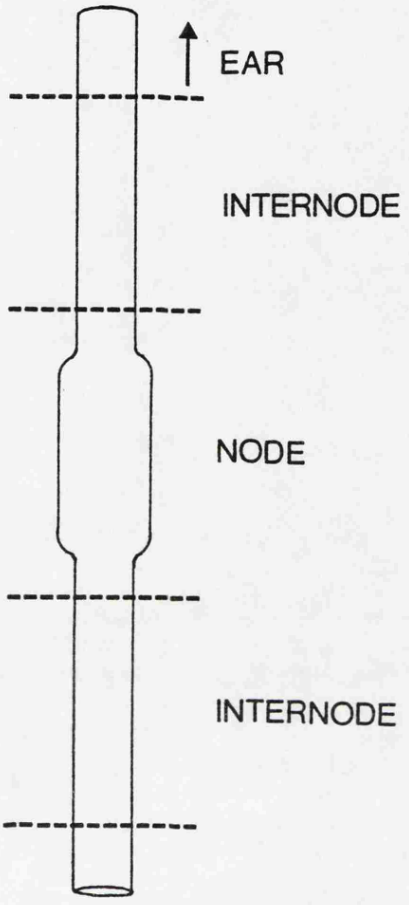
agent. After sterilising for at least 20 min, the segments were passed through a series of 4 petri dishes of sterile distilled water to rinse free from the sterilant.

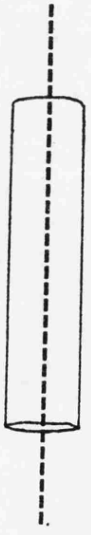
A sharp scalpel blade was used to remove both ends of the section of stem, and the remainder was cut into several pieces 2 - 6 mm in length. Some of these pieces were left as whole cylinders, while others were split (see Fig. 1.2). In the case of the internode segments, the cut was made longitudinally; the node segments were cut obliquely lengthwise. Thus, the explants had several cut surfaces. One end of the explant was pushed a little way into the agar, and, since petri dishes were used, the segments were sloping rather than upright.

As well as using the nodes and internodes, explants were taken from the wheat ear. Both enclosed and fully emerged ears were used. After sterilising the ear as above, the spikelets were stripped off, the remaining rachis cut into segments and the basal end pushed into the agar. Only a few rachis segments were put into culture but it was found that only the younger ones formed callus.

Callus formation occurred on the segments after a few days. It grew slightly faster than that from embryos and could be subcultured after 4 or 5 weeks. If left longer before subculture, roots were produced from the callus. Callus formed on cut surfaces, and sometimes all over the node region. The callus could be green or white, compact or fluffy. A description of the types of callus found appears later (P. 45). During subculture, the original plant material was cut away with a sharp scalpel and the callus transferred to fresh medium in a 100 ml flask. Often, a considerable amount of the original explant had

Fig. 1.2 : Diagrams to show Sectioning of Plant Stem Segments after Excision.





Internode
Cut Longitudinally

Node
Cut Obliquely.

to be left with the callus, but as the tissue grew, this could be seen as brown strands and easily removed.

Callus cultures were initiated from both 'diploid' and 'haploid' wheat plants, and maintained in culture over several passages of different duration.

(iv) Explants from young seedlings

Mature grain was imbibed for 16 h in tap water, then surface sterilised in 25% v/v Domestos for 10 min. After rinsing thoroughly, in sterile distilled water, the grain was placed whole on solid medium ("M" containing no auxin). Germination occurred, and when the coleoptile was about 5 cm long, segments were cut from the roots, hypocotyl and coleoptile. These were transferred to medium containing 1 mg/l 2,4-D to encourage callus production.

Only a few such cultures were set up, the object being to see whether all parts of the seedling were capable of callus production. Callus formed quite easily around the hypocotyl region and more slowly on root segments. Some callus formed at the cut end of the coleoptile. Callus obtained from different parts of the seedling was maintained in culture over several passages.

(v) Initiation of cultures in liquid medium

Cultures were grown in liquid medium in an attempt to obtain fine suspensions. About 1 g fresh weight of undifferentiated callus tissue was transferred to 30 ml of liquid medium in a 100 ml flask and agitated on a horizontal rotary shaker at 120 r.p.m. The callus soon broke up, and after 2 weeks a reasonable dispersion of fine aggregates made the solution appear cloudy. When this cloudy

solution was subcultured (by automatic pipette*, 2 mm aperture) it grew very slowly and after about 3x3-week passages, there were larger aggregates present again.

Cultures maintained from the larger aggregates grew more quickly and could be subcultured every 3 weeks over a long period of time, but unfortunately the aggregates in the cultures remained large.

An attempt was also made to form a suspension from excised embryos. Mature grain was sterilised in 25 % v/v Domestos for 10 min, rinsed well, and the embryos excised and transferred to 30 ml of liquid basic medium ("M") in 100 ml flasks. The cultures were agitated in a horizontal rotary shaker at 120 r.p.m. Callus formed on the embryos in a similar way to those cultured on solid medium. After a month, the whole culture was poured into fresh "M" in 250 ml flasks. The callus did not break away from the explant during the first few passages (once a month) and hence growth was very slow. Eventually aggregates appeared which had arisen from small pieces of broken-off tissue.

Once a culture had been established in liquid medium, it would nearly triple its mass in 3 weeks. Unfortunately, the growth rate could not be measured quantitatively since a cell count was not practical due to aggregate size. Liquid cultures were subcultured using a perforated spoon on a long handle (see Fig. 1.3), which filtered off most of the 'old' medium through 1 mm holes. Usually one "spoonful" (roughly 1 g fresh weight) was transferred to fresh medium.

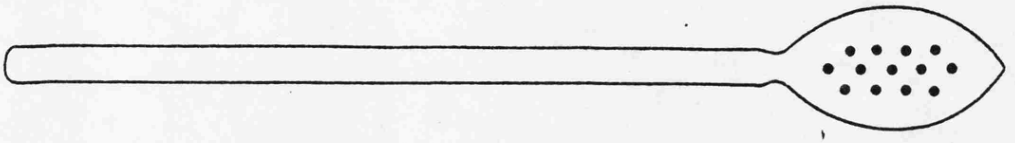
E. Methods for estimating the growth of cultures

(i) Percentage increase in fresh weight, and "doubling time"

As will be seen later, callus could be induced to grow

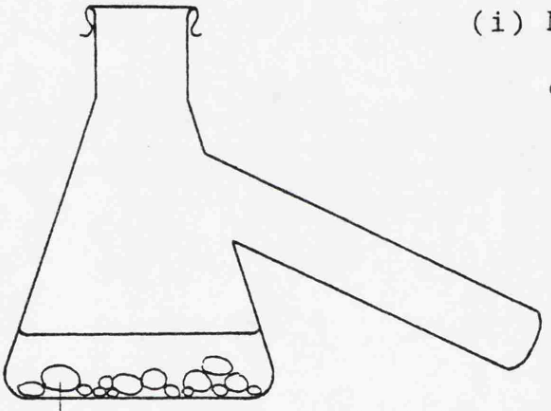
* A.R. Howell Ltd., Pipetting Unit

Fig.1.3



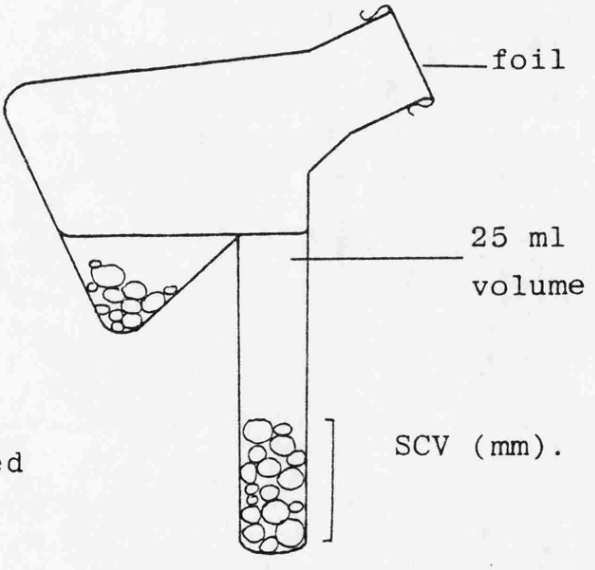
A. Long-handled 'spoon' with 1mm perforations, used for transfer of cultures in liquid medium.

B. "Arm-flask" used for culture of callus tissues in liquid medium.



(i) Normal position for culture.

aggregates of callus



(ii) Position used when measuring the 'Settled Cell Volume'.

either as a fairly homogenous mass of tissue, or as a mixture of callus and roots or shoots. The most suitable method of estimating growth involved measurement of the total fresh weight (F.wt.) at the beginning and end of a passage. Sometimes, the callus was simply sacrificed and the total F. wt. taken. More often, the growth was to be followed over several passages and so the weighings were taken aseptically. A top-loading balance (Sartorius, Werke G.M.B.H. Guttingen) was sprayed with alcohol and used in the sterile cabinet. With this balance, weights were taken to the nearest 10 mg. The sealed flask, containing the callus, was weighed, then the callus was subcultured to another flask, and the empty flask and foil reweighed. The percentage increase in fresh weight over a stated period of time was calculated from:

$$\frac{\text{Final fresh weight - original fresh weight}}{\text{Original F. wt.}} \times 100$$

The percentage increase in fresh weight gave a measure of the growth rate over one passage. In order to compare the rate of growth of cultures over several passages which might be of different lengths, the "doubling time" was used. This was the time taken for a callus to double its biomass (100% increase in fresh weight) and was calculated from:

$$\frac{\text{Passage length (weeks)}}{\text{Mean \% increase in F.wt. for that passage}} \times 100 \text{ \% increase in F.wt.}$$

(ii) Percentage undifferentiated tissue (% U)

In experiments where the effect of various substances on morphogenesis was being investigated, the amount of undifferentiated tissue ("U") was estimated. The method involved taking the fresh weight of the callus, as described above, and then measuring the weight of undifferentiated callus, after removing roots and shoots.

This removal was difficult to perform accurately when the roots were very small. No account was taken of the amount of differentiation at cellular level, so that "% U" is only a measure of the amount of visibly undifferentiated callus. Percentage undifferentiated callus was calculated from:

$$\frac{\text{F.wt. "U" callus}}{\text{Total F.wt.}} \times 100$$

While it is appreciated that the estimate of % U involved a level of error, this was standardised by being always determined by the same operator, and therefore the estimates are regarded as having comparative value.

(iii) Dry weight as percentage of fresh weight

In some experiments, the whole culture was sacrificed, and after taking the fresh weight the callus was placed on aluminium foil and dried in an oven at 80°C for 20 hours. The weight of dried callus was measured after cooling in a desiccator for 30 min. Since the original dry weight (D.wt.) was unknown, the results were expressed as a percentage dry weight:

$$\frac{\text{Dry wt.}}{\text{Fresh wt.}} \times 100$$

(iv) Settled cell volume (liquid cultures)

Cultures in liquid medium tended to grow as aggregates of various sizes, in some cases with roots attached. It proved very difficult to estimate the growth of such cultures, and few quantitative experiments were performed.

The main purpose of growing cultures in liquid medium was to study their morphogenesis, so that it was more important to know if the culture in a particular flask was continuing to grow, than to compare its growth with that in other flasks. To this end, specially made 250 ml flasks were used, which had a side-arm attached ("arm-flasks", Fig. 1.3). The capacity of the side-arm was 25 ml, and the volume of medium used in these flasks was 50 ml. The culture was swirled about in the flask, to disperse it through the medium, then quickly tipped into the side-arm. The pieces of tissue were allowed to settle and their height up the tube measured (2 cm = 5 ml approx.). This "settled cell volume" (SCV) was expressed as mm (height), and on each occasion was read three times for each flask and the values averaged. The SCV would be measured several times during each culture passage, and so the growth of the culture could be followed without disturbing the contents of the flask. This method of following the growth of the culture was not suitable if roots were present on the aggregates.

(v) Differentiation of the callus

During each passage in culture on various media, the appearance of the callus was scored every few weeks. For brevity, abbreviations were used for the main characteristics, and an explanation of these follows:

w	white colour
b	browning of some areas of callus
g	greening of callus and/or roots
gsp	dark green spots on the callus

R	rough texture to callus
S	smooth texture to callus
r	presence of roots
sh	presence of shoots or leaves
sh.p	possible shoot primordium present
U	whole culture visibly undifferentiated

Greening which occurred on the callus or roots, "g", was usually distinct from "gsp", being paler and less localised. Where greening was found it was usually associated with roots. "Gsp" on the other hand, refers to small dark green spots on the surface of the callus. However, in some cases it was difficult to distinguish between these two types of greening. "Sh.p" were thought to be possible shoot initials, since they appeared to have an organised structure, while otherwise being similar to "gsp". In some cases, shoots definitely formed from "sh.p" but this was not always so. Roots first appeared from the callus as tiny outgrowths covered with hairs. Two types of roots were produced: thin, white roots and fatter green roots, the green roots being common under conditions of high light intensity.

F. Histological methods

(i) Fixatives

Glacial Acetic Acid

Pieces of callus and aggregates from liquid culture were immersed for at least 24 hours in Glacial Acetic Acid at 8°C. This fixative was suitable for material later to be embedded in plastic, or stained with Feulgen.

Formalin-Aceto-Alcohol (FAA)

Composition:

70% ethanol	- 90%
Glacial acetic acid	- 5%
Formalin	- 5%
Cupric sulphate	- 0.2%

This fixative could be used before embedding in wax or plastic, or staining in Feulgen. Again, the material was fixed for at least 24 hours at 8°C.

12½% Glutaraldehyde in 0.1M Phosphate Buffer

This fixative was used only for material to be embedded in plastic. The specimens were usually fixed for 2 days at 8°C.

Phosphate Buffer : stock solutions

Stock A	31.2 g/l $NaH_2PO_4.2H_2O$
Stock B	28.4 g/l $Na_2H\ PO_4$ (anhydrous)

0.1M Phosphate Buffer

Stock A	78 ml
Stock B	122 ml
Distilled water	200 ml

50% Analar Glutaraldehyde was mixed with 0.1M Phosphate Buffer to make a 12½% proportion of Glutaraldehyde.

(ii) Method of embedding in wax

This technique was found useful for investigating early callus formation on excised embryos. The main advantage being that the size of the specimen was not limited.

(a) After fixing, two 10 min washes with 70% alcohol.

(b) Soaked for 2 hours each in:

I	Distilled water	50%
	95% ethanol	40%
	Tertiary butyl alcohol	10%
II	Distilled water	30%
	95% ethanol	50%
	Tertiary butyl alcohol	20%

(A small amount of Toluidine Blue in 70% alcohol may be added at this stage, to stain the specimen)

III	Distilled water	15%
	95% ethanol	50%
	Tertiary butyl alcohol	35%
IV	95% ethanol	45%
	Tertiary butyl alcohol	55%
V	100% ethanol	25%
	Tertiary butyl alcohol	75%
VI	Pure Tertiary butyl alcohol	

(c) After a second long soak in pure Tertiary Butyl Alcohol, the specimen was placed in a mixture of Pure Tertiary Butanol and Paraffin Wax for one hour in an oven at 55°C. The Butanol was gradually replaced, over the next few hours, by pouring off some of the mixture and adding more melted Paraffin Wax. When the specimen was in pure wax, the Butanol could no longer be detected by smell.

(d) Some molten wax was now poured into a mould, the specimen added, and its position adjusted with a warmed needle. Plunging the mould into cold water set the wax quickly.

(iii) Method of embedding in plastic

Plastic embedding was most suitable for small pieces of callus, roots, and aggregates and cells from liquid medium. The size of specimen which could be handled was limited by that of the Gelatin Capsules* used as moulds (7 mm x 20 mm).

(a) Where Glutaraldehyde had been used as fixative, this was removed by 3 half-hour washes in 0.1M Phosphate Buffer. The material was then dehydrated by passing through a series of alcohol (30,50, 70 and 90%), 30 min in each. (Where Glacial Acetic acid had been used as fixative, the material could be put in absolute alcohol straight away).

(b) After two fairly long washes in absolute alcohol, an equal volume of embedding medium (see below) was added for 2 h. This was then replaced with fresh embedding medium twice, each time for 2 h at least.

(c) The specimens were arranged in the Gelatin Capsules, filled with embedding medium. At least 40 h at 60°C was required to set the plastic.

Embedding Medium

n-butyl methacrylate	100 ml
methyl methacrylate	40 ml
Styrene	60 ml
Benzoyl peroxide	2.0 g

Left to stand for one hour, shaking occasionally, to dissolve the Benzoyl Peroxide. One inch of granular anhydrous $CaSO_4$ added to dry the solution. The proportions of methacrylate and styrene may be varied to make the plastic harder for electron microscopy.

*Parke-Davis & Co., Pontypool, Mon. U.K.

(iv) Clearing of explant tissues

This technique was used for embryos which had been excised and grown in culture for several days. The process allows the inner vascular strands to be seen, with little disturbance of the outer callus tissues.

(1) 5% Potassium Hydroxide solution at 60°C for at least 24 hours. The tissue becomes translucent.

(2) Wash in distilled water.

(3) 80% Chloral Hydrate solution for 24 hours.

(4) Wash with distilled water.

(5) Stain with 0.25% aqueous saffranin for a few min.

(6) Distilled water to remove excess stain.

(7) 50%, 95% alcohol, 15 min each.

(8) Glycerine alcohol preservative* until required (see below).

(v) Preparation of material for the scanning electron microscope

The S.E.M. was used to examine the leaf surfaces of 'regenerated' plants compared to plants raised from seed.

The lower leaf surface and mesophyll cells were scraped away and the remaining upper surface pressed, face upwards, onto double-sided tape adhering to the metal stub. The material was dried in a desiccator for 48 hours, then coated with fine gold under reduced air pressure and in the presence of Argon. The coated material could be covered and stored in the desiccator for several months before scanning.

*Glycerol	50 ml
50% alcohol	50 ml

The leaf surfaces were scanned for the presence of wax-needles, using the Cambridge S4-10 Scanning Electron Microscope (operated by Mr. G. McTurk).

(vi) Sectioning on the freezing microtome

Using this technique, fresh, unfixed pieces of tissue could be sectioned to show internal structure. The main disadvantage was that delicate material could not be used since the sections would break up too easily.

The specimen was placed onto the cold stage of the 'Pelcool' Freezing Microtome (M.S.E.) and covered with quick-freezing M-1 Embedding Matrix* to form a mound. When this was thoroughly frozen, thin sections (6 - 25μ) were cut with the sharp knife of the microtome. The sections melted once on the knife and were floated off onto glass slides or a watch glass.

The sections could be lightly stained before viewing under a light microscope. No attempt was made to make such slides permanent.

(vii) Staining methods

Staining of wax sections

Sections of less than 10μ in thickness were cut on a microtome (hand operated, Cambridge Instrument Co. Ltd.).

Slides were prepared with a smear of Haupts Adhesive solution. A 'ribbon' of sections was arranged on the slide, a few drops of Formalin added, and the slide gently heated to spread out the sections and remove wrinkles from the wax. The wax sections were dried onto the slide before staining.

*Lipshaw Manufacturing Co., Detroit, Mich. U.S.A.

(1) Pure xylol to dissolve the wax — 15 min.

(2) 1:1 xylol to alcohol — 5 min.

(3) 1:3 xylol to alcohol — 5 min.

(4) Absolute alcohol — 5 min.

(5) Rehydrated through a series of alcohols (95, 90, 80, 70%) — 5 min. in each

(6) Phenosaffranin stain in 70% alcohol for more than 6h.

(7) Dehydrated through 70, 80 to 95% alcohol, 5 min. each.

(8) 1% Fast Green stain in 95% alcohol for 1 min.

(9) 95% alcohol, absolute alcohol 5 min each.
1:3 alcohol to xylol; 1:1 alcohol to xylol, 5 min each

(10) Pure xylol for 15 min.

(11) Slides were made permanent by mounting in Canada Balsam.

Staining of plastic sections

Plastic-embedded material could be sectioned much more thinly than wax-embedded material. The sections were cut on a microtome (C. Reichart, Austria) using glass knives. The sections were floated onto gelatinized slides (see below) and were stained by adding a few drops of Paragon* stain. The slides could be made permanent by mounting in X.A.M. Paragon is a multiple stain, colouring the nucleus, cell wall and cytoplasm differentially (see Martin, Lynn & Nickey (1966); Spurlock, Skinner & Kattine (1966)).

Gelatinized slides

0.5% Gelatin (sheet or powder)

0.05% Chrome alum

Heated gently to dissolve in distilled water.

Slides dipped in solution, drained and stored at 4°C.

*Gurr. Searle Diagnostic, High Wycombe, Bucks.

Feulgen staining

Used for root-tips and fine aggregates from liquid culture, where either chromosome counts or DNA measurement of nuclei was required.

(a) Pretreatment - root tips were left in Bromonaphthalene solution (formed by shaking a drop of n-Bromonaphthalene in water) for 4 hours. Chromosomes could also be thickened by keeping the cells near $0^{\circ}C$ overnight.

(b) After fixing in Glacial Acetic Acid for at least 24 hours, this was removed and the material was washed with distilled water.

(c) Cells were hydrolysed with 1N HCl at $60^{\circ}C$ for 12 min.

(d) The acid was removed and replaced by Feulgen for 2 hours.

(e) The stained material was rinsed in SO_2 water.

Slides were prepared by squashing the cells in 25% acetic acid under a coverslip to spread the chromosomes. The coverslip was detached using dry ice, and the slides could be made permanent by dehydrating through a series of alcohols to absolute alcohol (10 min each stage), then passing through 1:1 and 1:3 alcohol to xylol, to pure xylol.

Slides were mounted in XAM if microdensitometry was to follow.

Staining of callus and explant

The specimen was put in concentrated hydrochloric acid and a few drops of red saffranin solution added. This changed the colour to blue. The material was left for several minutes, and was then placed

in water. The cell wall, cytoplasm and nucleus were stained contrasting shades of pink. The specimens could then be viewed under a light microscope (low power). Such preparations were not made permanent.

SECTION TWO : Experimental

A. Callus tissues on semi-solid medium

I. Initiation of callus from the embryo

Mature embryos from ripe wheat grain were used as the major source of callus in the present work because they proved a reliable and convenient source of callus tissue, and also the grain was easily stored and required little preparation for culture. One disadvantage was that the (usually) small percentage of inviable embryos could not be distinguished until they had failed to form callus, thereby reducing the number of replicates in any treatment in which they occurred. However, grain was obtained from reliable sources and inviability only proved a serious problem with the primitive Aegilops varieties of wheat. A second disadvantage was that individual embryos would not be uniform in their genetic constitution, even when obtained from the same population. This was clearly illustrated in experiments where few replicates were used and consequent variability in response to culture conditions made interpretation of results difficult. Eventually, a policy of using between 10 and 20 replicates per treatment was adopted. Experiments in which less replicates were used are, however, included in this work where they indicated basic trends, despite their unsuitability for establishing statistically significant differences.

Ideally, organogenetic studies should be carried out on a large number of uniform pieces of callus of common origin. To this end, attempts were made to optimise conditions for producing callus readily, and to maximise the formation of undifferentiated callus on the explants.

The immediately following sections are concerned with experiments investigating the effect of various factors on callus initiation from wheat embryos. A study was made of the differences to be found in callus production from 10 varieties of wheat. The minimum requirements necessary for the formation of callus on excised embryos, and also the effect of alterations to the organic components of the basic medium "M" were investigated. The effects of several auxins and cytokinins on callus initiation were studied. Finally, experiments were carried out using immature embryos at different stages of development to determine whether this would affect callus production.

(i) Variety of wheat

Mature embryos were excised from both the primitive and modern commercial varieties of wheat grain, and cultured on "M" plus 1.0 mg/l 2,4-D, in Controlled Environment Room II (low light). The following table lists the full name of each variety, plus ploidy level (n = 7).

Table 2.I

Variety of wheat	Ploidy
Aegilops speltoides	2n
A. squarrosa	2n
Triticum monococcum	2n
T. dicoccum	4n
T. aestivum 'Maris Ranger'	6n
T. aestivum 'Maris Ensign'	6n
T. aestivum 'Holdfast'	6n
T. aestivum '6 wuk 3' (dwarf)	6n
T. aestivum '6 wuk 4' (dwarf)	6n
MSxRanger (Male sterile cross with Maris Ranger)	6n

Fresh and dry weights were taken immediately after excision of the embryos (Table 2.2). It can be seen that ploidy level is not strictly correlated with fresh or dry weight of the embryo, and that, apart from the Aegilops varieties, all the fresh weights lie within

Table 2.2 : Fresh and Dry Weights (mg) of freshly-excised Embryos from 10 varieties of Wheat.

Variety *	Total number of Embryos	Fresh Weight	Dry Weight
Maris Ranger	10	1.9±0.19	0.47
T. dicoccum	10	1.9±0.26	0.48
MS x Ranger	10	1.8±0.16	0.36
Holdfast	10	1.6±0.11	0.34
Maris Ensign	11	1.4±0.11	0.42
6 WUK 3	8	1.4±0.11	0.32
T. monococcum	11	1.3±0.07	0.33
6 WUK 4	7	1.2±0.17	0.30
Aegilops speltoides	8	0.3±0.04	0.02
Aegilops squarrosa	8	0.3±0.05	0.05

* see text for further details of the varieties used.

All figures are means, standard errors are shown for Fresh Weights only.

Table 2.3 : Appearance of Excised Embryos after 5 days incubation on 'M' + 1mg/l 2,4-D.

Variety	Total replicates	% of Cultures with appearance indicated :			
		Callus only	C + S	C + R	C + S + R
Maris Ranger	16	88	6	0	0
T. dicoccum	34	21	21	3	3
MS x Ranger	14	64	28	0	0
Holdfast	34	59	38	0	3
Maris Ensign	32	91	0	0	0
6 WUK 3	30	90	7	0	0
T. monococcum	33	36	58	0	0
6 WUK 4	30	80	0	0	0
Ae. speltoides	31	45	13	0	0
Ae. squarrosa	8	50	38	0	0

C + S = Callus plus Shoot extension from the Embryo.

C + R = Callus plus Root extension from the Embryo.

C + S + R = Callus plus Shoot & Root extension from the Embryo.

Table 2.4 : Fresh and Dry Weights (mg) of Excised Embryos after 13 days incubation on 'M' + 1mg/l 2,4-D, and Percentage of Tissue Undifferentiated.

Variety	Total replicates	Fresh Weight	Dry Weight	% U
Maris Ranger	13	67.1±4.5	4.2±0.3	92.6±2.3
T. dicoccum	16	121.7±9.1	7.9±0.5	80.5±2.8
MS x Ranger	15	46.3±5.8	3.4±0.4	96.6±2.3
Holdfast	20	66.5±4.1	5.2±0.3	87.7±3.4
Maris Ensign	20	45.1±2.2	2.0±0.1	100.0
6 WUK 3	20	42.4±2.3	3.3±0.2	98.9±1.1
T. monococcum	24	80.1±6.0	5.7±0.4	86.5±1.9
6 WUK 4	20	48.9±2.7	3.5±0.2	100.0
Ae.speltoides	17	19.0±2.6	1.2±0.2	97.7±1.2
Ae.squarrosa	9	17.0±3.1	1.7±0.3	89.1±4.4

All data expressed as the mean ± standard error.

1 - 2 mg. After 5 days, the cultures were scored for callus formation (Table 2.3). If the excised embryo was now completely covered by callus, this was recorded as "callus only". In other cases, either the shoot, or more rarely, the roots, of the embryo had continued development while callus was being formed. In two varieties, Maris Ensign and 6 wuk 4, no extension growth of the embryo axis was observed. After 13 days, the cultures were sacrificed and the total fresh weight (F.wt.) and dry weight (D.wt.) determined (Table 2.4). The proportion of undifferentiated callus (%U) refers to the amount of visibly undifferentiated tissue left after removal of roots and shoot (see Materials & Methods). T. dicoccum had by far the greatest F.wt.(122 mg) but also had the lowest (80%) amount of undifferentiated callus. Maris Ranger, having just over half this F.wt. (67 mg), had more than 90%U. Both Maris Ensign and 6 wuk 4 were completely undifferentiated but had rather low F.wt. (45 and 43 mg respectively). The two Aegilops varieties had the lowest F.wt. (17 to 19 mg), although fairly high %U. The 4 varieties used most in the present studies, Maris Ranger, Maris Ensign, 6 wuk 3 and 6 wuk 4, all had a high %U (>90%).

The experiment illustrates the difference in response of the various genotypes on the chosen medium. The Standard Errors of the Mean do not rise beyond 10% of the fresh weight, and are much lower for the %U.

(ii) Effect of nutrients

Organic constituents

Maris Ranger embryos were excised and placed on semi-solid "M" media in which the nutrients had been progressively depleted, as shown overleaf:

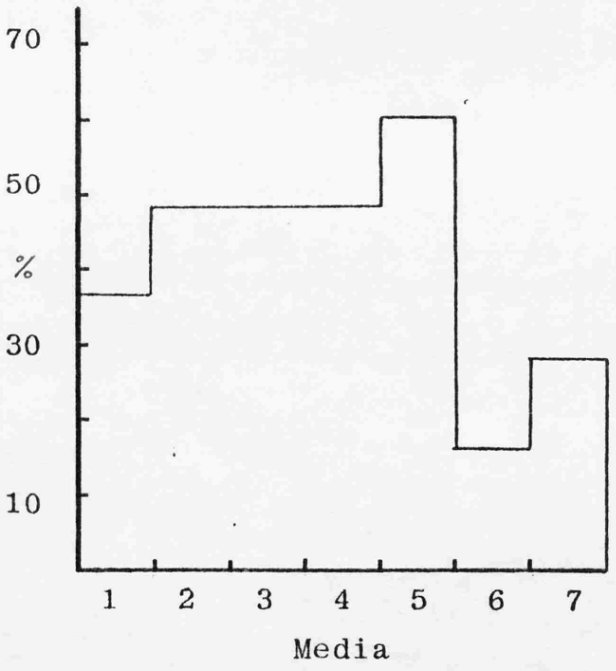
Medium	Mineral salts	Casein hydro-lysate	Inos-itol	Vitamins	Fe EDTA	Sucrose
1	+	+	+	+	+	+
2	+	-	+	+	+	+
3	+	-	-	+	+	+
4	+	-	-	-	+	+
5	+	-	-	-	-	+
6	+	-	-	-	-	-
7	-	-	-	-	-	+

All 7 media contained 1 mg/l 2,4-D.

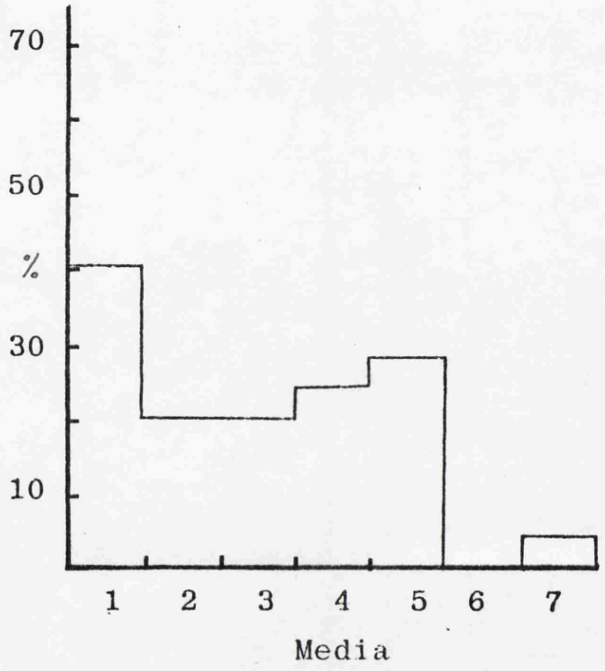
Cultures were placed in Controlled Environment Room II, under low light intensity. Each treatment had 25 replicates.

By 3 days, callus had already formed as a thin layer of light-coloured tissue over parts of the explant. Some callus had been produced on all 7 media. Fig. 2.1A shows the percentage of explants on each medium having callus development; the greatest percentage was on Med. 5 (2,4-D, mineral salts and sucrose). A few of the explants showed continued development of the embryo axis and this is recorded in Table 2.5. The initiation of callus even on severely depleted medium suggests that the presence of 2,4-D alone is enough to initiate callus on excised embryos. After 24 days, although the majority of explants showed some callus formation only those also showing development of the embryo shoot and/or roots had a healthy growth "Healthy Callus" (Table 2.6, Fig. 2.1B), those which had "Callus only" tended to be small and some were browning. After 34 days on depleted media, the need for extra nutrients is evident from the number of callus pieces which appear to have stopped growing. Only the complete medium I has a high percentage of cultures with

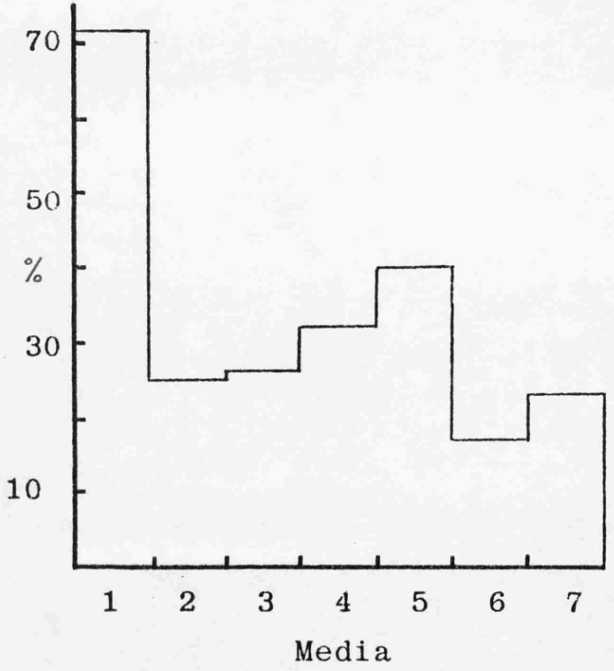
Fig.2.1 : Presence of Callus on Excised Wheat Embryos incubated on 7 Media differing in depletion of certain nutrients.



A. Percentage of Explants having Callus Development after 3 DAYS on 7 Media.



B. Percentage of "Healthy Callus" in each Treatment after 24 DAYS.



C. Percentage of "Healthy Callus" in each Treatment after 34 DAYS.

Table 2.5 : Characteristics of Cultures after 3 Days on 7 Media.

Medium	Callus only	Callus + Root	Callus + Shoot	No Devt.	Total % with Callus	Total N°. of Reps.
1	9	0	0	16	36	25
2	10	1	1	13	48	25
3	9	2	1	13	48	25
4	9	2	1	13	48	25
5	15	0	0	10	60	25
6	4	0	0	21	16	25
7	7	0	0	18	28	25

Table 2.6 : Characteristics of Cultures after 24 Days on 7 Media.

Medium	Callus only	Callus + Root	Callus + Shoot	No Devt.	Total % with Callus	% of Healthy Callus	Total N°. of Reps.
1	9	0	10	6	76	40	25
2	18	0	5	2	92	20	25
3	15	0	4	5	80	20	25
4	17	0	6	2	92	24	25
5	15	1	6	3	88	28	25
6	22	0	0	2	92	0	24
7	23	0	1	1	96	4	25

Table 2.7 : Characteristics of Cultures after 34 Days on 7 Media.

Medium	Callus only	Callus + Root	Callus + Shoot	Dying Callus	% of Healthy Callus	Total N°. of Reps.
1	7	0	8	6	71	21
2	2	0	3	15	25	20
3	3	1	2	13	26	19
4	5	0	3	17	32	25
5	4	0	4	12	40	20
6	1	0	0	5	17	6
7	2	1	2	17	23	22

healthy, white callus growing in an active manner (Fig. 2.1C). However, the number of cultures having "Healthy Callus" (Table 2.7) appeared to have increased, because this now included cultures referred to as "Callus only" (as well as "Callus and shoot"). By 34 days there was a sharp distinction between good healthy callus and that which was drying up and dying. Depletion of the media slowed down the growth of the callus but even media lacking casein hydrolysate, Inositol, vitamins and iron could still support a little growth of the callus, presumably by the supply of nutrients from the embryo tissue of the callus.

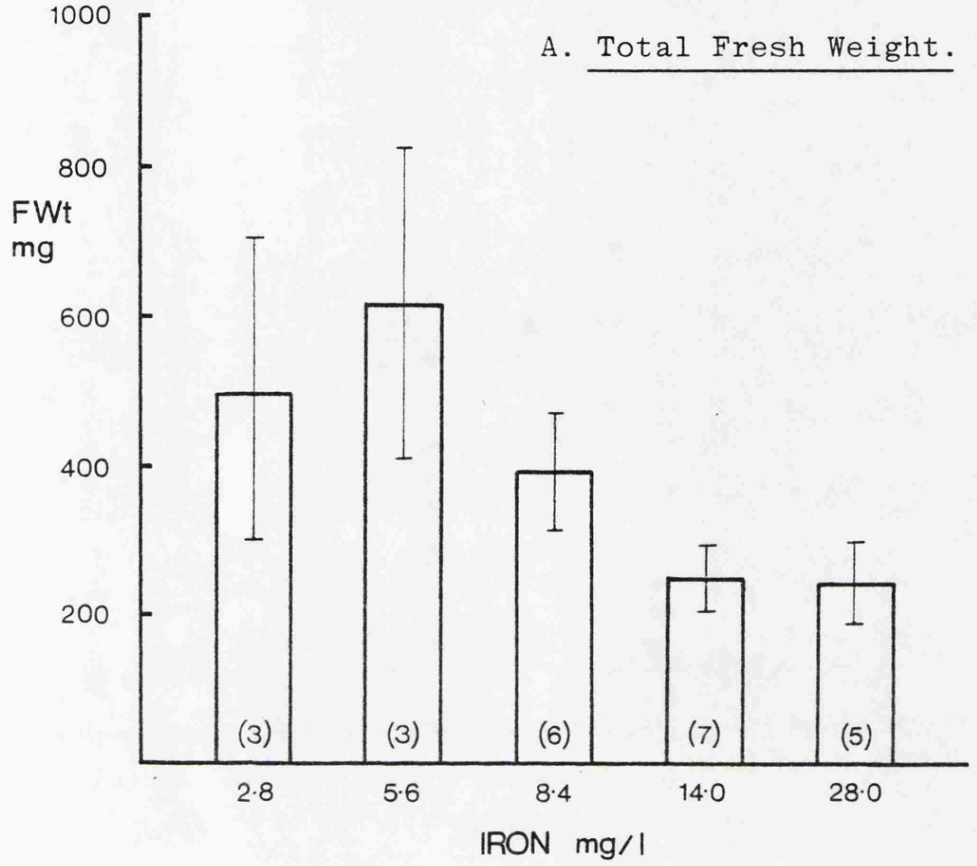
After a total of 11 weeks on these media, Med. I had the greatest percentage (44%) of cultures having callus of a reasonable size suitable for subculture, i.e. roughly 1 g F.wt. of callus. Less than 30% of the cultures on Media 2,3,4 and 5 had callus of this size. One culture on each of the Media 6 and 7 (containing only mineral salts or sucrose) had a viable piece of callus, and these pieces were capable of further growth when transferred to complete medium.

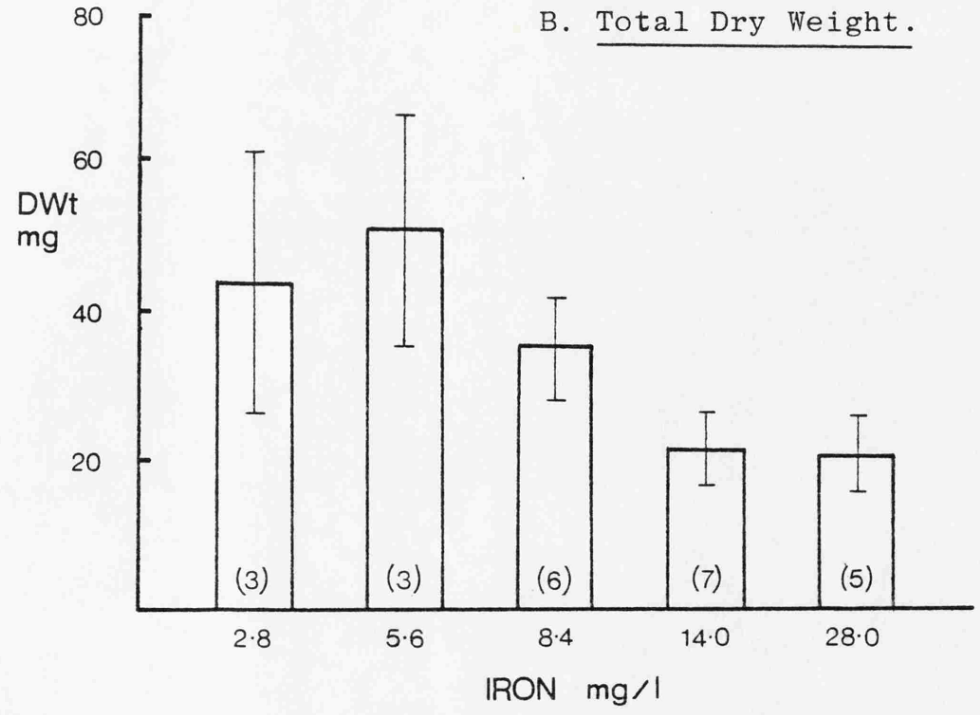
The experiment demonstrated that callus formation on excised wheat embryos takes place irrespective of organic nutrients, provided 2,4-D is present, but that the organic components of the standard medium are necessary for healthy callus growth.

Level of FeEDTA

This experiment was conducted to determine whether the level of iron (5.6 mg/l) recommended for Murashige & Skoog medium ("M") was in fact the optimum for growing wheat callus on this medium.

Fig.2.2 : Fresh and Dry Weights of Excised Wheat Embryos, after incubation for 11 weeks on "M" plus increasing levels of FeEDTA.(number of replicate cultures in brackets). $\bar{x} \pm SE$.





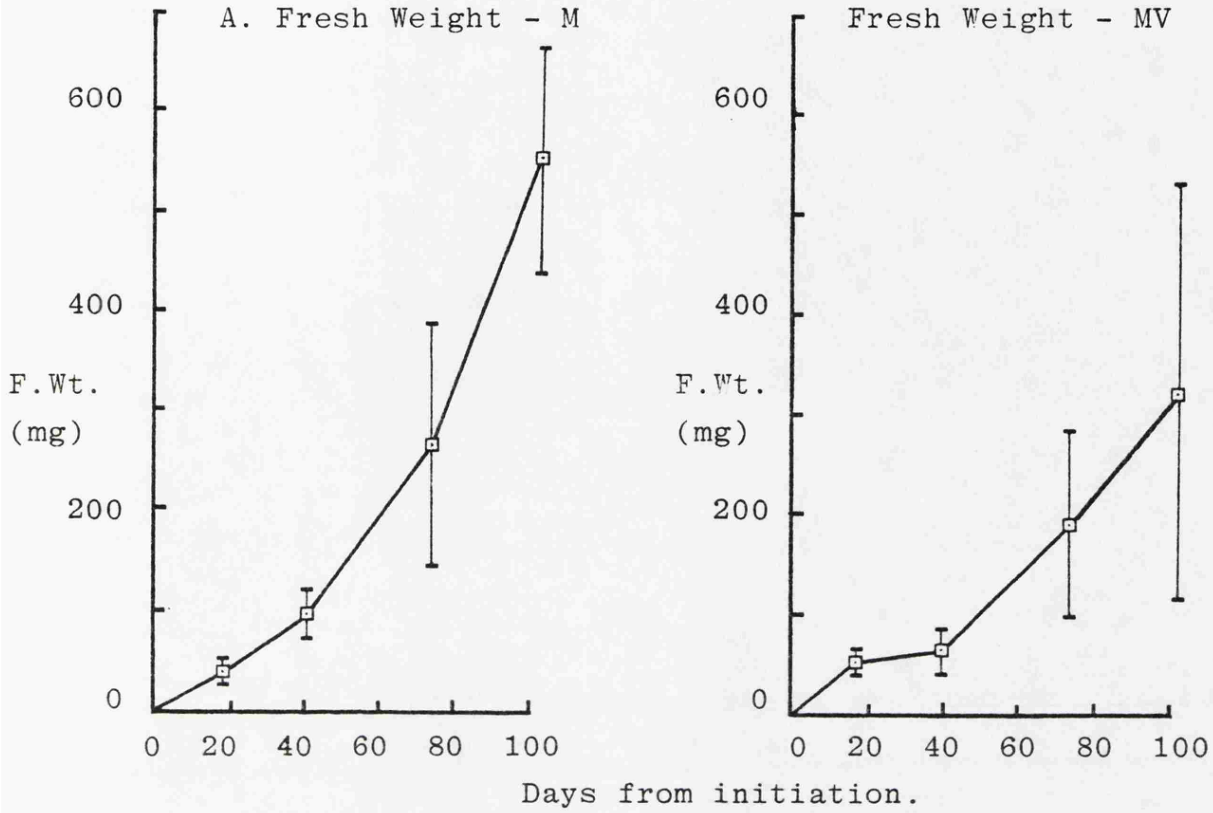
Ten replicates of Maris Ranger embryos were used for each level of iron tested, but several became contaminated, so the replicate numbers vary. The medium "M" contained 100 mg/l casein hydrolysate and 1 mg/l 2,4-D, plus one of the 5 different chosen levels of FeEDTA. Cultures were placed under low light intensity in Controlled Environment Room II.

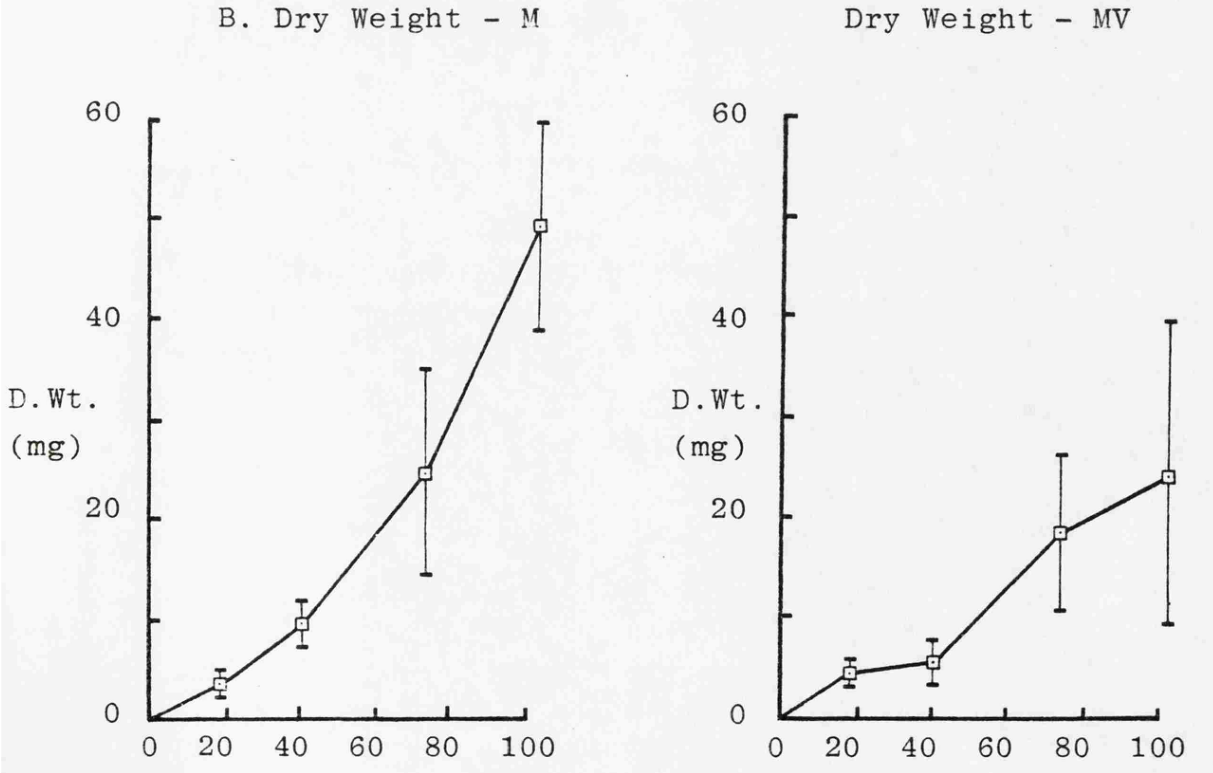
After 11 weeks, the total fresh and dry weights were taken (Fig. 2.2 A,B). Because of the small numbers of replicates, the standard errors of the mean are rather large, but the results suggest that increasing the iron content beyond 8.4 mg/l reduces the yield of callus tissue. For both F.wt. and D.wt. the medium giving the greatest mean value contains 5.6 mg/l iron, which is the level standard in Murashige & Skoog medium.

The vitamin content in media containing 100 mg/l casein hydrolysate

Maris Ranger embryos were excised and placed on "M" containing 1 mg/l 2,4-D and 100 mg/l casein hydrolysate. One batch of medium also contained 10 times the standard level of Whites vitamins ("MV"). The cultures were placed under low light intensity in Controlled Environment Room II. At intervals over thefirst passage of 102 days, 5 of the cultures were sacrificed and their total fresh and dry weights taken (Fig. 2.3). These weights included both callus tissue and any roots or shoots formed by further development of the embryo axis. Although the size of the standard error of the mean indicated a high level of variation among the replicates (especially later on in the passage), the results show that increasing the vitamin level 10 times did not significantly affect either fresh or dry weight.

Fig.2.3 :Growth over the First Passage of Excised Wheat Embryos, in the presence of the standard (M) and x10 the standard (MV) level of vitamins.





The vitamin content in media lacking casein

Maris Ranger embryos were transferred to "M" with the following vitamin and casein content:

"V" = M + normal level vitamins + no casein hydrolysate

"10V" = M + x10 vitamins + no casein hydrolysate

"CV" = M + normal level vitamins + 100 mg/l casein hydrolysate

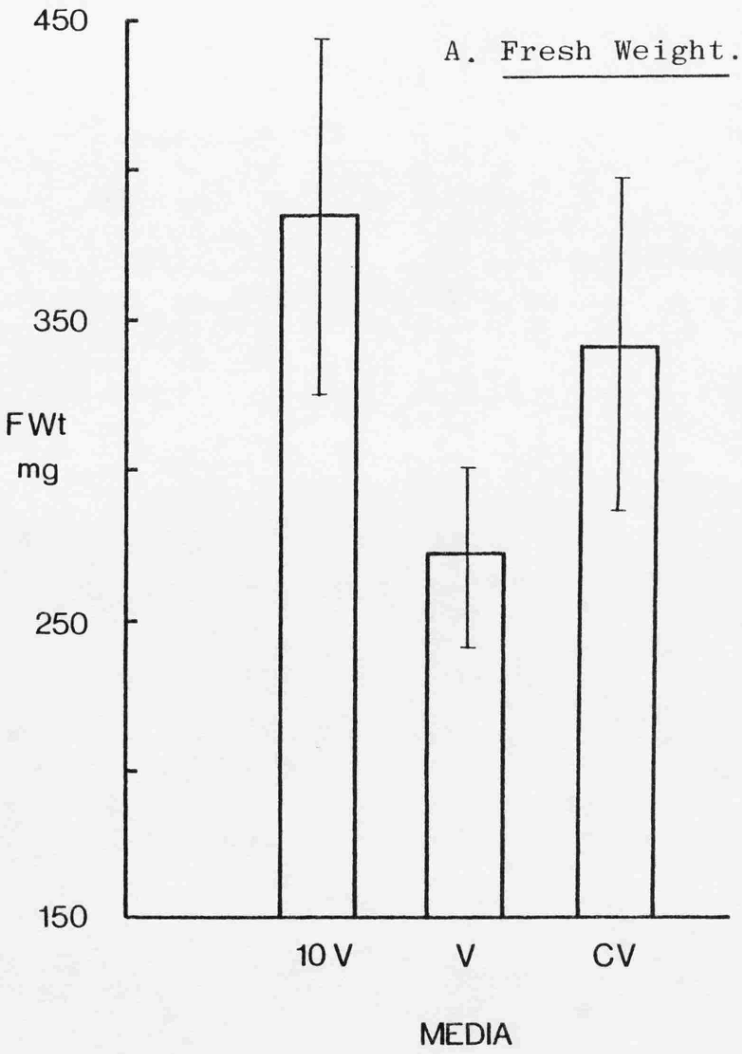
All media contained 1 mg/l 2,4-D.

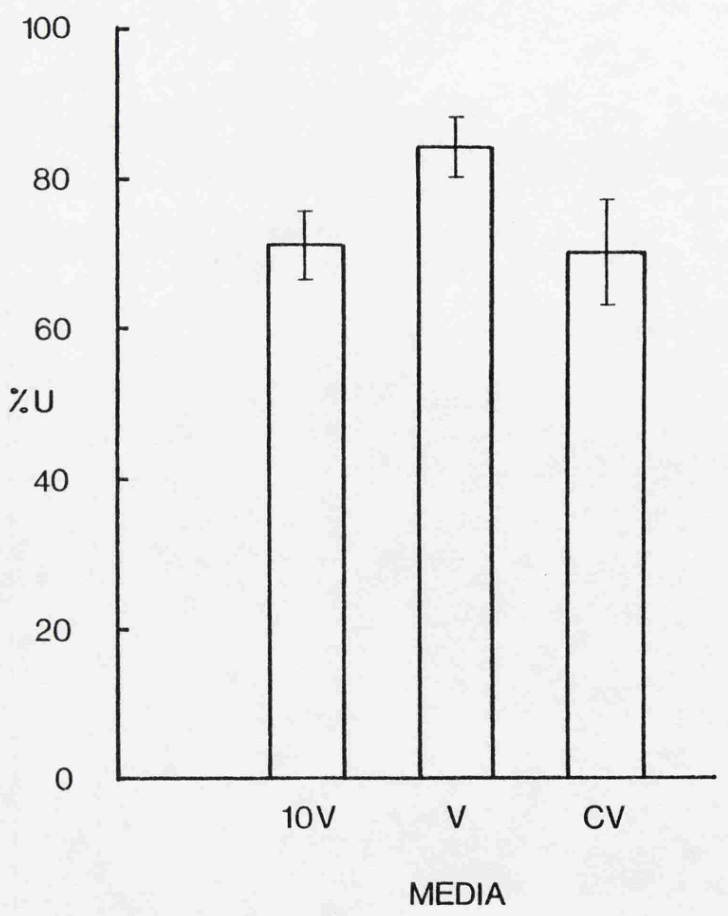
The cultures were grown in low light in Controlled Environment Room II. After 9 weeks, the explants were weighed to obtain the total fresh weight, and the percentage of undifferentiated callus was estimated after removal of any roots or shoots which had developed from the original embryo (Fig. 2.4 A,B). Addition of 100 mg/l casein hydrolysate to the medium did not significantly affect the F.wt. of tissue. However, increasing the vitamin content (in the absence of casein) did lead to a significant increase in F.wt. This is in contrast to the finding in the previous experiment, where increased vitamins in the presence of 100 mg/l casein hydrolysate, did not affect the F.wt. It can be suggested, therefore, that the presence of casein may in some way interfere with the enhancing effect, which increasing the vitamin content of the medium has on the fresh weight.

The percentage of undifferentiated tissue present was higher in the basic medium "V" than where either increased vitamins ("10V") or casein hydrolysate ("CV") had been added. Addition of these to the medium seemed to favour further extension of shoots and roots from the original embryo. This enhanced development of differentiated tissue could, at least partially, explain the increased mean F.wt. discussed above.

Fig.2.4 : Growth and Differentiation of Wheat Cultures after 9 weeks on "M" modified with regard to Casein Hydrolysate and Vitamins. ($\bar{x}$ ± SE).

"V" = normal level vitamins,no casein
"10V"= x10 vitamins, no casein.
"CV" = normal vitamins,100mg/l casein.





(iii) Growth substances

2,4-Dichlorophenoxyacetic acid (2,4-D), p-Chlorophenoxyacetic acid (CPA) and L-Tryptophan (Tryp.)

2,4-D has been much used as a dedifferentiatior in plant tissue culture, and the following experiment investigated the effect of this auxin on initiation of callus from excised wheat embryos. At the same time, the effects of CPA and L-Tryptophan on callus initiation were studied, either alone or in combination with 2,4-D. CPA has been little used as an auxin source for cultures of Monocotyledons, although its use was reported for Bromus inermis by Gamborg, Constabel & Miller (1970), and more recently, by Cheng & Smith (1975) for cultures of Hordeum. L-Tryptophan is known to be a precursor of IAA and has been used in the culture of excised wheat roots, where Carter & Street (1963) showed that only autoclaved Tryptophan is active in enhancing root growth: the autoclaving process caused the production of two active 'factors', one identified as IAA.

Excised Maris Ranger embryos were placed on modified "M", as shown below, containing 2,4-D, CPA, or Tryptophan in different combinations. Half of the replicates were cultured in complete darkness (D) and the rest in low light (L) in Controlled Environment Room II.

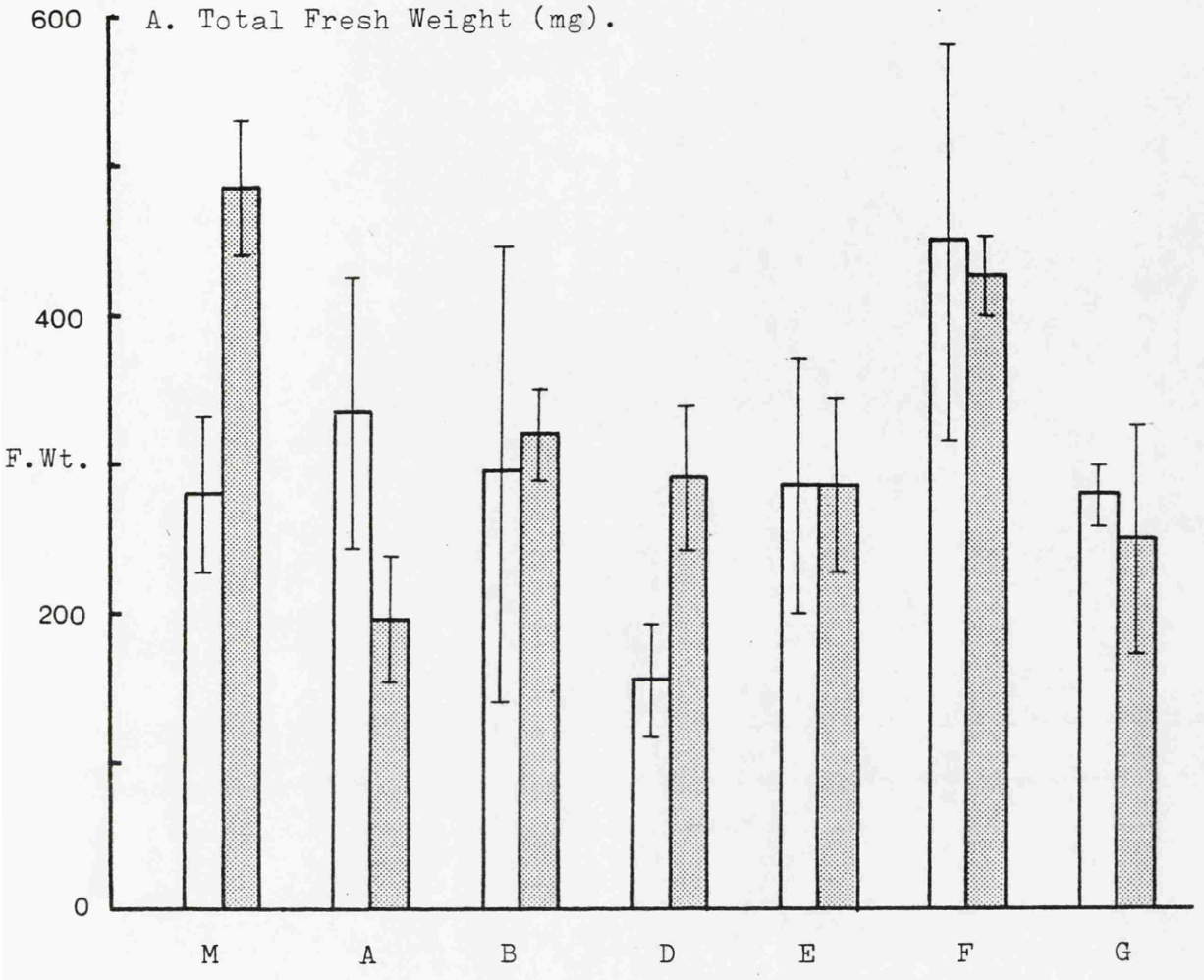
Medium	Casein hydrolysate (100mg/l)	2,4-D (1 mg/l)	CPA (5 mg/l)	Tryp. (4 mg/l)
M	+	+	-	-
A	-	+	-	-
B	-	-	+	-
D	-	+	+	+
E	-	+	+	-
F	-	+	-	+
G	-	-	+	+

After 11 weeks, the total fresh weight of the cultures was taken, and the proportion of undifferentiated tissue present calculated. Fig. 2.5 A,B, shows the results with 'L' and 'D' cultures plotted separately. Statistical analysis of these data showed that while the means were significantly different between the media, there was no significant difference between the cultures placed in Light or Dark. As the Standard Errors of the mean show, there is quite a high level of variation within each batch of replicates, particularly those grown in light.

Since there was no statistical difference between 'L' and 'D', these were combined and the results re-plotted (Fig. 2.6 A,B). Medium F, containing 2,4-D, and Tryptophan, seems to produce a markedly higher total F.wt. than all the other media (Fig. 2.6, A). The lowest mean F.wt. is found on Medium D (2,4-D, CPA and Tryp). The other media tested do not differ in their production of F.wt. However, in Fig. 2.5A, there is clearly a marked difference in F.wt. between 'L' and 'D' on Media M and D. In both cases, the callus had grown better in the dark. Because this is based upon so few replicates, these results are not reliable, but could be considered to suggest that 100 mg/l casein hydrolysate enhances, and the presence of '2,4-D + CPA + Tryptophan' inhibits, fresh weight growth in the dark.

The data for the percentage of undifferentiated tissue (Fig. 2.6 B) shows that Medium F (2,4-D + Tryp) gives a much lower %U than Medium G (CPA + Tryp). Comparing Medium A (2,4-D) and Medium B (CPA), the difference in %U is less, so it could be the presence of Tryptophan with 2,4-D which is promoting development of the embryo root and shoot. However, when comparing the effect of Tryptophan with and without CPA (Media G & B) there is very little difference in U%, so that it seems that Tryptophan in the presence of CPA has

Fig.2.5 : Total Fresh Weight and % of Undifferentiated Tissue present in Excised Wheat Embryos, incubated for 11 weeks on 7 Media (see text) in light (open) or dark (hatched column).($\bar{x} \pm SE$)



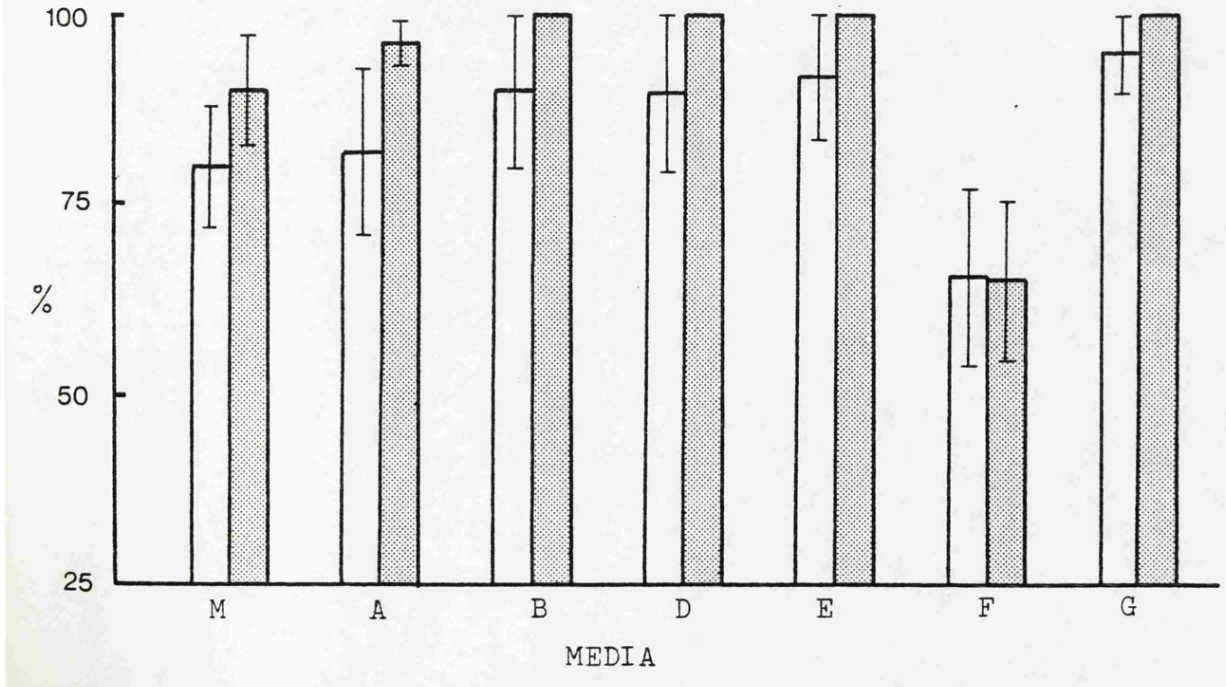
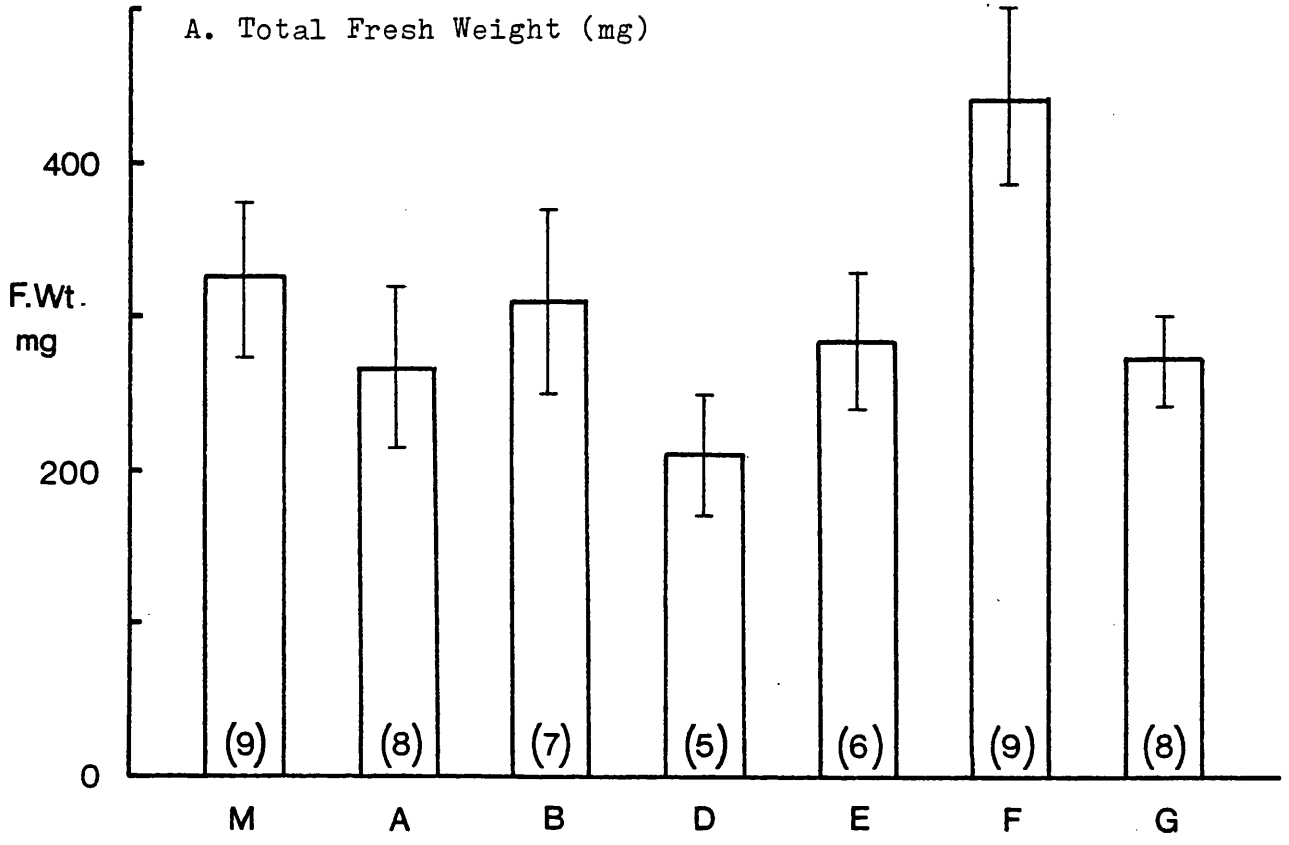
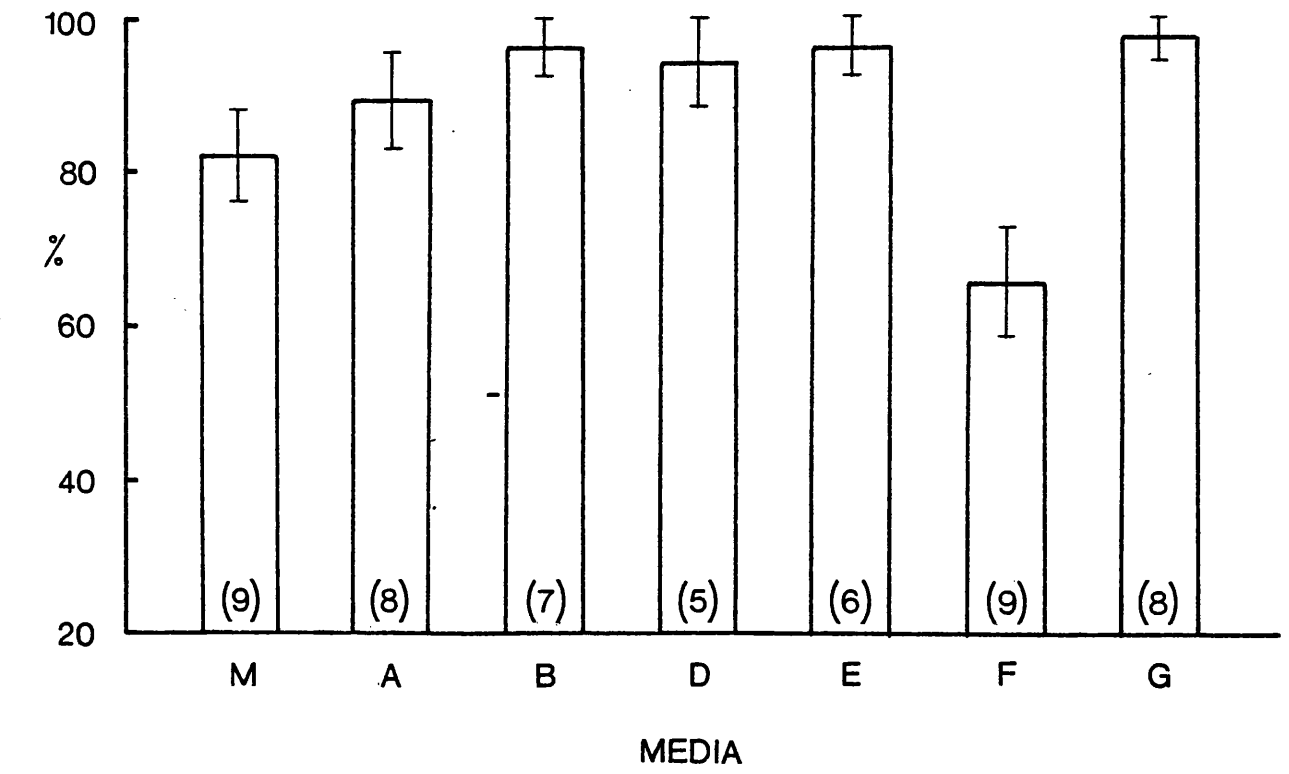


Fig.2.6 : Total Fresh Weight and % of Undifferentiated Tissue present on excised Wheat Embryos, incubated for 11 weeks on 7 Media (see text for details).
(number of replicates in brackets; $\bar{x} \pm SE$)



B. Percentage of Undifferentiated Tissue.



a different effect to that which it has in the presence of 2,4-D.

All the media containing CPA (B,D,E and G) have well over 90% undifferentiated tissue; a strong indication that the presence of 5 mg/l CPA is suppressing development of the embryo axis. Media containing 2,4-D (M,A,F) tend to have a lower mean %U, especially where Tryptophan is also present (F). Where both 2,4-D and CPA are present (D,E) the mean %U is again high, suggesting that the 5 mg/l CPA is the active factor in suppressing embryo axis elongation.

This preliminary experiment led on to experiments designed to determine the optimum levels of 2,4-D and CPA for producing the maximum amount of undifferentiated tissue:

2,4-Dichlorophenoxyacetic acid (2,4-D)

Maris Ranger embryos were excised and cultured on "M" with a range of 2,4-D levels from 0 - 20 mg/l (20 replicates each). They were placed in low light in Controlled Environment Room II.

The explants were observed after 5 days in culture and the presence of callus noted (Table 2.8). No callus was found on the medium lacking 2,4-D; these explants showed normal development of the embryo axis, having a strong green shoot and 3 or more primary roots. With only 0.1 mg/l 2,4-D or more, all of the explants had some degree of callus initiation. At concentrations of 2,4-D over 1.0 mg/l there was a suppression of development of the embryo axis. While extension of the embryo shoot could be observed at all concentrations up to and including 2.0 mg/l 2,4-D, root extension rarely occurred above 0.3 mg/l 2,4-D. The development of the shoot represented further development of the plumule, which is already well formed when the embryos are excised (Fig. 1.1, Materials & Methods). The suppression of elongation of the

Table 2.8 : Appearance of Excised Embryos after 5 days on 'M' plus increasing levels of 2,4-D.

2,4-D (mg/l)	Total replicates	% of Cultures with appearance indicated			
		Callus only	C + S	C + R	C + S + R
0	20	0	0	0	0
0.1	19	5.4	36.9	0	57.9
0.3	15	13.3	73.3	6.7	6.7
1.0	19	79.0	21.1	0	0
2.0	20	100.0	0	0	0
5.0	19	100.0	0	0	0
10.0	19	100.0	0	0	0
20.0	19	100.0	0	0	0

Table 2.9 : Appearance of Excised Embryos after 13 days on 'M' plus increasing levels of 2,4-D.

2,4-D (mg/l)	Total replicates	% of Cultures with appearance indicated			
		Callus only	C + S	C + R	C + S + R
0	19	0	0	0	0
0.1	19	5.4	21.0	0	73.7
0.3	15	6.7	80.0	0	13.3
1.0	19	57.9	42.1	0	0
2.0	19	94.7	5.3	0	0
5.0	19	100.0	0	0	0
10.0	19	100.0	0	0	0
20.0	19	100.0	0	0	0

C + S = Callus plus Shoot extension from the embryo.

C + R = Callus plus Root extension from the embryo.

C + S + R = Callus plus Shoot & Root extension from the embryo.

Plate I

Characteristics of excised Wheat Embryos, incubated for 13 days on "M" containing increasing levels of 2,4-D.

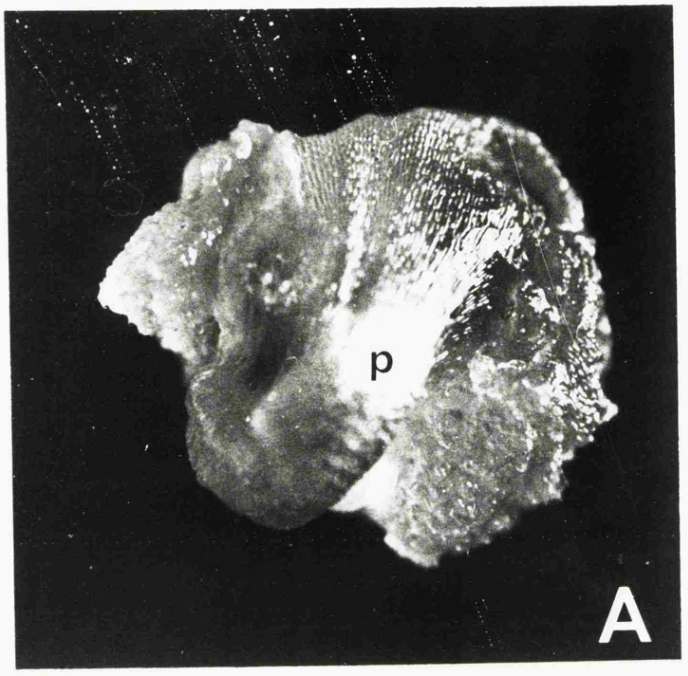
A. Callus covering the entire explant; the plumule (p) can be seen just below the surface of callus tissue. (0.1mg/l 2,4-D)

B. The plumule (p) shows very little extension and the epidermal layer of tissue (t) can be seen as a loose outer layer attached to the callus (c) at the base of the plumule. (1.0mg/l 2,4-D)

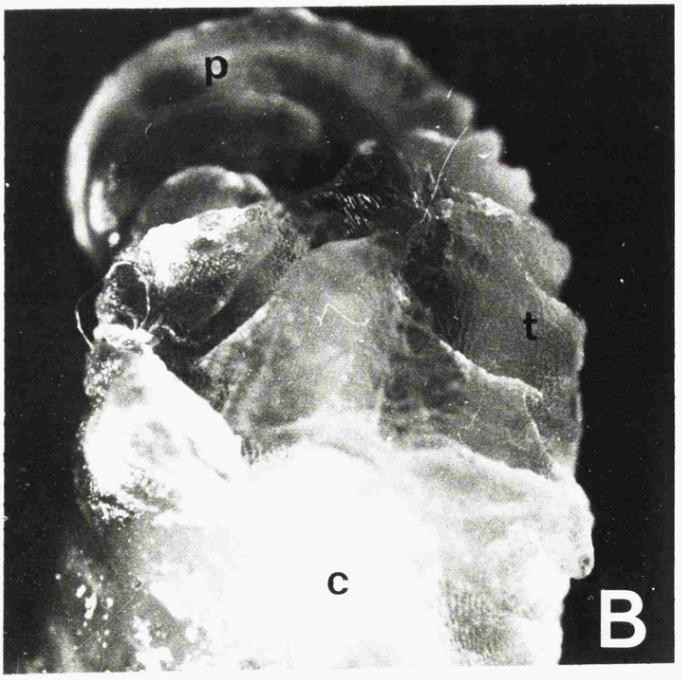
C. The plumule of the excised embryo has continued extension growth, while callus covers the rest of the explant. (0.3mg/l 2,4-D)

D. Small shoots (s) have continued development from the excised embryo, and also one root has extended (r); callus formation has occurred at the base of the plumule. (0.3mg/l 2,4-D)

PLATE I

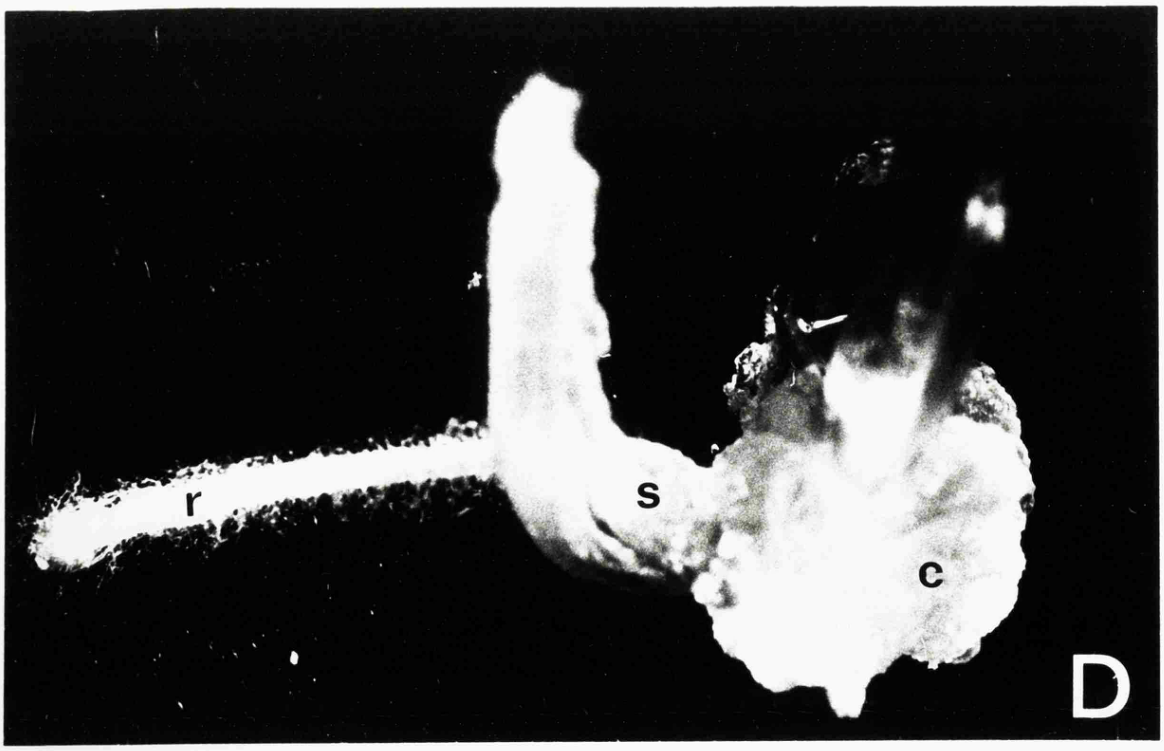


0·5 cm



0·25 cm

0·5 cm



0·5 cm

Fig.2.7,A :Fresh Weight (□ - □) and % undifferentiated Tissue (△ - △)of Excised Wheat Embryos,after 13 days on "M" containing increasing levels of 2,4-D. ($\bar{x}$ ± SE)

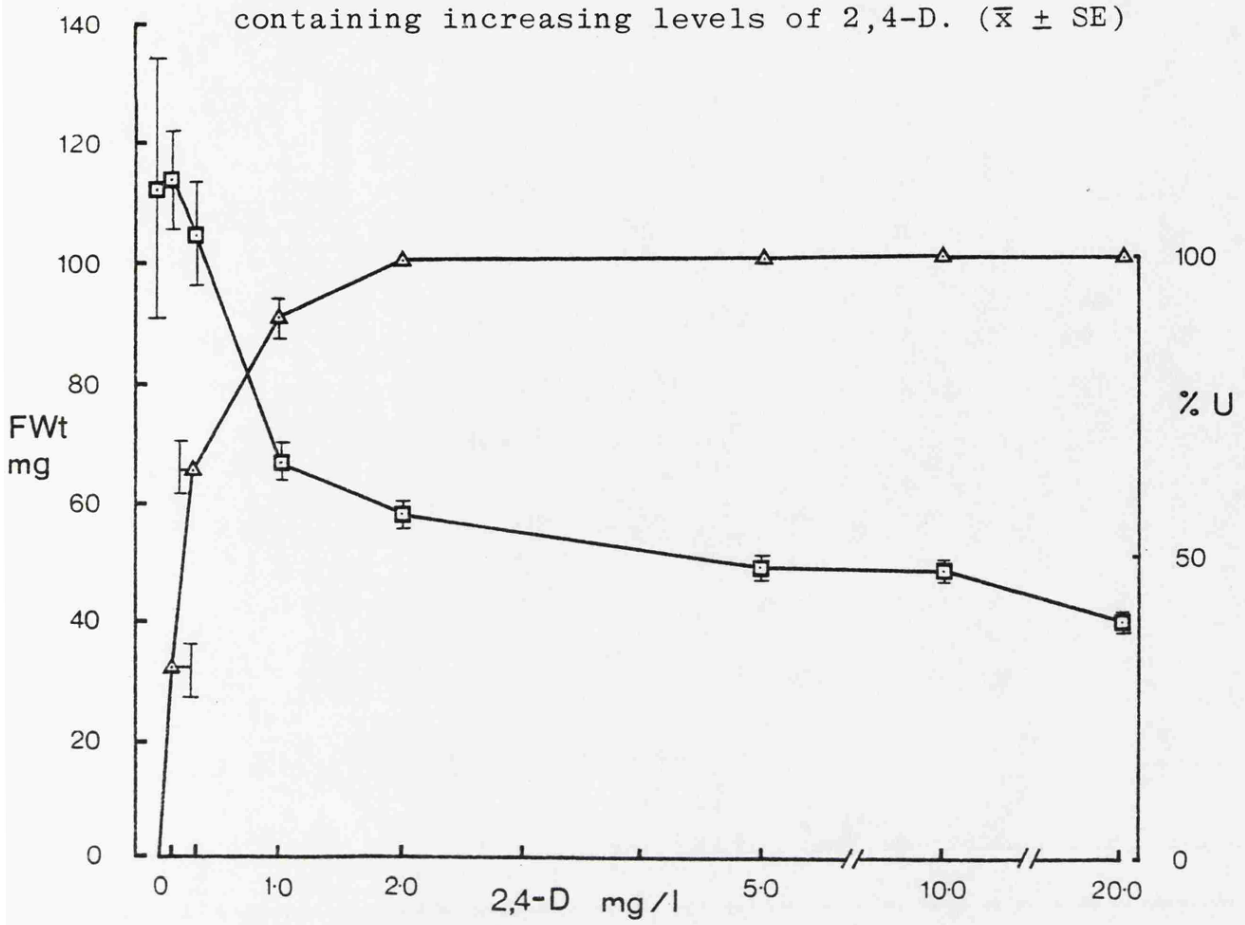
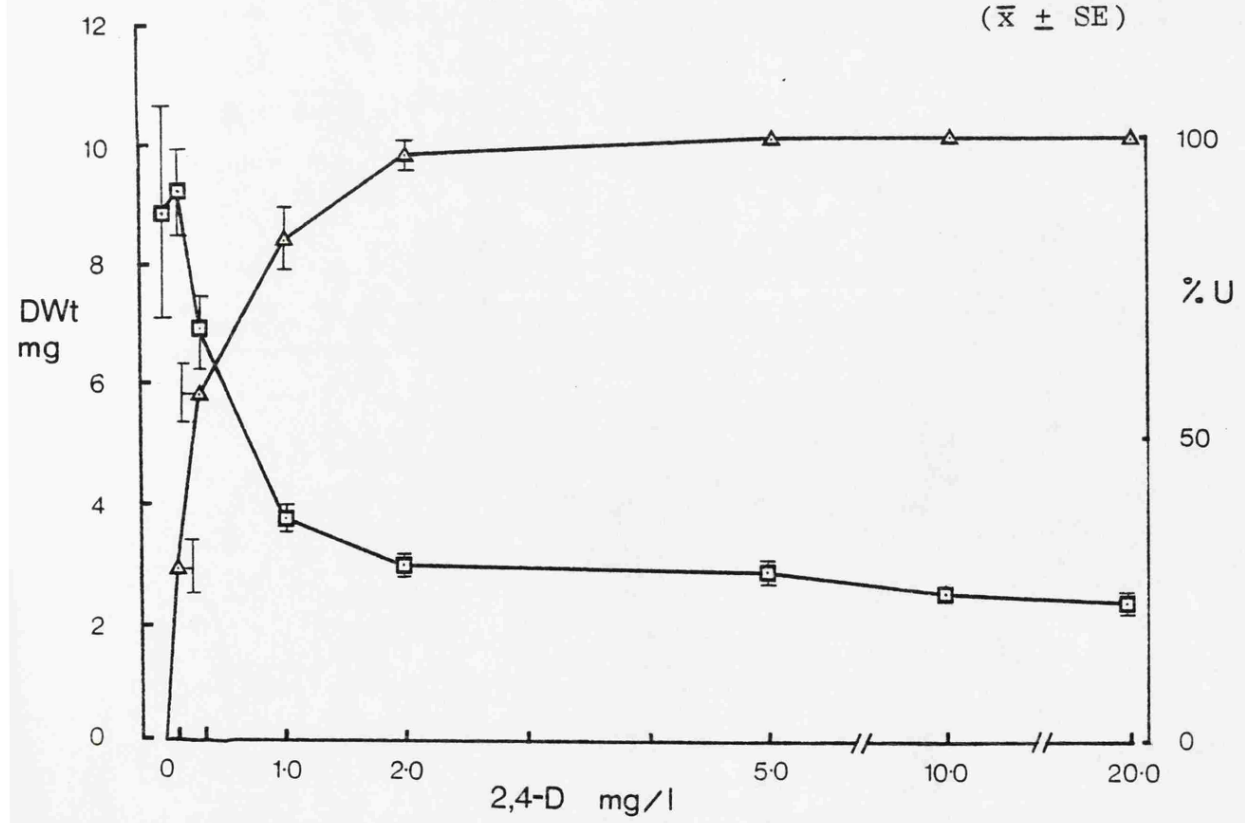


Fig.2.7,B : Dry Weight (□ - □) of Excised Wheat Embryos, after 13 days on "M" plus increasing levels of 2,4-D.

($\bar{x}$ ± SE)



seminal roots is in line with the generally encountered sensitivity of roots to auxin inhibition of their elongation. Above 2.0 mg/l 2,4-D, the explants were entirely covered by callus tissue and could be referred to as "undifferentiated", although a core of original material of the embryo was still present (Plate I).

After 13 days in culture (Table 2.9), more of the explants showed development of shoots and roots, but above 2.0 mg/l 2,4-D well over 90% of the explants showed callus formation only. At this time, the cultures were sacrificed and the fresh and dry weights determined (Fig. 2.7 A,B). "%U" was determined from the visibly undifferentiated tissue remaining after removal of shoots and roots. Both F.wt. and D.wt. follow a similar pattern. The highest mean weights were found at the lowest levels (0.1, 0.3 mg/l) of 2,4-D, but these explants also showed the greatest development of the embryo axis. Between 1.0 and 2.0 mg/l 2.4-D, the mean weight values reached a plateau, which remained until 10.0 mg/l as regards fresh weight, and up to the highest level of 2,4-D tested for dry weight.

This experiment shows that levels of 2,4-D between 1.0 and 10.0 mg/l are suitable for obtaining callus of an undifferentiated nature from the wheat embryo. At higher levels, the yield of tissue decreases, at lower levels, the proportion of undifferentiated callus decreases. On the basis of these findings, 1.0 mg/l 2,4-D was adopted for regular use, because although it allows some development of the embryo axis, it permits a high yield of suitable callus to be obtained.

p-Chlorophenoxyacetic acid (CPA)

Excised Maris Ranger embryos were placed on "M" containing increasing levels of CPA (0.5 - 20.0 mg/l), 20 replicates per treatment. The cultures were grown in low light in Controlled Environment Room II. Table 2.10 summarises the character of the cultures after 10 days incubation. With concentrations above 3.0 mg/l CPA, over 80%

Table 2.10 : Characteristics of Excised Wheat Embryos after 10 days incubation on 'M' plus increasing levels of CPA.

CPA (mg/l)	Total replicates	% of Cultures with appearance indicated:			
		Callus only	C + S	C + R	C + S + R
0.5	19	15.8	68.4	0	15.8
1.0	18	38.9	55.6	0	5.6
2.0	17	41.2	58.8	0	0
3.0	19	79.0	21.0	0	0
4.0	18	72.2	27.8	0	0
5.0	18	77.8	22.2	0	0
6.0	17	88.2	11.8	0	0
10.0	15	100.0	0	0	0
15.0	17	100.0	0	0	0
20.0	19	100.0	0	0	0

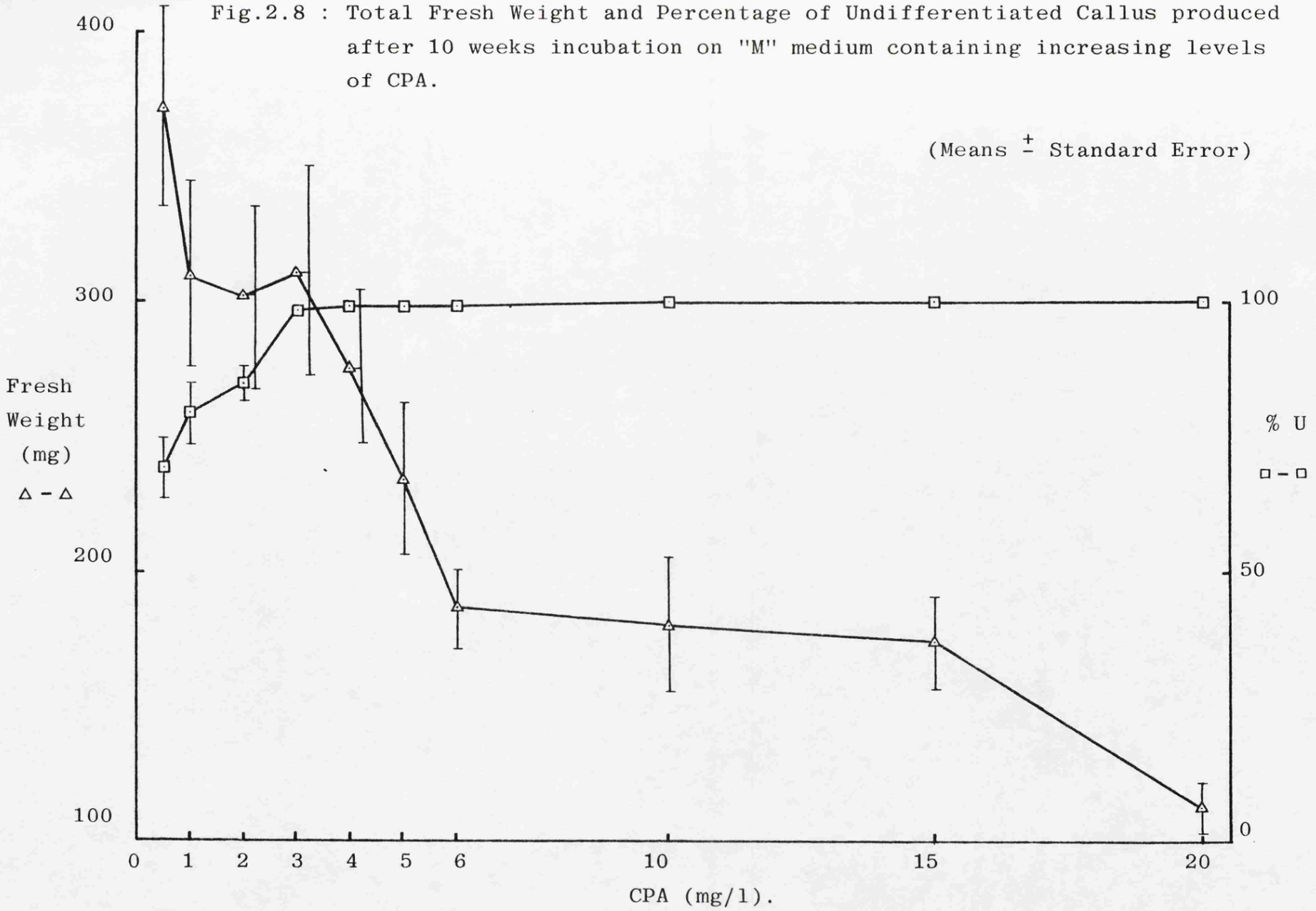
C + S = Callus plus Shoot extension from the embryo.
C + R = Callus plus Root extension from the embryo.
C + S + R = Callus plus Shoot & Root extension from embryo.

Table 2.11 : Characteristics of Excised Embryos after 4 days incubation on 'M' containing different Auxins or Cytokinins.

Medium	Total replicates	% of Cultures with appearance indicated:			
		Callus only	C + S	C + R	C + S + R
'M'	42	83	12	0	5
'MC'	26	100	0	0	0
'MP'	38	34	18	5	42
'MK'	42	19	36	0	29

C + S, C + R, C + S + R -abbreviations as above.
'M', 'MC', 'MP', 'MK' - see text.

Fig.2.8 : Total Fresh Weight and Percentage of Undifferentiated Callus produced after 10 weeks incubation on "M" medium containing increasing levels of CPA.



of the explants showed callus formation alone. Some shoot development from the plumule was found in low frequencies even up to 6.0 mg/l CPA, but any shoots elongating in the presence of 0.5, and 1.0 mg/l CPA were well developed and occurred on cultures also showing roots. No roots were seen above 1.0 mg/l CPA, and roots were only observed on cultures also showing shoots.

After 10 weeks, the explants were transferred to fresh medium, taking undifferentiated callus tissue only, and discarding visibly organised parts (as described in Materials & Methods, P10), and the total fresh weight and percentage of undifferentiated tissue were determined (Fig. 2.8). Concentrations of CPA above 3.0 mg/l almost completely suppressed development of the embryo axis (hence the total F.wt. is almost 100%U). At 0.5 mg/l CPA, ca. 70% of the fresh weight represented undifferentiated callus. The fresh weight of the cultures decreased sharply as the concentration was increased from 3.0 to 6.0 mg/l CPA and a further decrease occurred above 15.0 mg/l CPA. These decreases with increase in concentrations of CPA were due to inhibition of growth of the callus. The use of between 2.0 and 3.0 mg/l CPA produced the optimum amount of undifferentiated tissue.

Comparing these findings with the previous experiment with 2,4-D, the level of CPA required for optimum F.wt. plus embryo axis suppression (2.0 - 3.0 mg/l CPA), is higher than the optimum level of 2,4-D (1.0 - 2.0 mg/l). 2,4-D gives complete suppression of the embryo axis above 2.0 mg/l, and CPA above 3.0 mg/l. Hence the effect of CPA is that much weaker than 2,4-D.

2,4-D and cytokinins

Maris Ranger embryos were excised and placed on "M" containing the following:

"M" = control medium

"MC" = 5 mg/l CPA

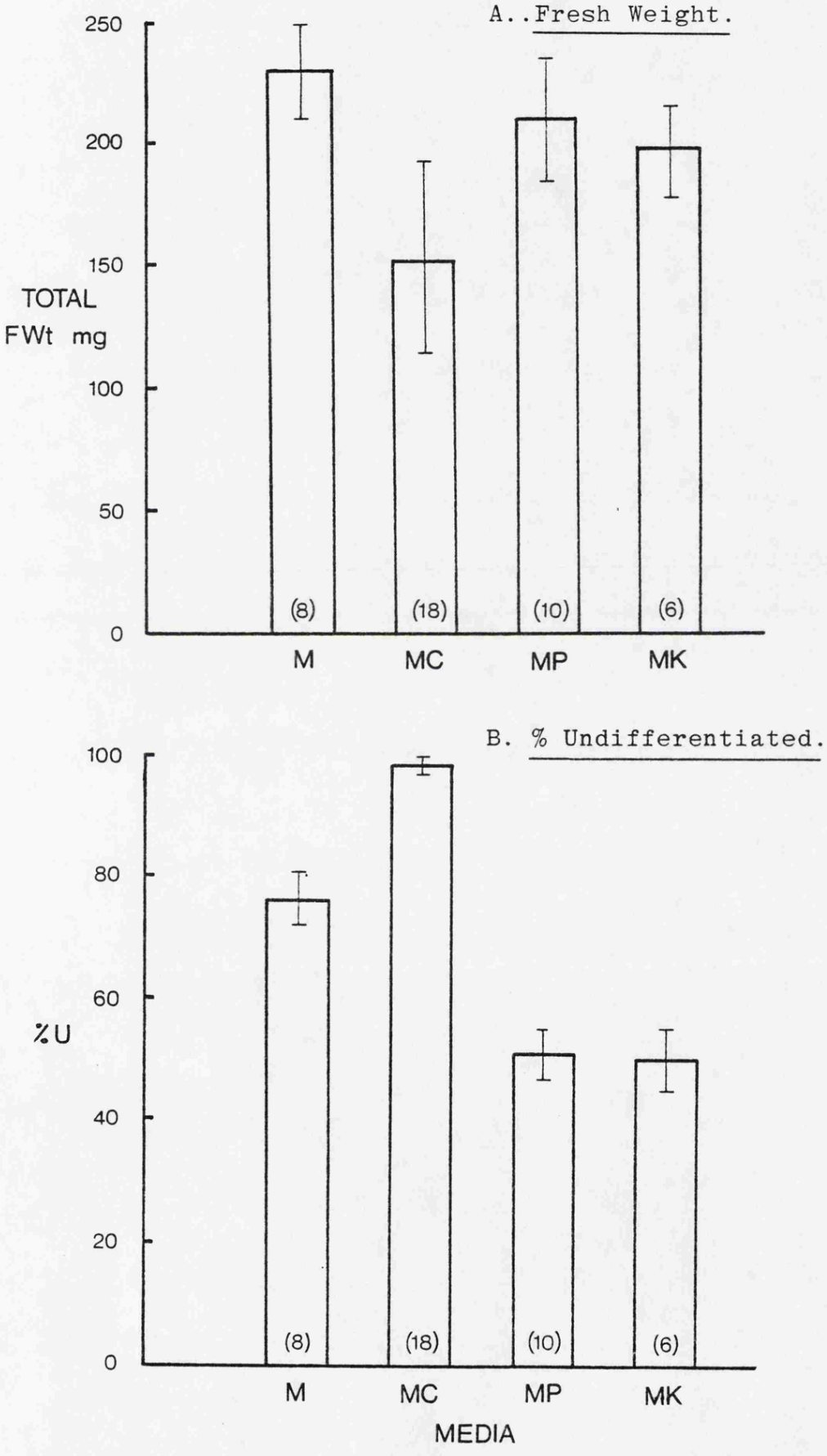
"MP" = 2.5 mg/l 6-(3-methyl-2-butenyl-amino)purine (2iP)

"MK" = 5 mg/l Kinetin

All media contained 100 mg/l casein hydrolysate and 1.0 mg/l 2,4-D. 50 - 60 replicates were used for each treatment, but unfortunately about half became contaminated. The cultures were placed in Controlled Environment Room II (low light). After 4 days in culture, the appearance of the explants was noted (Table 2.11), after removal of infected cultures. Media with Kinetin or 2iP both had low numbers of explants with callus formation alone, more than half had either shoot or root extension from the embryo, or both. Medium with CPA had no explants showing development of the embryo axis. We have seen in the previous experiment that 5.0 mg/l CPA gave nearly 100% undifferentiated callus on excised embryos which had been in culture for 10 weeks.

After 7 weeks, all explants showing no development of callus were discarded. Of the remaining cultures, those on "MC" still showed no development of the embryo axis, while on the other media more explants now showed shoot extension. Fig. 2.9 A,B shows the total fresh weights and percentage undifferentiated tissue after 7 weeks. Explants on media containing cytokinins ("MP", "MK") behaved very much alike, the %U being ca. 50% and the mean F.wt. being ca. 200 mg. Explants on "MC" had by far the highest %U (98.6%) but the F.wt. was lower than with 2,4-D alone ("M"). The addition of 5.0 mg/l CPA to 1.0 mg/l 2,4-D increased suppression of the embryo axis and lowered the yield of callus. Addition of 5 mg/l Kinetin or 2.5 mg/l 2iP to 1.0 mg/l 2,4-D, did not alter the fresh weight yield but favoured further development of the embryo axis.

Fig.2.9 : Total Fresh Weight and Amount of Undifferentiated Tissue of Excised Wheat Embryos, after 7 weeks on 4 Media. (number of replicates in brackets) $\bar{x} \pm SE$.



The experiment demonstrated how addition of cytokinins to 1.0 mg/l 2,4-D antagonised the dedifferentiating effect of the auxin, while addition of another auxin (CPA) reinforced this auxin action.

(iv) Stage of maturity of the embryo

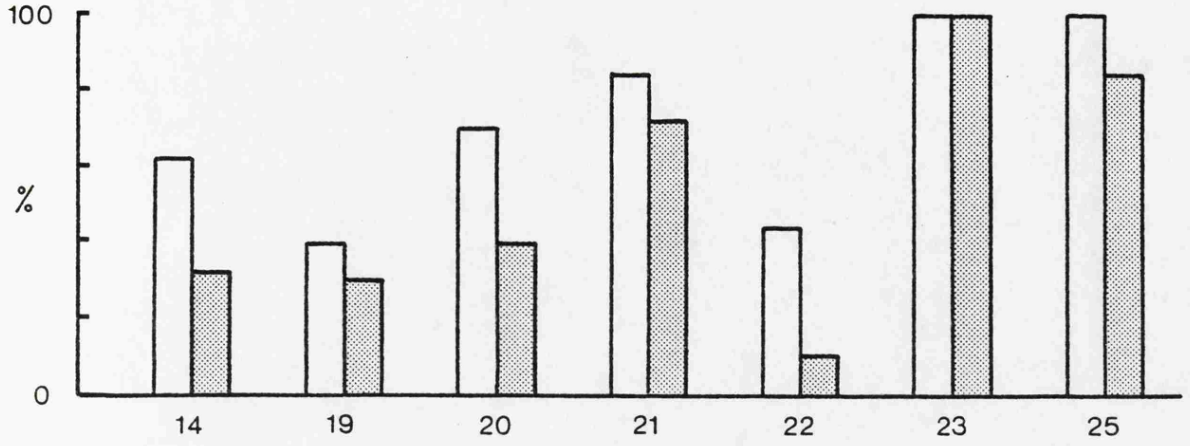
In the first experiment of this series, the initiation of callus from immature embryos was investigated, using medium, as developed by Green & Phillips (1975)*, with 2 mg/l 2,4-D (Medium "G"), or 2 mg/l CPA (Medium "GC"). Immature embryos of 6 wuk 4 Wheat were used, at several stages ranging from 14 - 25 days after anthesis. By Day 25, the grain had less fluid endosperm and the colour of the scutellum was changing from green to yellow (the last stages of ripening had begun). Because the number of plants available was limited, as much grain as possible was taken from each ear. There was a range of sizes within each ear and the specification of age in the ear as 'days of anthesis' is therefore an indication of the average age. The embryos were excised with the scutellum attached and placed in sterilin tubes containing media 'G' or 'GC'. The cultures were incubated in the Growth Cabinet under high light intensity and with a 16 hr light/8 hr dark cycle.

After 4 days in culture, the explants were scored for presence of callus (Fig. 2.10 A). The general trend was for the percentage of explants with callus to increase with age from anthesis (the behaviour at 22 days being, however, anomalous). Apart from Day 23, less callus production occurred after 4 days on the medium containing CPA (GC).

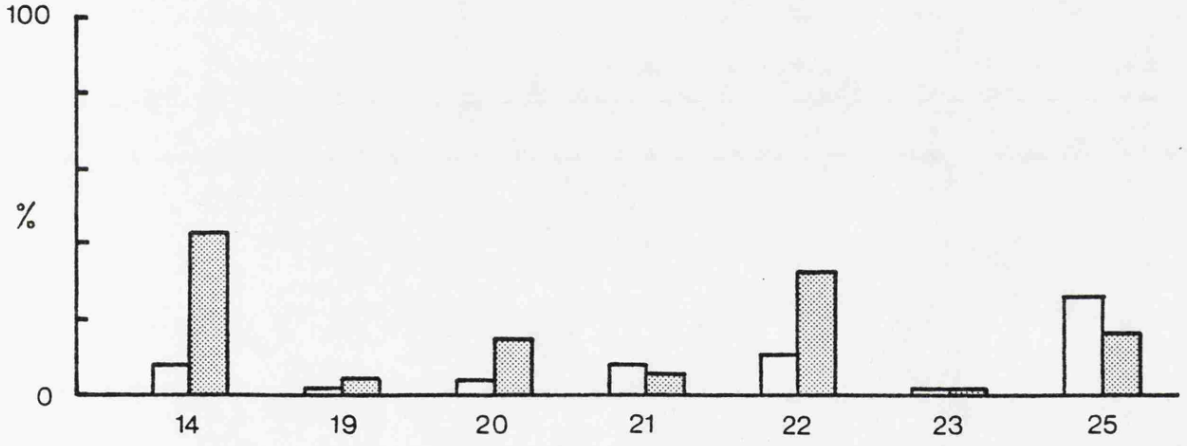
*The medium differed from that of Green & Phillips in containing 1.98 mg/l of asparagine instead of 1.98 g/l.

Fig. 2.10

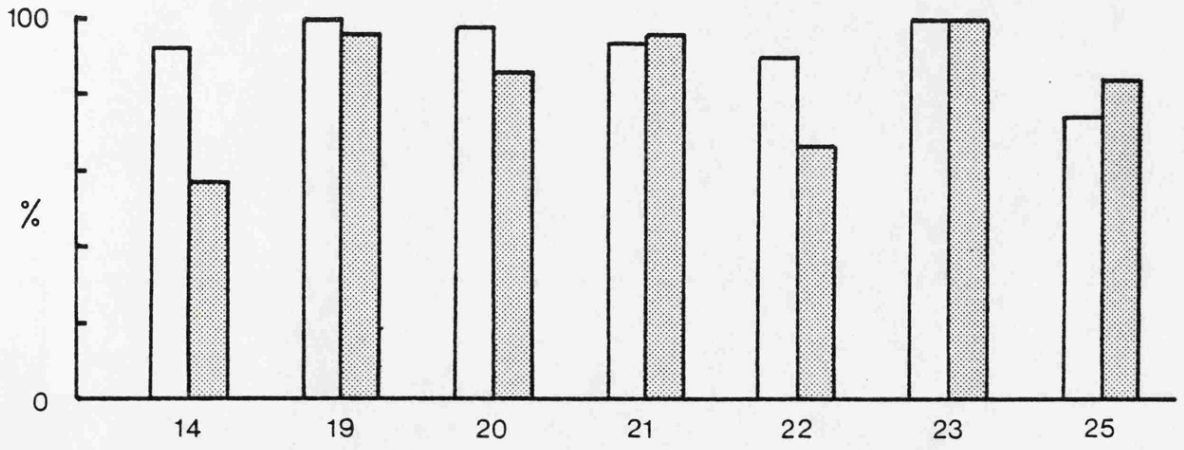
A. Precentage of Excised Immature Wheat Embryos having callus formation after 4 days incubation on 'G' □ or 'GC' ▨ Media.



B. Percentage of Excised Immature Embryos completely covered by Callus, after 5 weeks incubation on Media 'G' or 'GC'.



C. Percentage of Excised Immature Embryos having both Callus formation and Shoot extension, after 5 weeks on 'G' or 'GC'.



Age of Embryos when Excised (Days from Anthesis)

After 5 weeks in culture (Fig. 2.10 B,C) the majority of the explants of all ages showed development of the embryo shoot. Roots were usually present when shoot development occurred, and if the scutellum was visible (visibility depending upon orientation and development of the culture), it looked brown. The Day 14 and Day 22 explants on 'GC' medium showed the highest percentage of cultures with callus only; otherwise the cultures showed a high percentage having both callus and shoot extension from the embryo. Apart from the exceptions mentioned, the experiment does not show a clear difference in response to the 2 media over different stages of development of the embryo, nor any consistent response in relation to embryo age. Callus initiation did seem to occur more quickly in older embryos (Day 23, 25) but, although not quantified by measurement of weight, no difference in the amount of growth with time, as compared with callus more slow to form, was observed.

A second experiment was conducted, using 2 varieties of wheat 6 wuk 3 and 6 wuk 4 which were 9, 10, 12 and 14 days from anthesis. This time, as far as possible only the grain from the middle portion of the ear was used, but as Table 2.12 shows, there was still some variation in size within the ear. Green & Phillips' medium with the standard asparagine level (2 g/l) was used, supplemented with 3 levels of 2,4-D and plus or minus 5.0 mg/l 2iP, A 7th

Table 2.12

Variety	'Age'(days)	Embryo length* (mm)	Scutellum length* (mm)
6 wuk 3	10	0.99 ± 0.06	1.24 ± 0.10
	14	2.03 ± 0.06	2.62 ± 0.10
6 wuk 4	10	1.26 ± 0.11	1.48 ± 0.10
	14	1.88 ± 0.08	2.29 ± 0.09

* ($\bar{x} \pm$ S.E.)

medium with low asparagine (2 mg/l) and 2.0 mg/l 2,4-D, was used for comparison with the previous experiment (Table 2.13).

Table 2.13

Medium	Asparagine	2,4-D (mg/l)	2iP (mg/l)
1	1.98 g/l	0.1	-
2	"	0.1	5.0
3	"	0.5	5.0
4	"	0.5	-
5	"	2.0	-
6	"	2.0	5.0
7	1.98 mg/l	2.0	-

Cultures were incubated in the Growth Cabinet under high light intensity and on 18 hr light/6 hr dark cycle. Table 2.14 shows the characteristics of the cultures after 6 days incubation. The Day 9 embryos were very tiny, so it was difficult to be certain whether any callus had formed. Two of these explants had produced a very small shoot, showing that even at this very immature stage the embryos were capable of germination. Germination was also found on Medium 2 (0.1 mg/l, 2,4-D + 5.0 mg/l 2iP) for the Day 10 explants. Among the Day 12 and Day 14 explants, some germination without callus production ('root + shoot only' in Table 2.14) was found on levels of 2,4-D below 2.0 mg/l (Media 1,2,3,4). Where callus was present, it was of two types; transparent 'fluffy' callus, or more compact, yellowish callus (it is considered that this probably originated from the scutellum). The 'fluffy' callus tended to surround the embryo region.

After 26 days in culture (Table 2.15) more of the Day 9 explants tended to have callus formation, especially on Media 5 & 6 (high 2,4-D). For embryos of all ages, more cultures on all media showed development of the embryo axis. Further, more of the explants had germinated without producing callus (mainly on media containing the lower levels of 2,4-D).

Table 2.14 : Characteristics of Excised Immature Embryos after 6 days incubation on Media 1-7.

'Age' + Variety +Medium	Total Explants	% of Cultures with appearance indicated:				
		Callus only	Callus + Shoot	Callus + Root	Callus +Shoot +Root	Shoot or Root only
DAY-9						
6 WUK 3						
1	5	40.0	-	-	-	-
5	4	25.0	25.0	-	-	-
6	4	100.0	-	-	-	-
6 WUK 4						
2	10	40.0	10.0	-	-	-
3	9	33.3	-	-	-	-
5	4	50.0	-	-	-	-
6	5	60.0	-	-	-	-
DAY-10						
6 WUK 3						
1	21	61.9	4.8	-	-	-
2	20	-	5.0	-	-	75.0
3	20	35.0	35.0	-	-	-
4	19	57.9	10.5	-	-	-
5	21	76.2	4.8	-	-	-
6	15	26.7	26.7	-	-	-
6 WUK 4						
1	25	44.0	12.0	-	-	-
2	26	23.1	30.8	-	-	11.5
3	25	36.0	24.0	-	-	-
4	19	42.1	42.1	-	-	-
5	22	59.1	9.1	-	-	-
6	26	53.9	19.2	-	-	-

(contd.)

Table 2.14 (cont.)

'Age' + Variety +Medium	Total Explants	% of Cultures with appearance indicated:				
		Callus only	Callus + Shoot	Callus + Root	Callus +Shoot +Root	Shoot or Root only
DAY-12						
6 WUK 3						
1	20	65.0	-	-	-	35.0
3	10	20.0	30.0	-	-	30.0
4	19	42.1	47.4	-	-	-
5	21	66.7	14.3	-	-	-
6	20	35.0	40.0	-	-	-
6 WUK 4						
1	21	42.9	9.5	-	-	9.5
2	21	4.8	23.8	-	4.8	52.4
3	21	33.3	42.9	-	-	14.3
4	20	55.0	20.0	-	-	-
5	20	65.0	10.0	-	-	-
6	20	50.0	25.0	-	-	-
DAY-14						
6 WUK 3						
1	20	55.0	10.0	-	30.0	-
4	20	70.0	15.0	-	15.0	-
5	20	85.0	10.0	-	-	-
6	16	37.5	50.0	-	-	-
6 WUK 4						
1	20	-	15.0	-	10.0	45.0
2	20	-	10,0	-	5.0	85.0
3	20	5.0	45.0	-	-	45.0
4	19	47.4	15.8	5.3	-	-
5	20	45.0	45.0	-	-	-
6	20	20.0	75.0	0	5.0	-
7	25	40.0	56.0	-	-	-

(- = 0%)

Table 2.15 : Characteristics of Excised Immature Embryos after 26 days incubation on Media 1-7.

'Age' + Variety +Medium	Total Explants	% of Cultures with appearance indicated:				
		Callus only	Callus + Shoot	Callus + Root	Callus +Shoot +Root	Shoot or Root only
DAY-9						
6 WUK 3						
1	5	20.0	60.0	-	-	20.0
5	4	100.0	-	-	-	-
6	4	100.0	-	-	-	-
6 WUK 4						
2	10	60.0	30.0	-	-	-
3	9	77.8	11.1	-	-	-
5	4	100.0	-	-	-	-
6	5	100.0	-	-	-	-
DAY-10						
6 WUK 3						
1	21	61.9	14.3	-	9.5	14.3
2	20	15.0	5.0	-	5.0	80.0
3	20	30.0	30.0	-	5.0	25.0
4	19	31.6	52.6	-	15.8	-
5	21	71.4	23.8	-	4.8	-
6	15	60.0	40.0	-	-	-
6 WUK 4						
1	25	36.0	-	4.0	20.0	36.0
2	26	15.4	34.6	-	3.8	42.3
3	25	48.0	36.0	-	8.0	8.0
4	19	10.5	31.6	5.3	52.6	-
5	22	59.1	36.4	-	4.5	-
6	26	53.8	30.5	7.7	3.8	-

(contd.)

Table 2.15 (cont.)

'Age' + Variety +Medium	Total Explants	% of Cultures with appearance indicated:				
		Callus only	Callus + Shoot	Callus + Root	Callus +Shoot +Root	Shoot or Root only
DAY-12						
6 WUK 3						
1	20	10.0	5.0	-	-	85.0
3	10	-	60.0	-	30.0	10.0
4	19	15.8	42.1	-	36.8	5.3
5	20	35.0	60.0	-	5.0	-
6	20	30.0	55.0	-	10.0	5.0
6 WUK 4						
1	21	9.5	-	-	19.0	71.4
2	20	5.0	15.0	-	-	80.0
3	20	5.0	25.0	-	25.0	45.0
4	20	20.0	10.0	5.0	50.0	15.0
5	20	45.0	50.0	-	5.0	-
6	20	40.0	45.0	-	-	5.0
DAY-14						
6 WUK 3						
1	18	5.6	-	-	22.2	72.2
4	20	10.0	35.0	-	35.0	20.0
5	20	25.0	45.0	-	30.0	-
6	16	12.5	62.5	-	18.8	6.8
6 WUK 4						
1	19	5.3	-	-	15.8	78.9
2	20	-	5.0	5.0	-	90.0
3	18	-	16.7	-	27.8	55.8
4	19	-	21.1	-	68.4	10.6
5	20	25.0	65.0	-	5.0	5.0
6	20	5.0	80.0	-	15.0	-
7	25	8.0	68.0	-	20.0	-

(- = 0%)

The experiment shows that embryos older than 10 days from anthesis tend to form callus more quickly than younger embryos. It was noted that callus which did form on Day 9 embryos seemed to grow more slowly than that on older embryos, and did not reach a similar size. 2.0 mg/l 2,4-D seems to be better than 0.1 or 0.5 mg/l 2,4-D for callus initiation, since these lower levels allowed many of the embryos to germinate without forming callus. Addition of 5.0 mg/l 2iP (media 2,3 & 6) to the medium does not appear to induce a different response with regard to callus initiation, although more browning of tissue was noticed on these media.

No quantitative data was taken during these experiments involving the use of immature embryos, since the cultures were intended for studies on morphogenesis and regeneration of plants, which are described in a later section (P. 80).

II. Initiation on other parts of the plant

Although the use of stored mature grain had proved a convenient and reliable source of wheat callus tissue, it was felt necessary to investigate callus formation on other parts of the plant. The experiments which follow in this section, describe the initiation of callus on explants from mature wheat plants (both from normal hexaploid plants, and from "haploid" plants having half this chromosome number (n = 7)). A qualitative study was made of which parts of the plant stem were most active in producing callus, and also of the effect of auxins and cytokinins on callus initiation.

A brief investigation was also made into callus formation on explants from young seedlings.

(i) Parts of the mature plant stem

Initiation on basic medium "M"

"Haploid" plants of the hexaploid American dwarf wheat varieties 6 wuk 3 and 6 wuk 4 were used. The "haploid" plants had been generated (via both callus and embryoid formation) from plated anthers, by Dr. L. Ayre (Lord Rank Research Centre), and were used 1 - 2 months after potting when they were just coming into ear.

Stem segments were surface-sterilized and cut into 2 - 6 mm lengths, some including the node, others consisting of only the internode. The "M" medium contained 100 mg/l casein hydrolysate and 1 mg/l 2,4-D. Explants were pushed partway into the semi-solid medium and were orientated in a random manner with regard to the 'top' and 'bottom' of the segment. They were incubated in the low light of Controlled Environment Room II.

After 2 weeks, small pieces of callus had formed on most node explants, and at the cut ends of some of the internode explants. Plate II shows the appearance of the callus after 2 weeks, and also a month after, when the roots have started to form. After 2 weeks, some node explants had callus both surrounding the node and also as a mass of tissue at the cut end (Plate II,A). In other explants, callus was formed as a ring of tissue at the cut end of the internode (Plate II,C); sometimes, only a very small part of the explant was actively producing callus (Plate II,B). Size of the explant did not appear to affect the amount of callus produced. Callus could be either white and 'fluffy' (Plate II,A) or pale green (Plate II,E). Photographs on Plate II, F - M, compare the appearance of 4 explants after 2 weeks and 4 weeks. F shows a 2 week explant having very small green nodules of callus,

Plate II

Callus formation from explants of the mature plant stem of two varieties of wheat : 6 WUK 3 and 6 WUK 4.

A - E : 6 WUK 3; callus formation after 2 weeks incubation.

A. Callus formation at the node (n) and, in greater abundance, at the cut end.

B. Only a very small piece of callus (c) has formed at the cut end of the internode segment.

C. A ring of white callus formed at the cut end of the internode.

D. Callus proliferation over the whole surface of the explant

F - M : callus formation after both 2 and 4 weeks incubation.

F, G, L & M = 6 WUK 4; others are explants from 6 WUK 3.

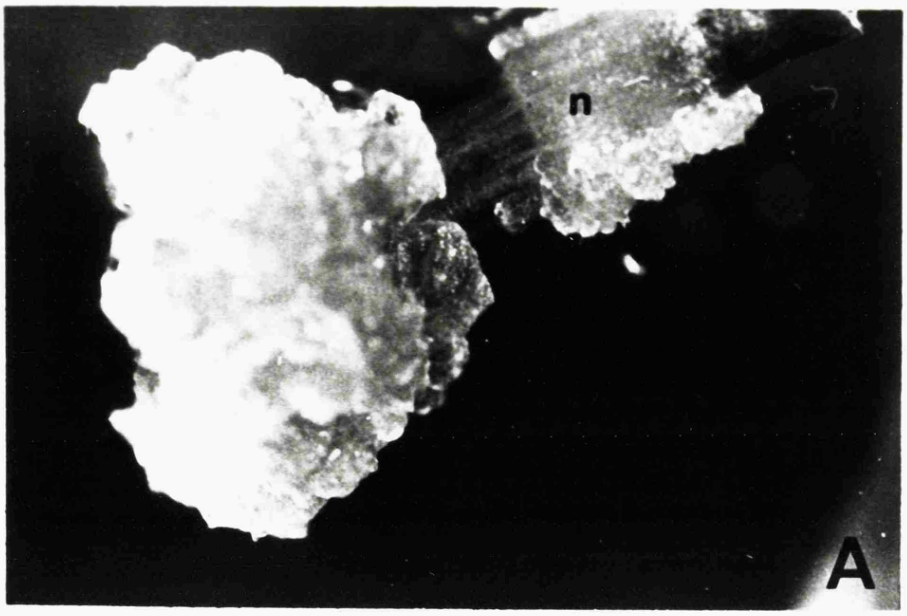
F. Small nodules of green callus (g) have formed at one end of the internode explant after 2 weeks in culture; G. the same explant after 4 weeks incubation now has long roots (r), and little callus development.

H. After 2 weeks in culture, white fluffy callus has formed at one end of the internode segment. I. The same explant after 4 weeks is completely covered with callus and roots (r) have been produced.

J. A small area of the node region has started to form callus, after 2 weeks in culture. K. After a further 2 weeks incubation, the callus has spread over the entire node region.

L. White fluffy callus at one end of the internode segment after 2 weeks incubation. M. After 4 weeks, the white callus has hardly increased in size but has formed roots (r), while green callus (g) is starting to proliferate at the other end of the explant.

PLATE II



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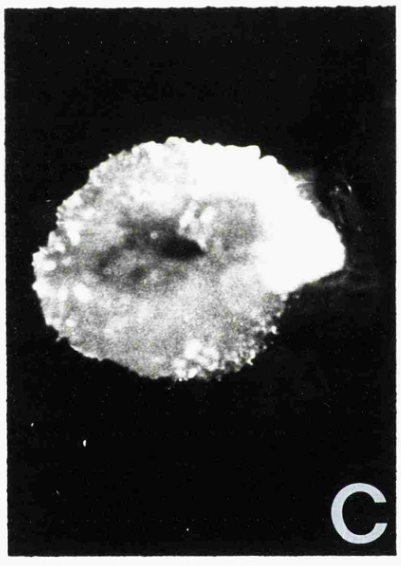

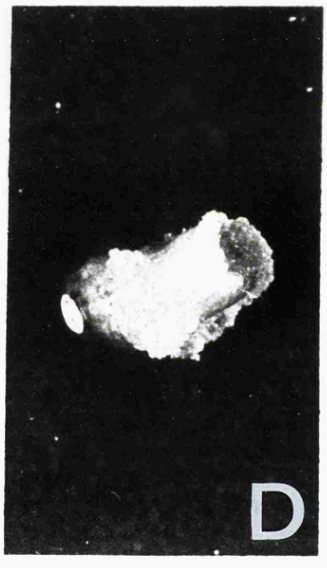

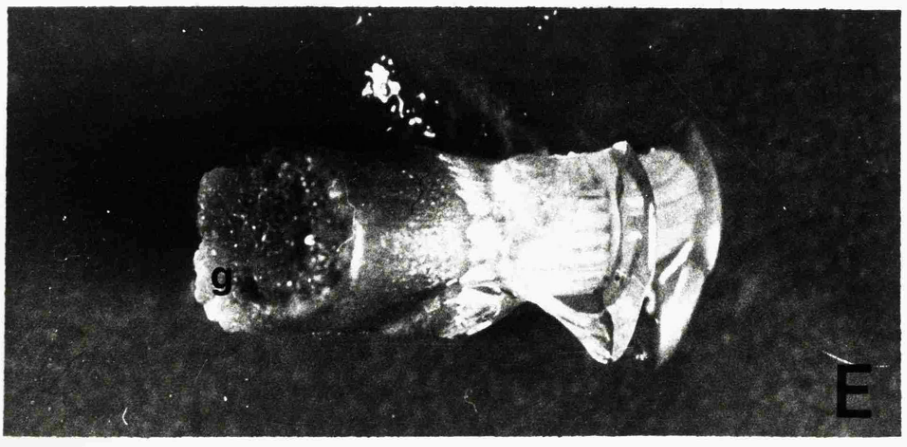

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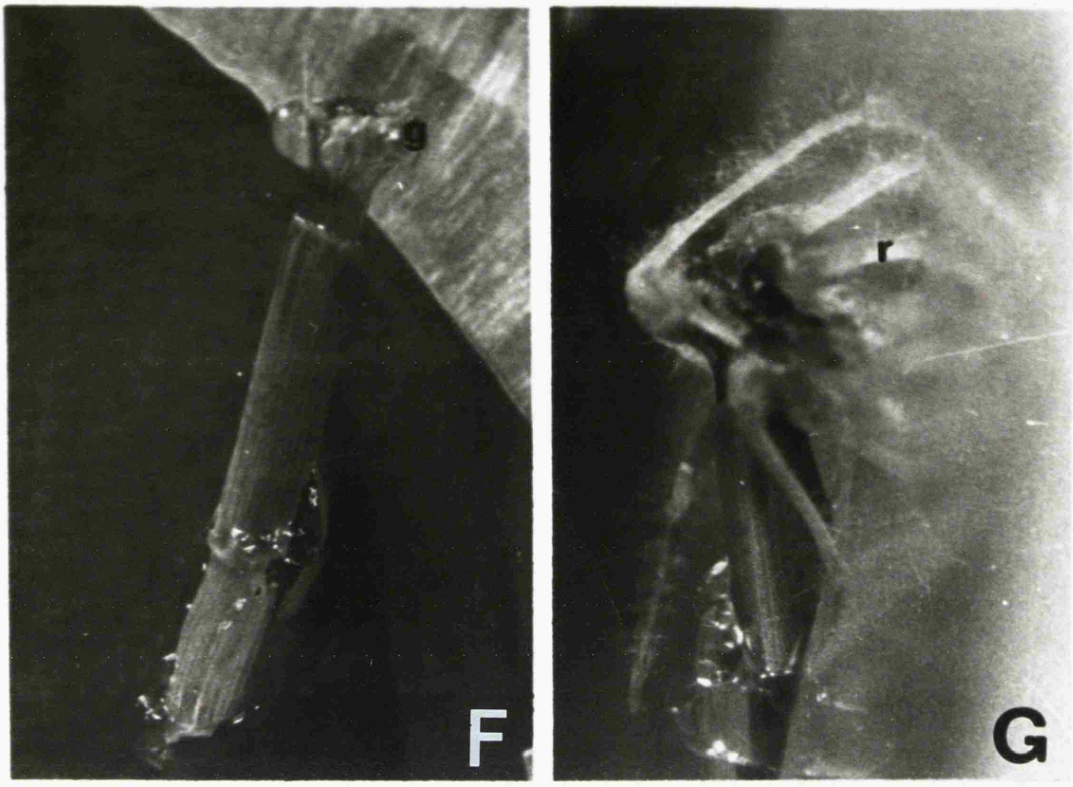
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F
G
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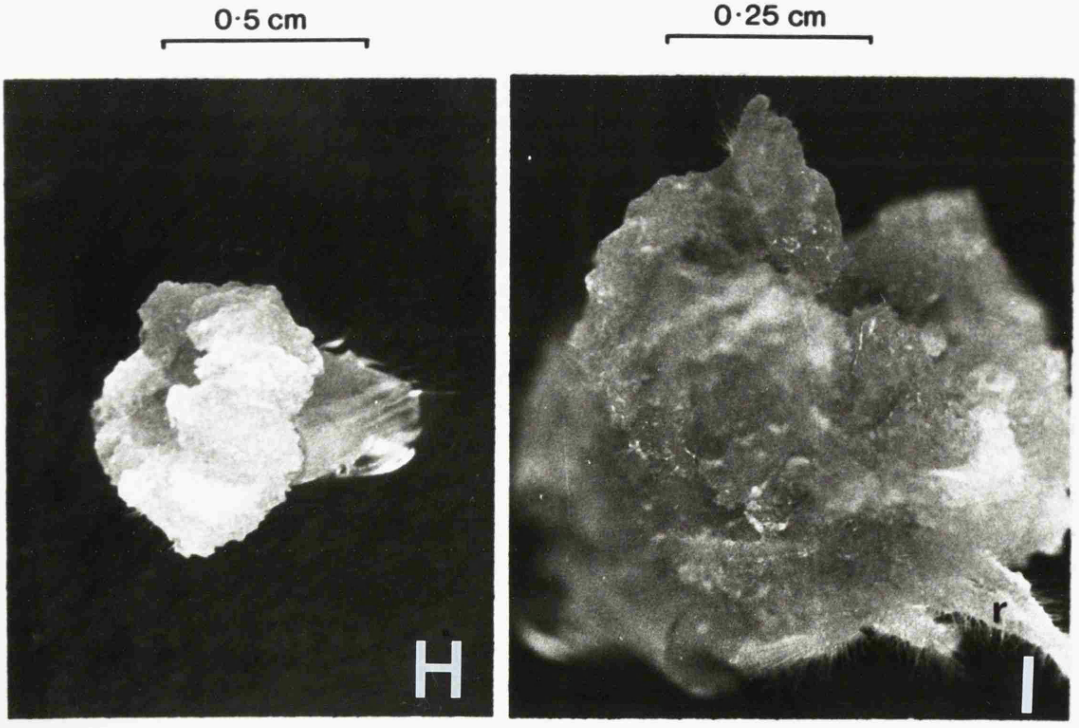
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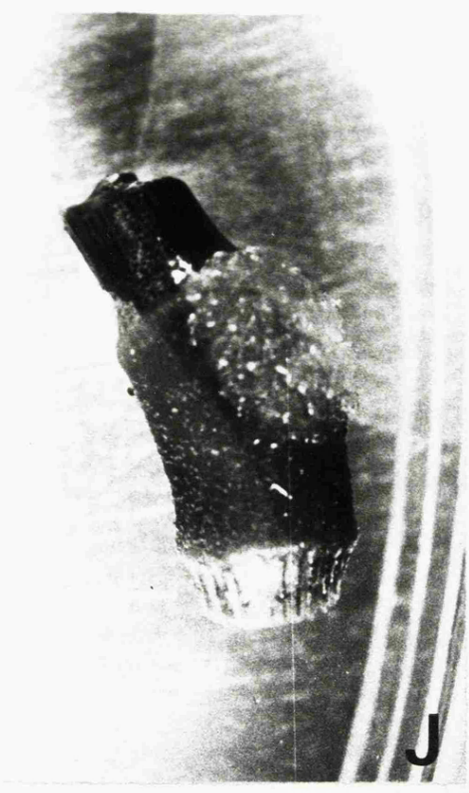

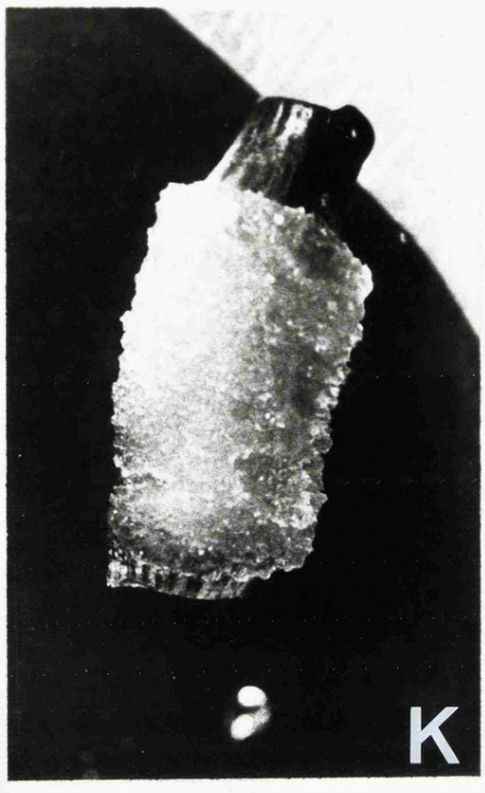

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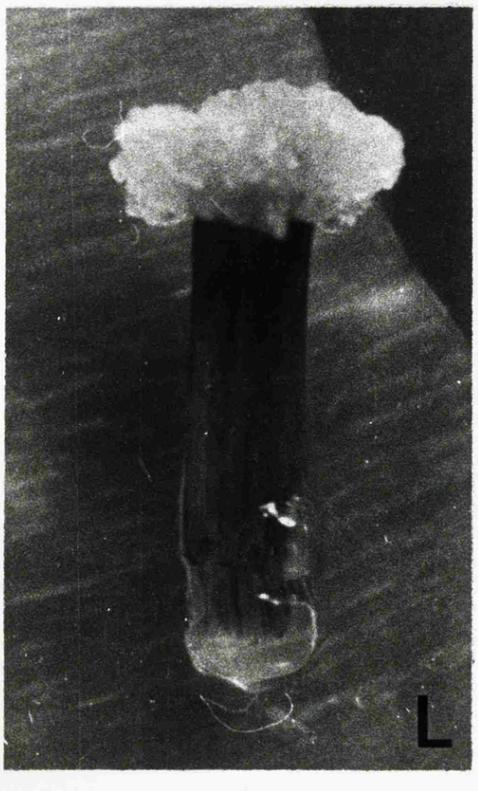
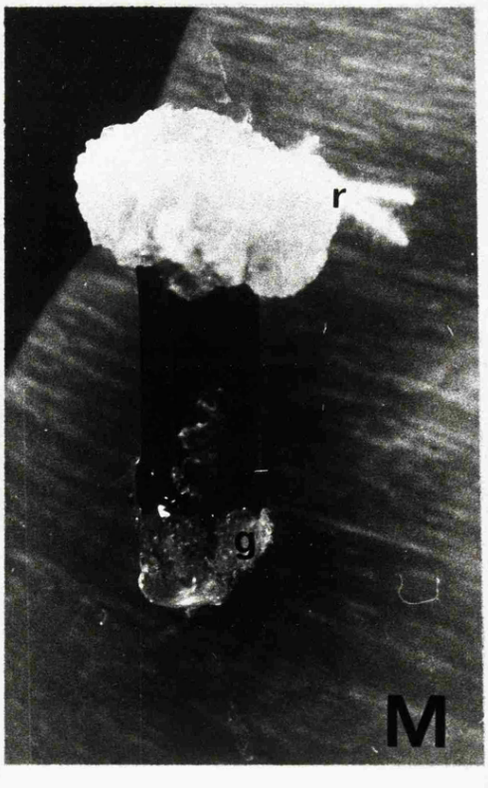




which, after 4 weeks (G), developed many roots from the callus region and showed no further proliferation of the callus. In H,I, callus has completely overgrown the explant by 4 weeks, and several roots have formed. In J,K, callus which had begun as a small patch of tissue on one side of the node, had spread all over the node region by 4 weeks. L and M show little increase in the size of the callus, but after 4 weeks, pale green callus has formed at the other end of the explant, and roots have developed.

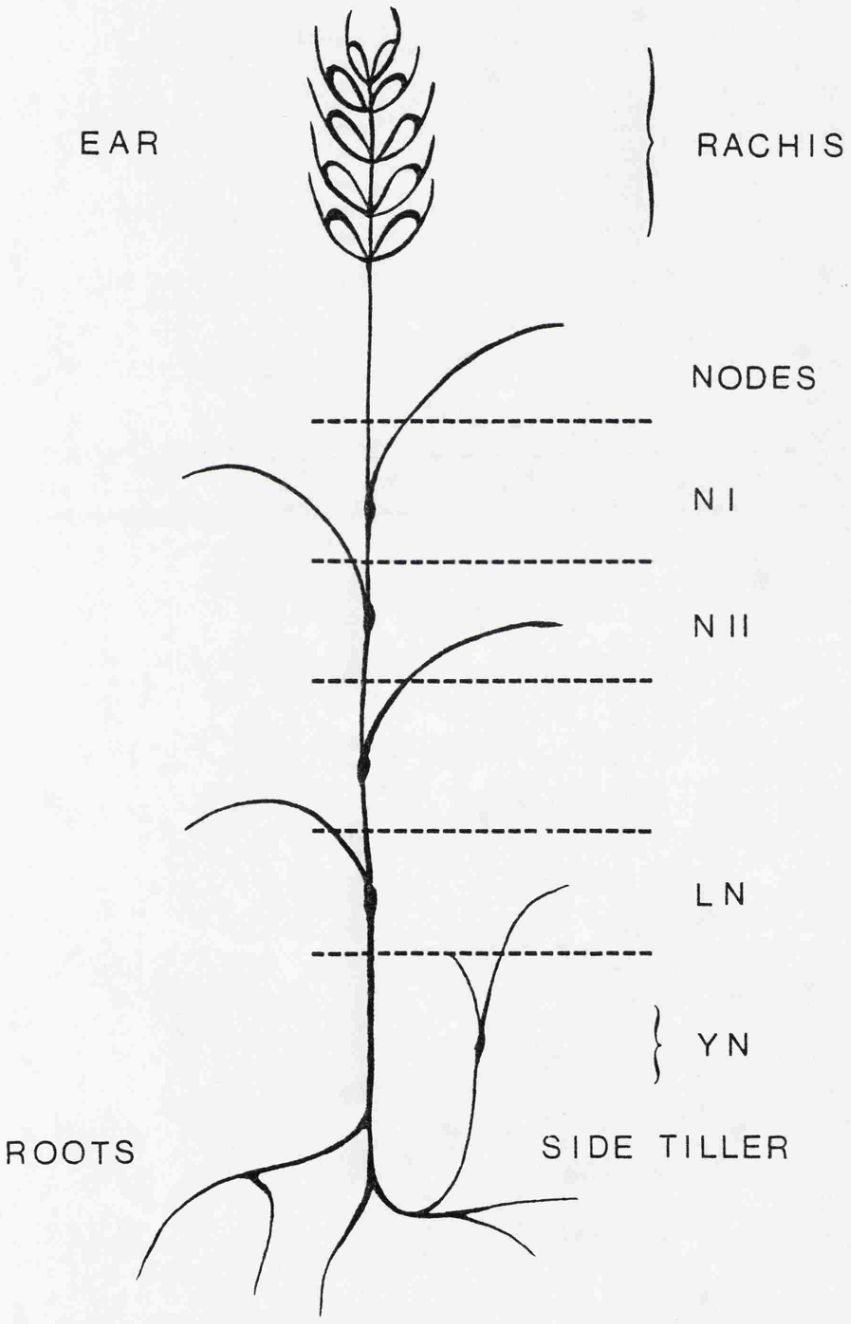
No difference was apparent in callus initiation from the two varieties of wheat used. The amount of callus produced was generally small, and roots soon began to form; the callus needed to be subcultured 2 - 3 weeks after initiation.

Initiation on different levels of 2,4-D

The previous experiment showed that callus formed quite readily on stem explants; the present experiment investigated, in more detail, the factors affecting this callus initiation, but using the normal hexaploid plants instead of the "haploids" used in the previous experiment.

5 week old plants of 6 wuk 4 were used. Sections of stem were cut from each of the positions shown in Fig. 2.11, to compare callus production of nodes and internodes from different points of origin on the plant. Rachis explants were excised after removal of the spikelets from the ear, and positioned in the agar with the basal end either up or down, to see the effect of orientation of the explant. All other explants were orientated in the same direction as they had been on the plant.

Fig.2.11. : Diagram of Wheat plant showing position of the Explants cultured in vitro.



Explants were placed on "M" containing 100 mg/l casein hydrolysate and one of a range of 2,4-D levels (1.0, 5.0, 10.0, 15.0, and 20.0 mg/l).

It had been observed in the previous experiment, that callus formed on the cut surface of explants, so some segments on each medium were split longitudinally, thus providing a large amount of wounded tissue. The rachis segments were left whole.

Cultures were incubated in the low light of Controlled Environment Room II. After 3 weeks, the explants were scored for presence of callus. Table 2.16a (below) shows the level of 2,4-D on

Table 2.16a: Presence of callus after 3 weeks on "M" plus increasing levels of 2,4-D

a) Media with "best" callus initiation

2,4-D (mg/l) \ Explant	R	NI	NII	LN	YN
1.0	-	-	-	-	-
5.0	-	-	+	-	-
10.0	-	+	+	+	-
15.0	-	+	-	-	+
20.0	+	+	-	-	-

(+ = "best" callus, - = less callus)

b) Presence of callus on nodes and internodes

	NI	NII	LN	YN
Upper internode - s	+	-	-	+
- w	+	-	-	+
Node - s	+	+	+	+
- w	+	+	+	+
Lower internode - s	-	-	-	-
- w	-	-	-	-

(s = split; w = whole; - = no callus detected)

which the most active ("best") callus proliferation was observed, (as observed by visual comparison of size) for both the split and the whole explants. Some degree of callus initiation occurred on all levels of 2,4-D. In general, the highest level of 2,4-D (10.0 to 20.0 mg/l) produced the "best" callus on the first nodes below the ear (NI) and on the very young nodes from side tillers (YN). 20.0 mg/l 2,4-D appeared to produce the "best" callus on rachis (R) explants, although very little callus formed on any of the media from the rachis explants. For the two older nodes (NII, LN), 5.0 to 10.0 mg/l 2,4-D produced the "best" callus. In general, the amount of callus produced from explants on the "best" media in one month seemed to be greater (by visual comparison) than that produced by excised embryos over the same period of time.

Table 2.16b shows that while node segments from any position on the plant formed callus, the segments of internode from below the node never produced callus. Also, only upper internode segments from the first node below the ear (NI) or young side tillers (YN) produced callus. Splitting the explants did not make any difference to whether the segment was capable of producing callus, but it did make a difference to the amount of callus formed. Splitting the segment usually increased callus production; although again this was not quantified, the effect was especially marked on NI and YN nodes.

To summarise the findings of this experiment:

a. Explants from the rachis (R) formed very little callus; segments orientated as they had been on the plant did better than those which were upside-down, suggesting the influence of polarity. 20.0 mg/l 2,4-D produced the "best" callus.

b. Splitting the explant increased callus production.

c. The position on the plant from which the explant was taken proved important: the youngest nodes (NI, YN) gave the most callus. Upper internode segments taken from these positions were the only internode segments to produce callus.

d. The optimum level of 2,4-D varied according to the position on the plant from which the explant originated.

Plates III and IV illustrate the variety in appearance of the callus tissue, in texture, colour and site of formation. At the node, callus tended to form from the outer layers of tissue and eventually covered the node region (Plate III, B). On internode segments, it was usually the inner layers of leaf material which callused (Plate III, C,D). The upper internode segments from the young nodes of the side tillers (YN) contained an immature floral spike, and when these segments were split, this inflorescence tended to produce nodules of callus (Plate IV, D,E). Splitting the segment allowed callus formation along the length of the explant (Plate IV, A, B,C). Pieces of callus were transferred to fresh medium after a month, after removing any explant material which had not callused.

The effect of growth substances on initiation

In this experiment, an attempt was made to quantify the amount of callus produced by explants on medium with different growth substances.

Hexaploid Maris Ranger plants were used which were 2 months from seed. Not all the plants used were at the same stage of development; some of the ears being enclosed in the flag leaf and others fully emerged. However, this difference was not observed to

Plate III

Callus formation from explants taken from different parts of the mature plant stem of 6 WUK 4 wheat, after incubation for 3 weeks (see text for details of medium).

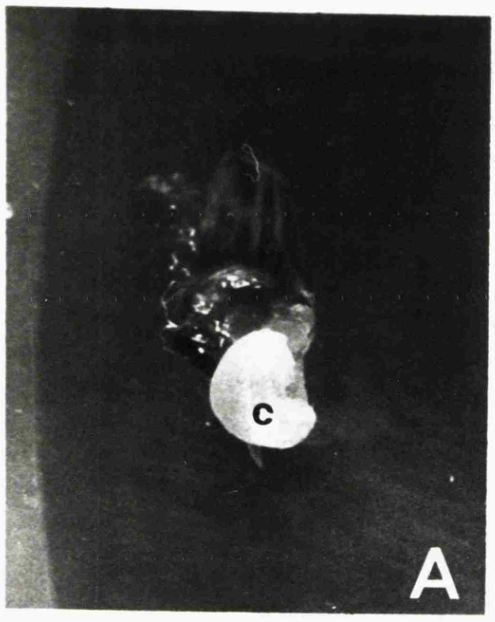
A. Rachis explant : a small piece of white, smooth callus (c) has formed from the cut end.

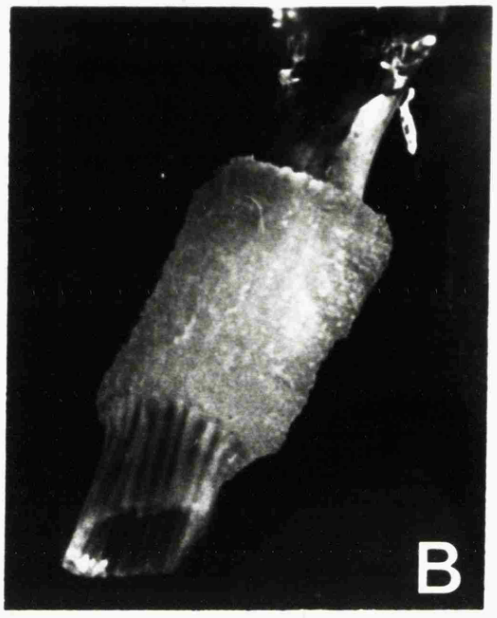
B. Node segment : the outer cells of the node region have proliferated to form callus, while the part of the stem at either side of the node remains unchanged.

C. Internode segment : White callus has proliferated from the inner leaf material of the internode causing the tissues to splay out at one end.

D. Split internode segment : the inner tissue exposed by splitting the explant has become covered with callus.

PLATE III





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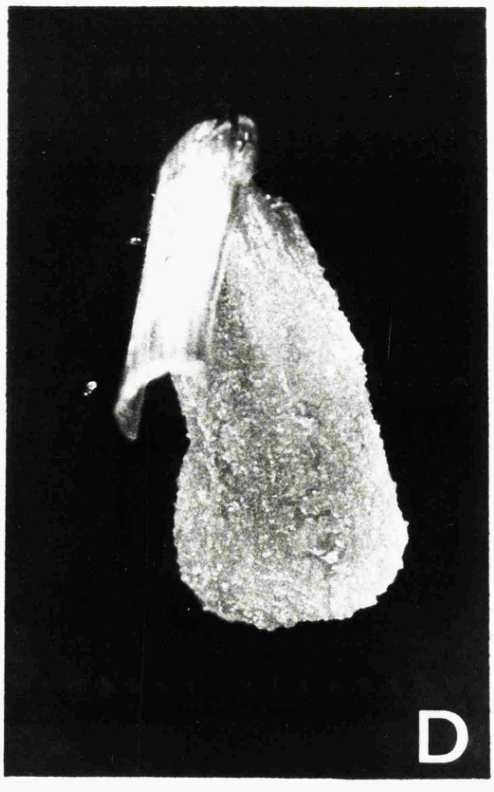


Plate IV

Callus formation from explants of the mature stem of 6 WUK 4 wheat, incubated on medium containing different levels of 2,4-D, for 3 weeks.

A. Internode segment taken from just above the young node of a side-tiller (YN) shows callus formation at both ends.

(2 mg/l 2,4-D)

B. The inner tissues of the split internode segment, taken from just above the last node on the stem before the roots (LN), have proliferated to callus (c).

(5 mg/l 2,4-D)

C. The split node (the second node below the ear, NII) has formed callus at the node region (n) only.

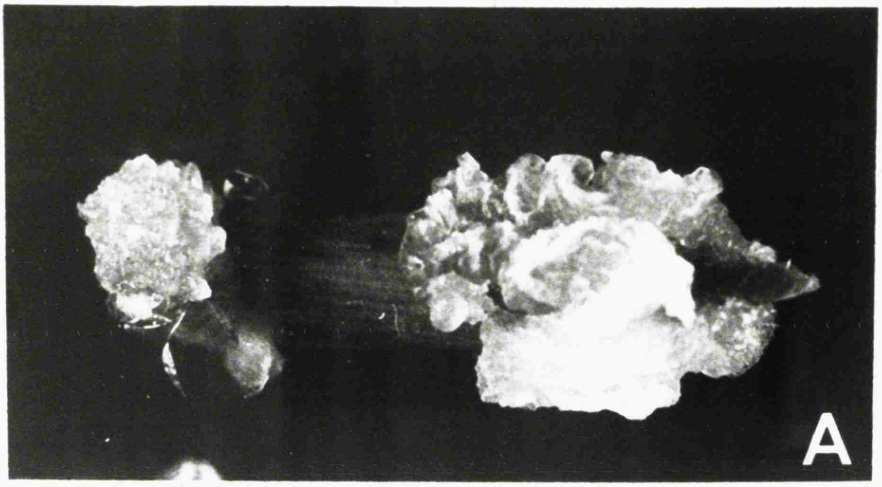
(5 mg/l 2,4-D)

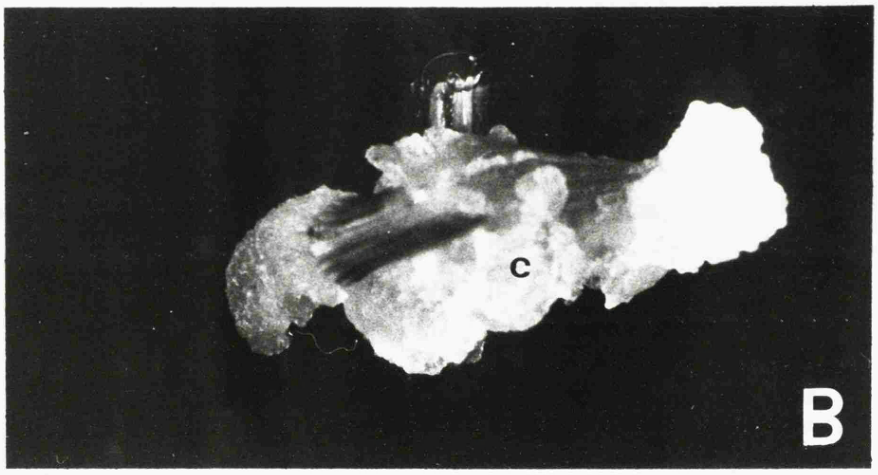
D. Nodules of callus (c) have formed from the young inflorescence (f) exposed by splitting the internode segment, taken from a side-tiller (YN).

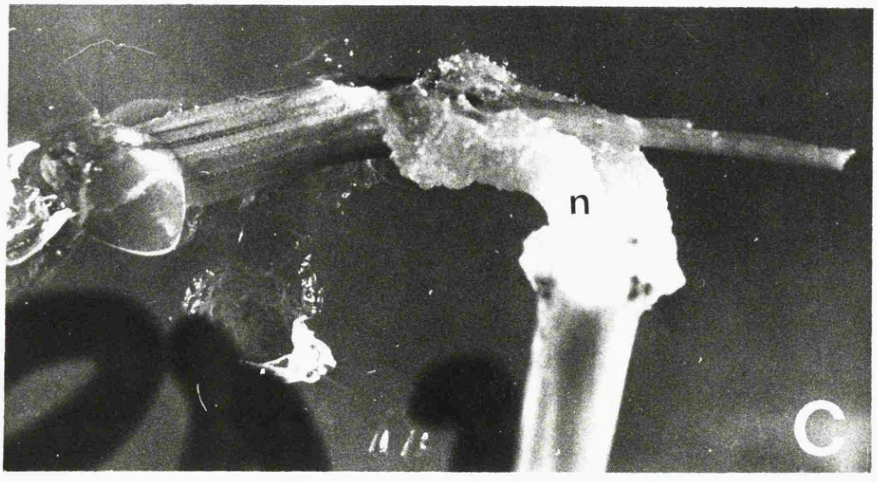
(2 mg/l 2,4-D)

E. Many nodules of callus have formed along the immature inflorescence (f) within the split internode segment taken from a young side-tiller (YN); root formation has also occurred (r). (1 mg/l 2,4-D)

PLATE IV







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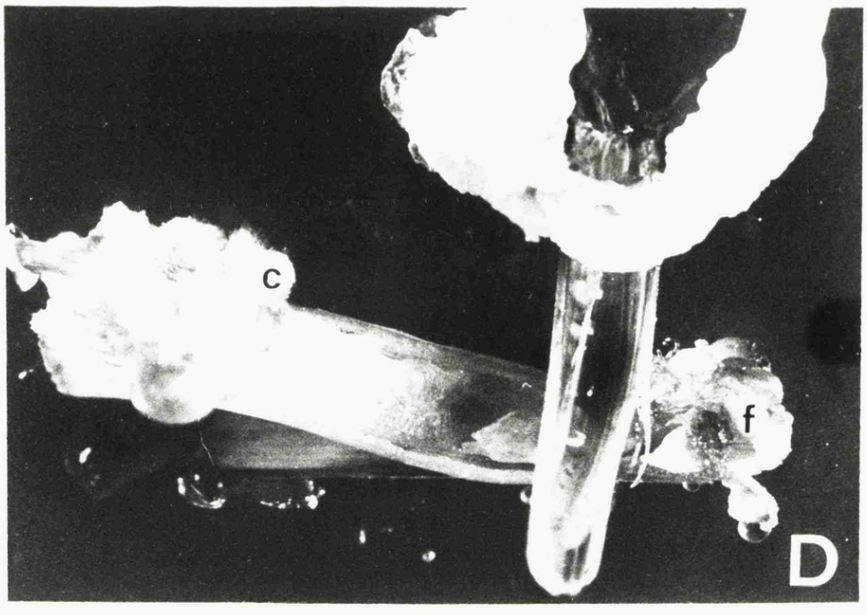

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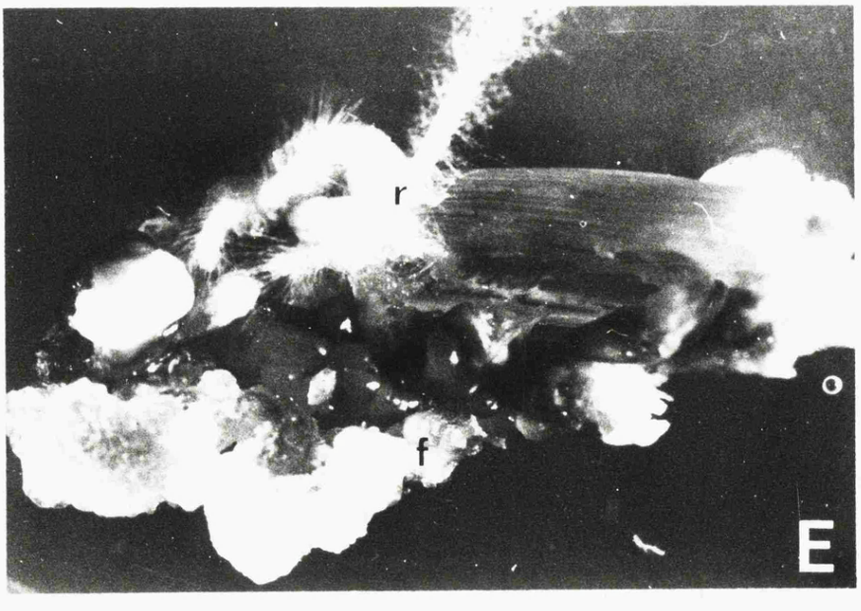


cause a difference in response.

Only that segment of stem including the first node below the ear was used. 3 types of explant were taken: the node itself, and segments of internode from immediately above and below the node. Each of these was split longitudinally, so that in all 6 explants were placed in each petri dish of medium. Ten replicate petri dishes were used for each medium, consisting of "M" containing different auxins and cytokinins (see Table 2.17). The cultures were incubated in the low light of Controlled Environment Room II.

After one month, the amount of callus produced was measured by taking the fresh weight of callus produced per petri dish. Of the 10 replicate plates per treatment, several had become contaminated and therefore their contents were not weighed. Table 2.17 (below) shows the total fresh weight of callus obtained from each medium, and the mean weight of callus per petri dish.

Table 2.17: Fresh weight (g) of callus after one month

Growth substance in medium*	No. replicate plates per medium	Total F. wt. of callus per medium	Mean F. wt. of callus per plate
2,4-D only	6	6.983	1.16 ± 0.19
2iP	7	3.795	0.54 ± 0.08
Triacanthine	7	4.915	0.75 ± 0.11
CPA	8	6.202	0.76 ± 0.08
Kinetin	5	2.068	0.41 ± 0.10
**1-NAA	8	6.654	0.83 ± 0.12

*All media contained 1.0 mg/l 2,4-D and 5.0 mg/l of other auxins or cytokinins
**1-Naphthaleneacetic acid

While most of the segments from the node and upper internode produced callus, none of those from the internode below the node formed callus. Therefore, in Table 2.17, the mean F.wt. of callus per

plate represents the callus produced from a maximum of 4 explants. The amount of callus produced on each explant varied widely, so that the total weight of callus tissue produced per petri dish was thought to give a better indication of how the media were affecting the yield of callus. Also, only the callus tissue was weighed, so that no account was taken of any roots present, or of the weight of explant material which had not callused.

Explants on medium containing Kinetin produced the least callus, closely followed by those on medium containing 2iP. Many of the explants showed browning, especially those on media containing the cytokinins, and again, Kinetin seemed to cause most browning. The medium containing only 1.0 mg/l 2,4-D produced the highest yield of callus, and also had the least browning. This suggests that the concentration of the other auxins and cytokinins tested was too high, since they caused a decrease in callus production and toxic symptoms (browning).

The appearance of the pieces of callus formed was similar to those already described in the two previous experiments.

(ii) Initiation of callus from parts of the seedling

An experiment was carried out to determine whether callus could be initiated from all parts of the seedling. Seeds were germinated on "M" lacking 2,4-D and casein, for 2 weeks, in which time the shoot and roots developed normally. Sections (5 - 10 mm long) were cut from the plumule, root (including the tip region) and the 'hypocotyl' (the part remaining after removal of the plumule and roots) as shown in Fig. 2.12. The explants were placed on "M" containing 1.0 mg/l 2,4-D and 5.0 mg/l of either CPA, Kinetin or Triacanthine.

Fig.2.12 : Diagram of a young Wheat seedling to show position of Explants cultured in vitro.

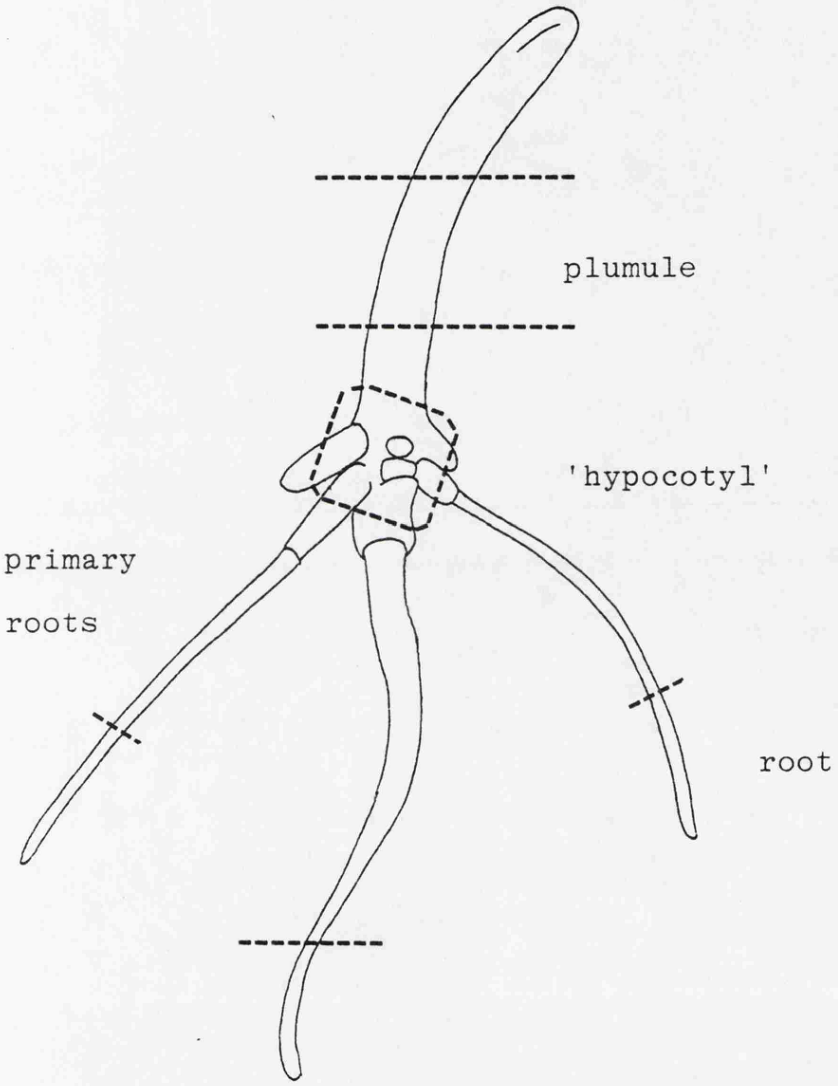


Plate V

Callus formation after 4 weeks, from different parts of a 2 week-old seedling of Maris Ranger wheat.

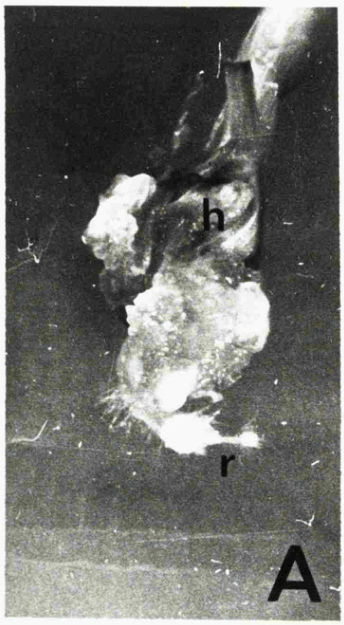
A. Explant from the hypocotyl (h) region : callus has formed at the cut surface, and a root (r) has developed. ("M" + 1mg/l 2,4-D + 5mg/l CPA)

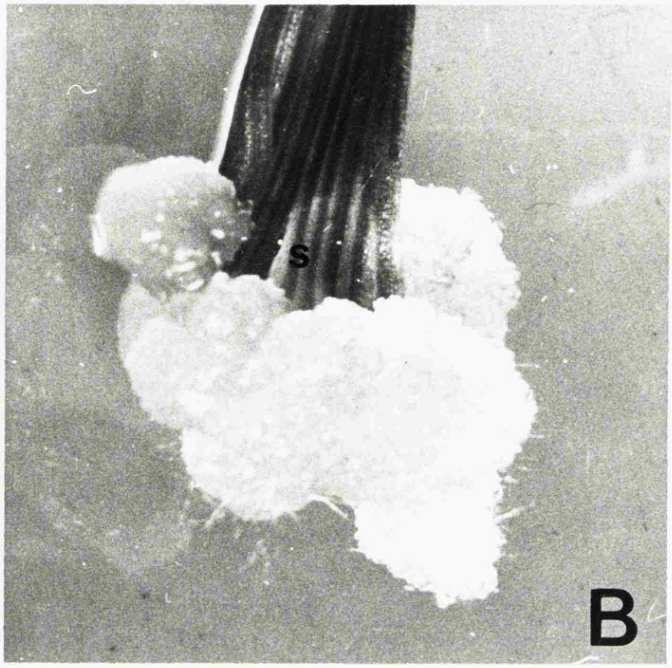
B. Callus formation from the cut end of the plumule and enclosed shoot (s).
("M" + 1mg/l 2,4-D + 5mg/l Triacanthine)

C. Excised root entirely covered by callus, and a 'new' root (r) has developed.
("M" + 1mg/l 2,4-D + 5mg/l Kinetin)

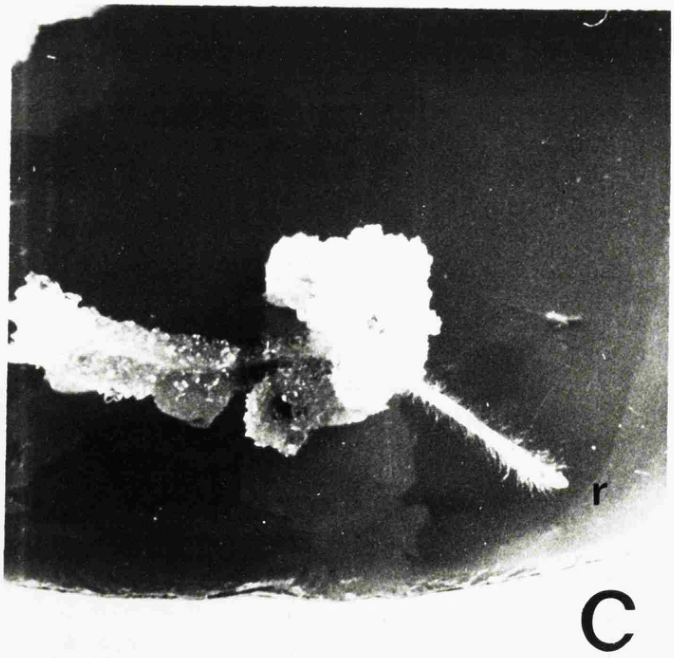
D. Excised root having a different form of callus from C above, being nodules of smooth callus at intervals along the explant.
("M" + 1mg/l 2,4-D + 5mg/l CPA)

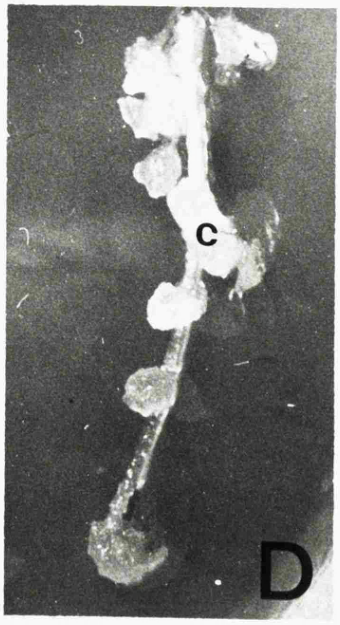
PLATE V





0·5 cm





Materials & Methods). However, if no differentiated tissue was visible, then the whole culture was transferred. Thus, the amount of callus present at the beginning of each passage (excepting the first, where the embryos were all much the same size) was different for each of the replicates, and it depended both on how rapidly the culture had grown in the previous passage and also on how much differentiated tissue had been produced. To overcome these differences in subculture size, the % increase in fresh weight was used as a measure of the growth rate, since it refers any increase in fresh weight to the original fresh weight of the culture. It is recognised that initial growth on transfer may be affected by inoculum size, but in these subcultures the size of the inoculum was considered to be above the critical value (which from published work with other callus cultures has normally proved to be less than 50 mg fresh weight).

At the end of the first passage, the mean total F.wt. of cultures to be transferred at 3 weekly intervals was 86 mg; for those with a passage length of 5 weeks the mean total F.wt. was 123 mg; and for passage length 8 weeks it was 238 mg. Thus, callus was continuing to grow on the embryo explant for periods up to 8 weeks. Fig. 2.14 shows that the percentage of undifferentiated callus was very similar for each of the three passage lengths (55 - 65%U), therefore the amount of extension growth of roots and shoot from the embryo axis was increasing at roughly the same rate as that at which undifferentiated tissue was forming. When transferred every 3 weeks (Fig. 2.13) the % increase in F.wt.of the callus decreased over the first 3 x 3 week passages and then appeared to reach a plateau where the rate of growth was very similar for each passage (30 - 40% increase). Thus, although

The behaviour of a piece of callus on fresh medium will, to some extent, depend upon its recent past history, for example, there may be carry-over effects from a passage on medium with high concentrations of hormones. To aid interpretation of results, a brief history of the callus concerned will be found at the beginning of each experiment, stating source of original explant, number of transfers (passages) the callus has undergone, and the content of the previous medium, e.g. 6 wuk 4, embryo, 1 passage on "M" + 1 mg/l 2,4-D, which means that the callus originated from excised embryos of 6 wuk 4 Wheat, and had one passage on "M" before the experiment on callus growth.

Studies were made of the effect of altering the composition of the medium, mineral salts, carbon source and organic components. Other experiments were concerned with following the growth of the callus over several passages, on media containing different auxins and cytokinins.

(i) Passage length

Maris Ranger embryos were placed on "M" containing 100 mg/l casein hydrolysate and 1 mg/l 2,4-D. The callus was transferred to fresh medium every 3,5 or 8 weeks (10 replicates per treatment) and at each subculture, the total fresh weight and amount of undifferentiated tissue (represented as %U) were measured. In this way, the % increase in fresh weight was recorded for each passage, to determine whether differences in passage length affected the growth rate. Cultures were incubated in the low light of Controlled Environment Room II.

At the end of each passage, any differentiated tissues were removed (after taking the total fresh weight) and only undifferentiated callus was transferred to fresh medium (as described in

Plate V shows callus formation after one month. Callus formed from explants on all media. The root explants formed callus most readily, but it grew very slowly as assessed after 2 months incubation. By this time more roots had started to be produced from the callus. Callus also formed at the cut surface of the plumule and 'hypocotyl' explants.

The experiment illustrated that callus may be initiated on root, plumule or 'hypocotyl' of the young seedling.

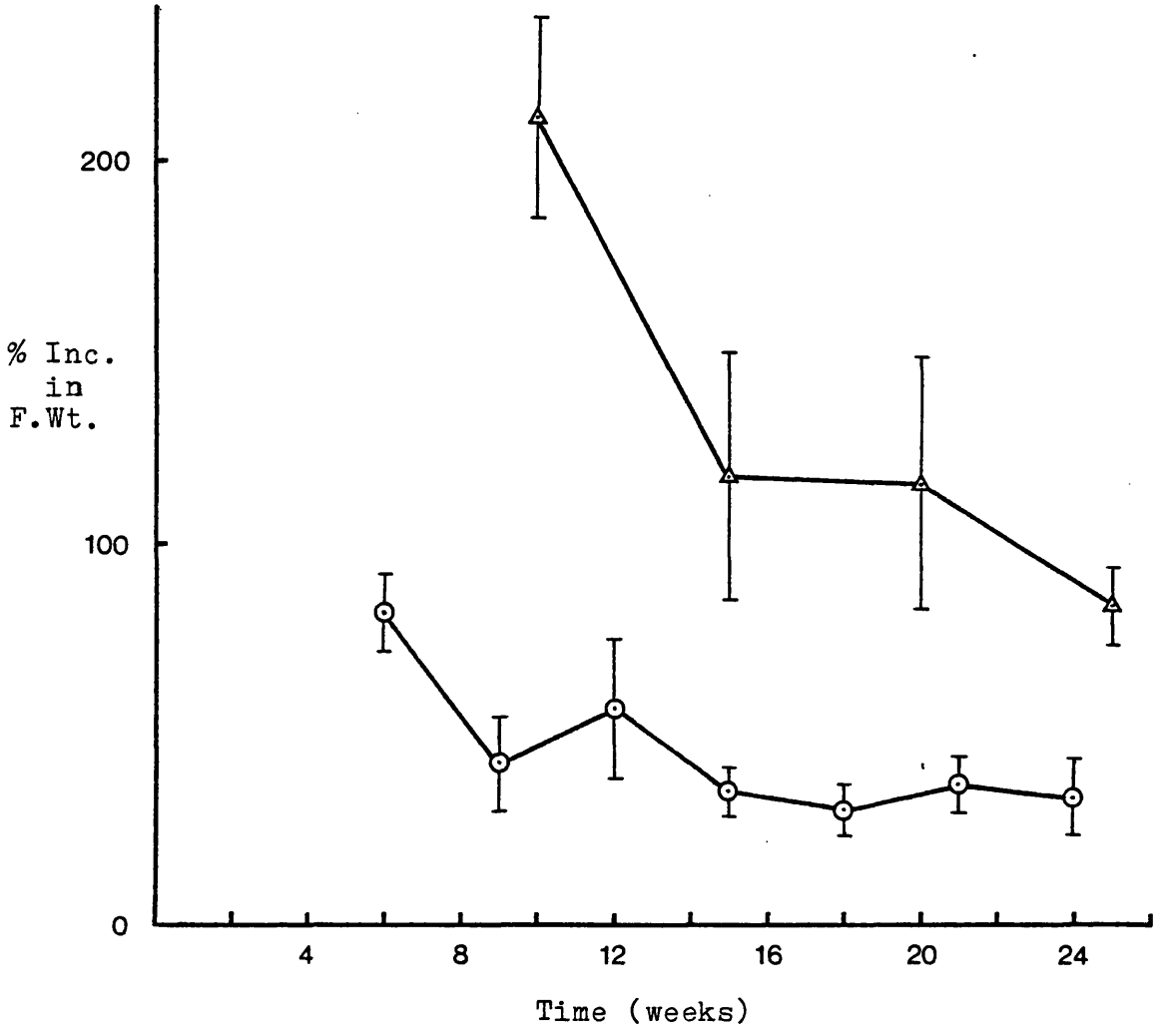
III. Growth of callus tissue on semi-solid medium

In the previous sections we have looked at some of the factors affecting the initiation of callus on explants. The present section investigates the growth of the callus after it has been separate from the original explant material, and so can no longer depend upon this tissue as a possible source of nutrients. The experiments immediately following are mainly concerned with growth of the callus, in terms of increase in total fresh weight. Other sections later in the thesis will deal with factors influencing morphogenetic changes in the cultures, and also the occurrence of plant regeneration.

The cultures were always subcultured by separating the undifferentiated tissue from any visibly organised parts (as described in Materials & Methods) and transferring only this undifferentiated callus to fresh medium. Thus, in effect, a selection was imposed for undifferentiated tissue, the aim being to obtain large numbers of homogeneous pieces of callus, with a reasonably high rate of growth. A study was made of how the passage length affected the rate of increase in fresh weight, by transferring cultures to fresh medium at different frequencies over a considerable period of time.

Fig. 2.13 : Changes in % Increase in Fresh Weight of Wheat Cultures transferred to "M" + 1mg/l 2,4-D every 3, 5 or 8 weeks. ($\bar{x} \pm SE$)

A. 3 (⊙) and 5 (△) week Passage.



B. 8 week Passage (note difference in scale from A).

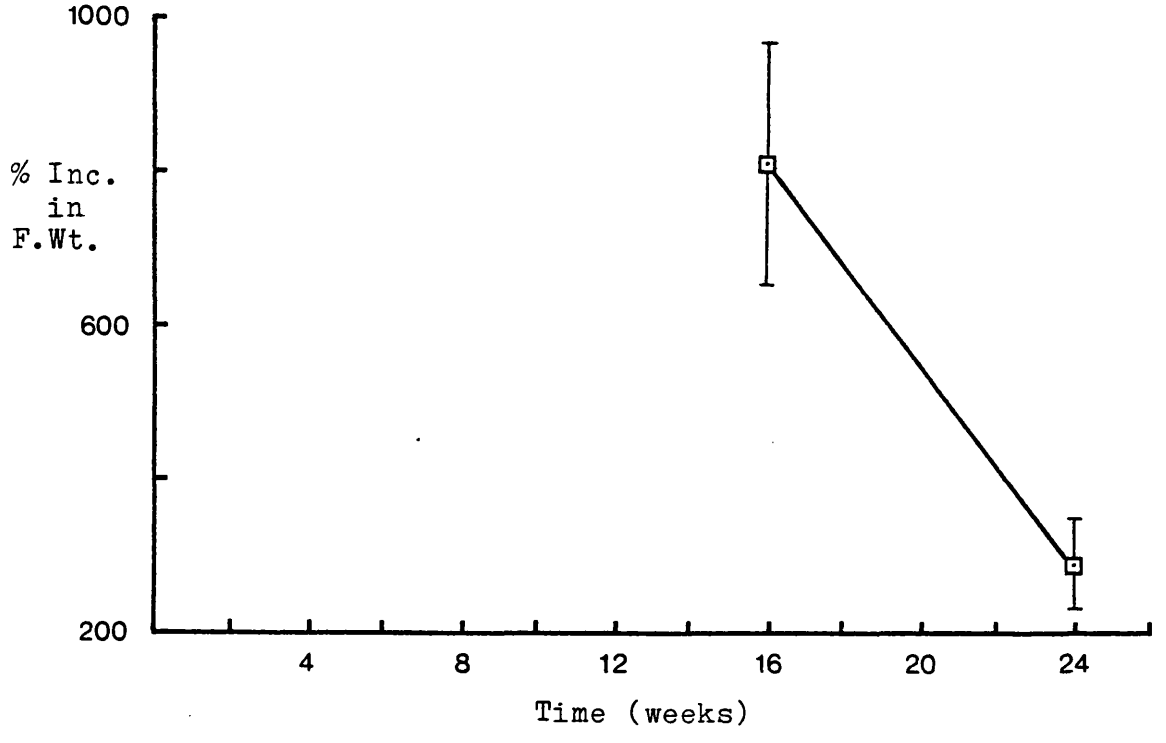
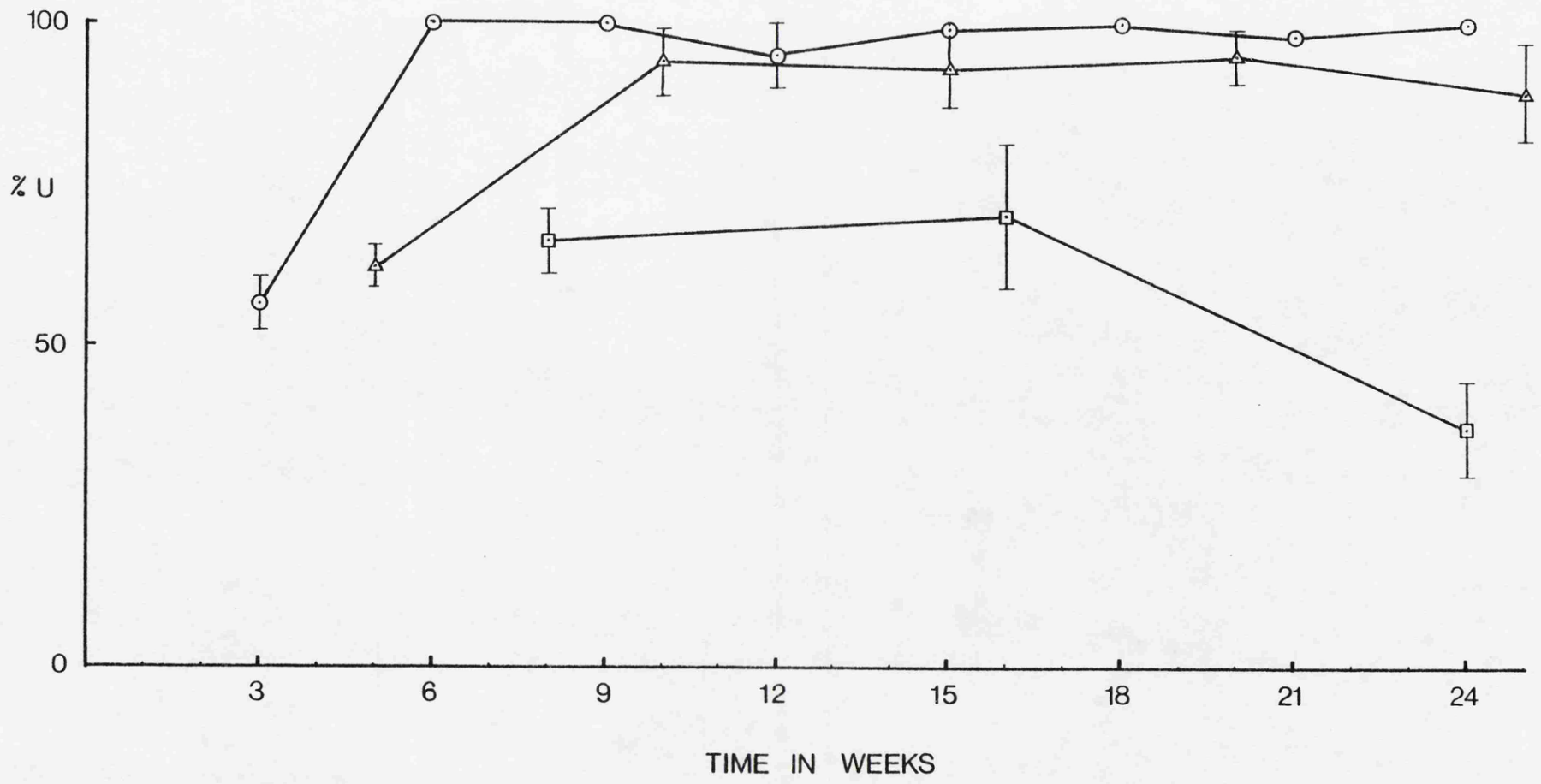


Fig.2.14 : Percentage of Undifferentiated Tissue in Wheat Cultures incubated on Basal Medium, and Transferred at 3 (○ - ○), 5 (△ - △) or 8 (□ - □) week intervals. ($\bar{x} \pm SE$).



the actual biomass of the callus was increasing with each transfer to fresh medium, the rate of increase in F.wt. was falling during this initial 9 week period, indicating that growth rate was slowing down. A similar decrease was found with cultures transferred every 5 weeks, but here no plateau was found. It would be interesting to know if a plateau would be reached if the cultures were grown over several more passages. Cultures transferred every 8 weeks showed a much more dramatic decrease in % increase in F.wt. over two passages (from 800% to 290%). Again, the experiment was not carried on for long enough to show whether a stable growth rate would eventually be reached. The experiment was curtailed after 25 weeks because contamination had reduced the number of replicates.

One possible explanation for the decline in growth rate over several passages, could be that as the initial subculture size increases with each passage, so the proportion of callus in direct contact with the medium becomes smaller. Thus, the outer layers of actively growing tissue become further away from the nutrient source and their growth rate is limited. With those cultures transferred every 8 weeks, another factor could be nutrient depletion in the medium due to long passage length.

Fig. 2.14 shows the percentage of undifferentiated tissue present at the end of each passage. As previously mentioned, at the beginning of each passage only undifferentiated callus was present, but as the passage proceeded some of the tissue became organised into organs (usually roots, but one culture produced shoots also, and this will be further discussed in the section dealing with 'regeneration', P.87).

When transferred every 3 weeks,,the cultures soon became nearly 100%U, and stayed like this throughout the experiment. When transferred every 5 weeks, again the %U quickly rose to over 90%U and did not fall below this level over several passages. However, transferring the cultures every 8 weeks seemed to allow a much larger proportion of the callus to produce roots, and after 3 passages the %U shows no sign of rising.

The results show that passage length does have an effect on the amount of differentiated tissue produced by the callus. The shorter the passage length, the less differentiated tissue was present. One possible explanation of this is that it could be a hormonal effect due to the 1 mg/l 2,4-D originally present in the medium, becoming depleted and reaching a low level during the longer passage, which allowed the organisation and growth of roots. In this case, transferring to fresh medium every 3 weeks would not allow the 2,4-D to decrease to a low enough level. Another possibility could be the presence of an endogenous factor which builds up in the cultures with increase in time between subculture and that this factor promotes root differentiation. The general trend for the %U to rise with the number of passages could be due to selection pressure, since only undifferentiated tissue was selected at each transfer.

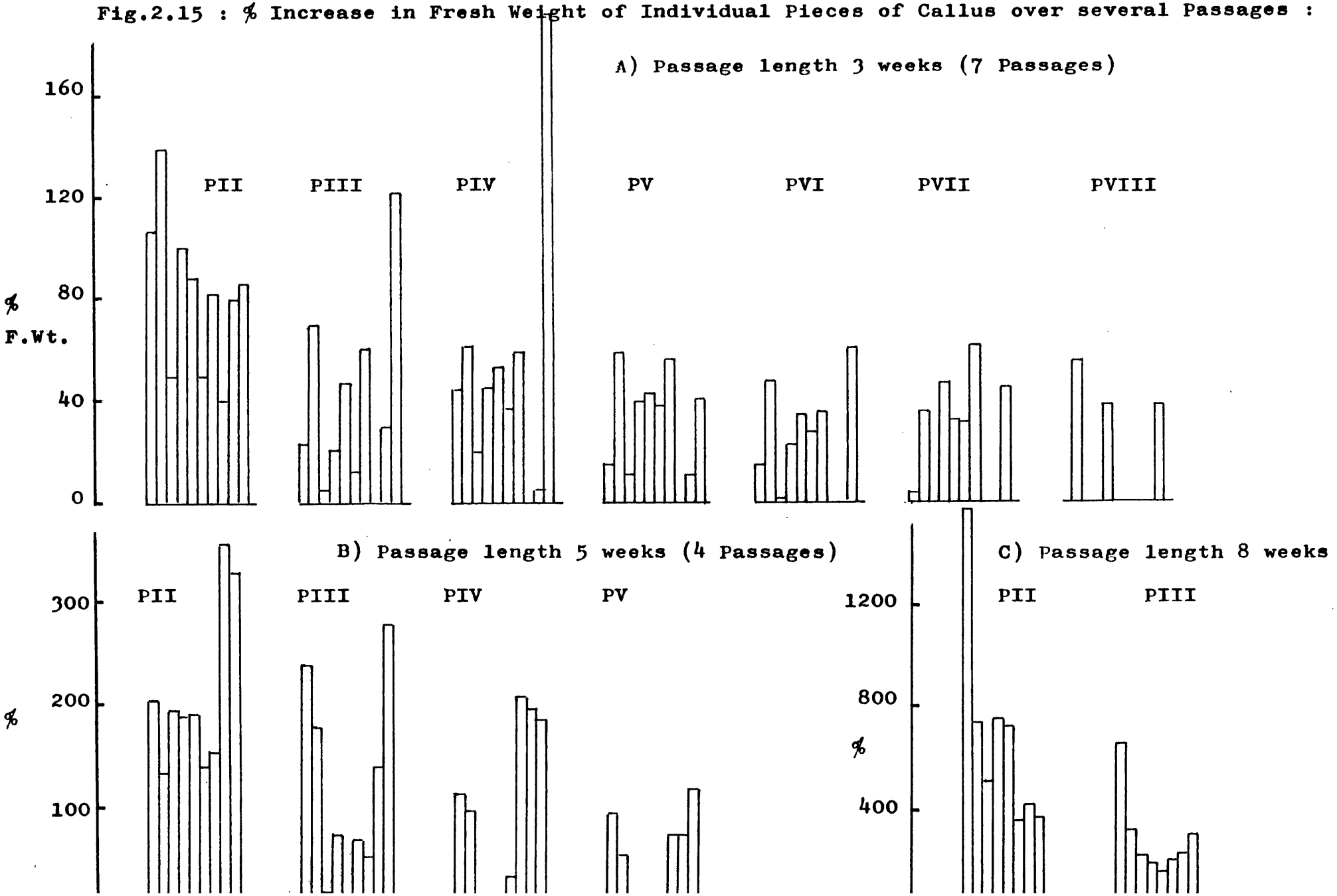
The experiment suggests that the optimum passage length for serial subculture is 5 weeks, since this gives a higher % increase in fresh weight than a 3 week passage, and the callus is over 90% undifferentiated.

The experiment also highlights a problem which recurs throughout these studies with wheat callus tissue, namely the inherent variability of the callus. We have seen in a previous experiment how

the genotype can affect response to culture conditions; the present experiment illustrates how individual callus pieces, from the embryos excised from one batch of grain, vary in growth rate over several passages on the same medium (Fig. 2.15). The histograms follow the % increase in F.wt. for individual pieces of callus. These are arranged in the same order for each passage so that the progress of each can be followed. The variability among the replicates is striking; a particular callus may have a very much higher % increase in F.wt. than the others, though this is not necessarily the same callus in each passage. Certain calluses which showed a decline in % increase in F.wt. with passage stopped growing due to contamination. Such contamination was not always visible in the culture flask, so that in some cases it was difficult to tell whether a decrease in growth was due to infection or was a response to culture conditions.

The experiment showed that growth rate may be influenced by subculture and may or may not reflect a genotypic difference between individual calluses. Also, a much larger number of replicates is necessary when it is desired to compare media or other culture variables, partly because of 'passage to passage' variation of the callus, and also beeause disturbing the callus regularly by frequent subculture raises the risk of contamination. If a technique could be formed to ensure that at each subculture, a uniform, actively-growing callus was transferred, then this experiment could be repeated to see whether the growth rate would stabilise for passages of different lengths. Also, additions could be made to the medium to try to optimise the growth rate or stop it declining from its originally high level (first 2 passages).

Fig.2.15 : % Increase in Fresh Weight of Individual Pieces of Callus over several Passages :



(ii) Nutrients

Mineral salts

In this experiment, pieces of undifferentiated callus were placed on media differing in their mineral salt composition.

History of callus used:" 6 wuk 4, node explants from several plants, 3 passages, the last one on 3 media: "M" + 1 mg/l 2,4-D, "M" + 1 mg/l 2,4-D + 5 mg/l CPA, and "M" + 5 mg/l 2,4-D (all media plus 100 mg/l casein hydrolysate). The callus had been growing for 5 months on these media, and had grown to such a size that at least two pieces of undifferentiated callus could be obtained from each.

Weighed pieces (mostly 1.5 - 2.0 g fresh weight) of visibly undifferentiated callus were transferred to 4 different media, at the same time ensuring that pieces of callus from the 3 previous media were randomly distributed among each of the 4 fresh media. The media varied in their mineral salt and vitamin content: "M" (Murashige & Skoog, 1962), "B5" (Gamborg,** 1968), "G" (Green & Phillips, 1975) and "S" (Smith, 1967*). The complete formulae for each of these may be found in the Appendix. The media were modified where necessary so that all 4 had the same level of FeEDTA, meso-inositol, sucrose (3.0%), and 2,4-D (1.0 mg/l), and the same pH 5.5. In "G" the normally present asparagine was omitted. The nitrate content of "M" and "G" was higher than that of "B5", which in turn was higher than "S". Phosphate was at the same level in "M", "B5" and "G" and lower in "S". Vitamin content was lowest in "M"; "B5" contained no glycine; "G" contained pantothenic acid. These were the main differences between the 4 media. The

*Smith's medium as modified by Mascarenhas (1971).

** see Gamborg, Miller & Ojima (1968).

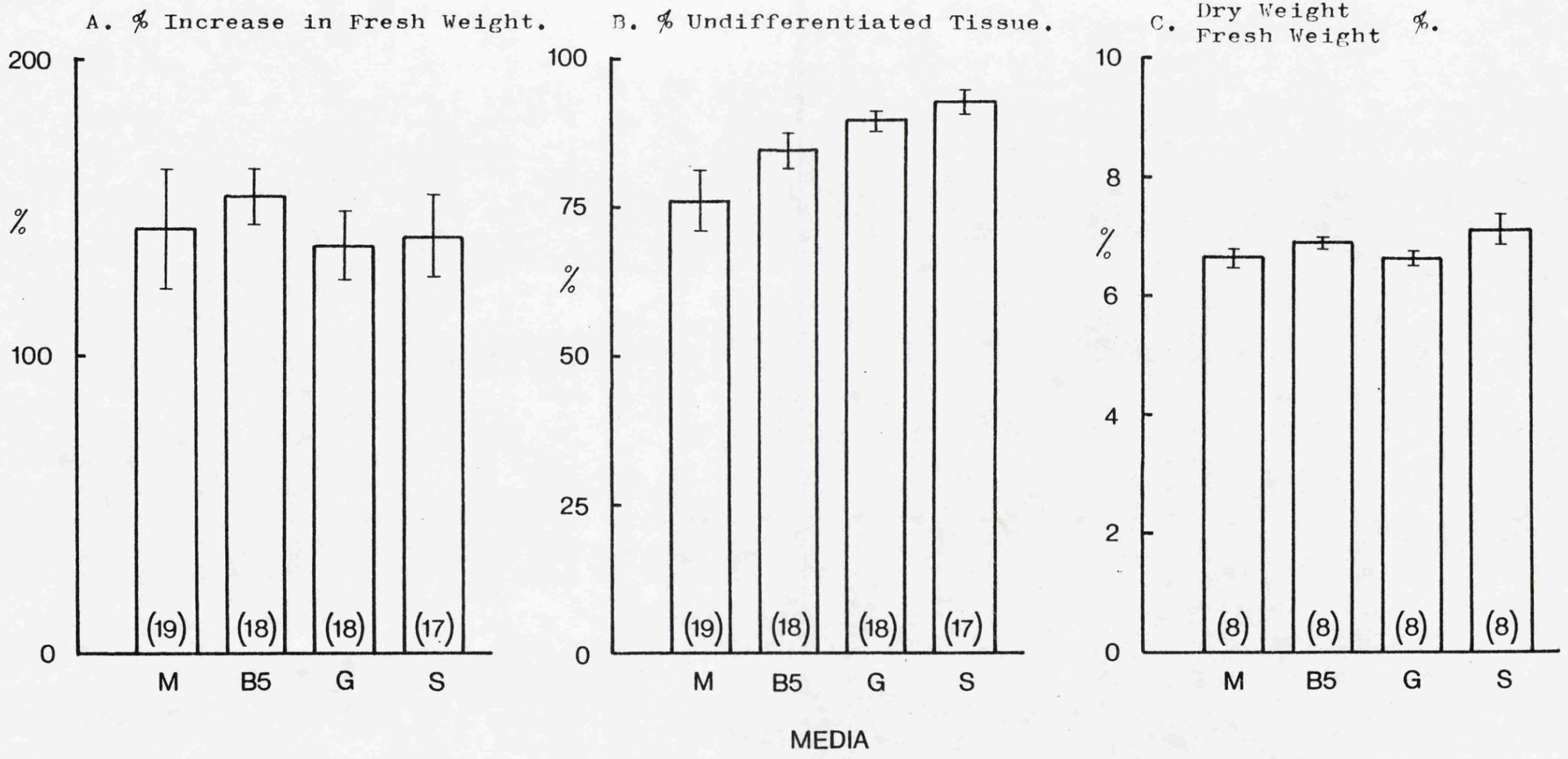
cultures (20 replicates per treatment) were incubated for 5 weeks in the low light of Room II. After this time, they were weighed to obtain the total fresh weight and the percentage of undifferentiated tissue. Some of the cultures were dried and the dry weight expressed as a percentage of the fresh weight (Fig. 2.16). Using 4 different media had very little effect on any of the parameters measured. No significant difference was present for the increase in fresh weight between the 4 media. There was very little difference between the D.wt./F.wt. ratios, although "S" had a slightly higher mean. Most of the cultures had produced a few roots, but again there was little difference in the %U. The fact that the standard errors of the mean were small in all cases indicated that the previous history of the callus pieces was not affecting the response to treatment.

The mean 'doubling time' (Table 2.19, G) for all 4 media is roughly 3½ weeks (a 100% increase in F.wt. means the callus has doubled its original weight - see Materials & Methods). The experiment showed that wheat callus will grow equally slowly on many different media differing in mineral salts and vitamins and this suggests that some other factor is responsible for either the slow division rate or the small percentage of actively growing cells.

Additions to the medium

A second experiment was carried out in which as well as using two media: "M" and "B5", the carbon source was varied, and organic substances were added to some of the media, as shown overleaf:

Fig. 2.16: Growth and Differentiation of Wheat Cultures incubated for 5 weeks on 4 Media differing in Mineral Salt and Vitamin content. ($\bar{x} \pm SE$)
(number of replicates in brackets)



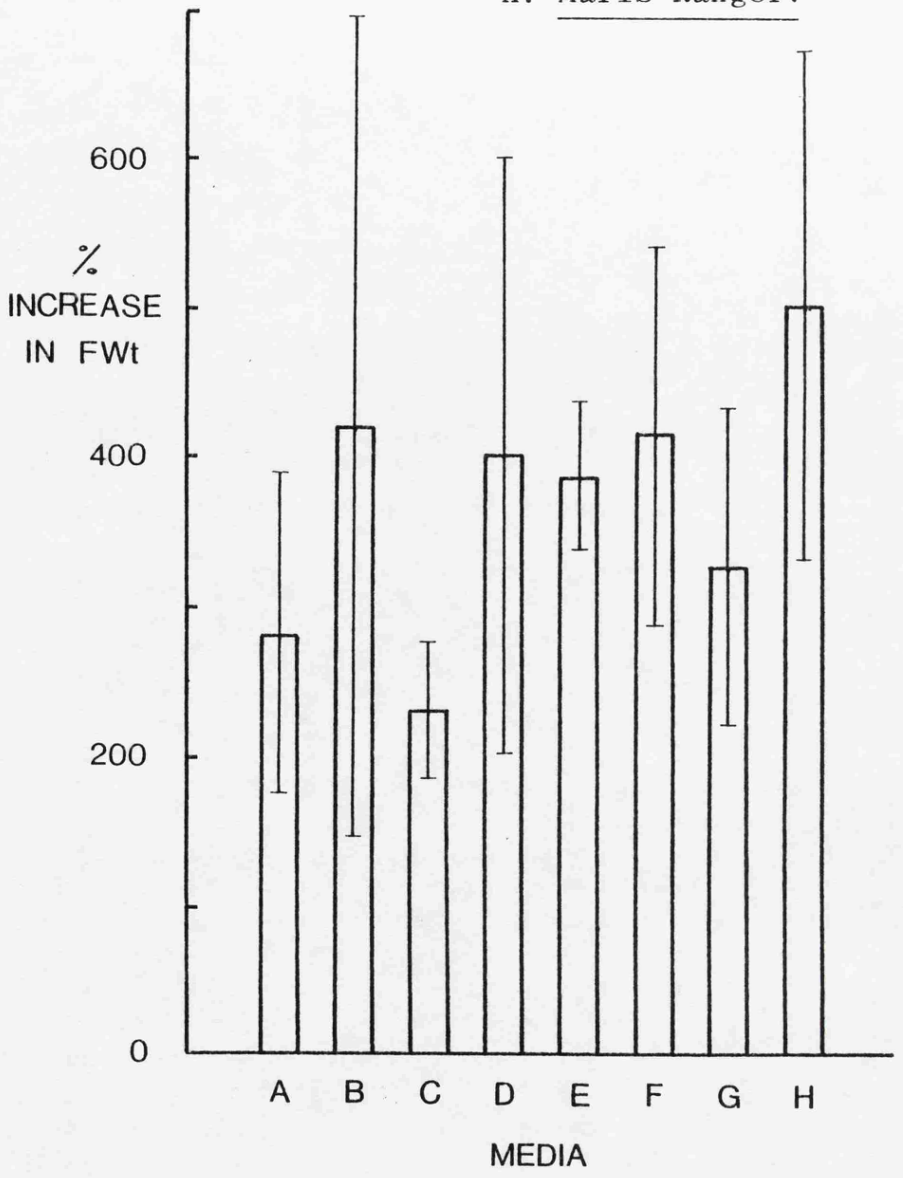
Media	Mineral salts + vitamins	Casein Hydro-lysate 100mg/l	Sucrose 3.0%	Glucose 3.0%	Coconut Milk 10%	Yeast Extr-act 25mg/l	2,4-D 1mg/l
A	M	+	+	-	-	-	+
B	M	+	-	+	-	-	+
C	M	+	+	-	+	-	+
D	M	-	+	-	-	-	+
E	M	+	+	-	-	+	+
F	B5	+	+	-	-	-	+
G	B5	-	+	-	-	-	+
H	B5	-	-	+	-	-	+

Callus tissues from the excised embryos of two varieties of wheat, Maris Ranger and Maris Ensign were used, both of which had undergone one passage (of 7 weeks) on "M" + 100 mg/l casein + 1 mg/l 2,4-D. Undifferentiated tissue from around the original embryo, was transferred to the media A - H (5 replicates from each variety). The mean fresh weight of callus transferred was higher in Maris Ranger (203 mg) than Maris Ensign (118 mg). The cultures were incubated in the low light of Room II, for 4 weeks, after which time the total fresh and dry weights were taken. The % increase in fresh weight (Fig. 2.17) was highly variable for the replicates on each medium and no clear differences could be seen between the media. However, the magnitudes of the % increase in F.wt. were nearly the same for both varieties of wheat, showing that they had been growing at a similar rate despite the Maris Ensign pieces of callus being of a smaller size. It is interesting to note that the 'doubling time' for both varieties ranges from 2.0 to 0.8 weeks - see Table 2.19, D - much higher than that found in the previous experiment (3.5 weeks 'doubling time') although the passage length was nearly the same. Variations in 'doubling time' from experiment to experiment will be discussed at the

incubation on several Media (A - H).

($\bar{x} \pm SE$)

A. Maris Ranger.



B. Maris Ensign.

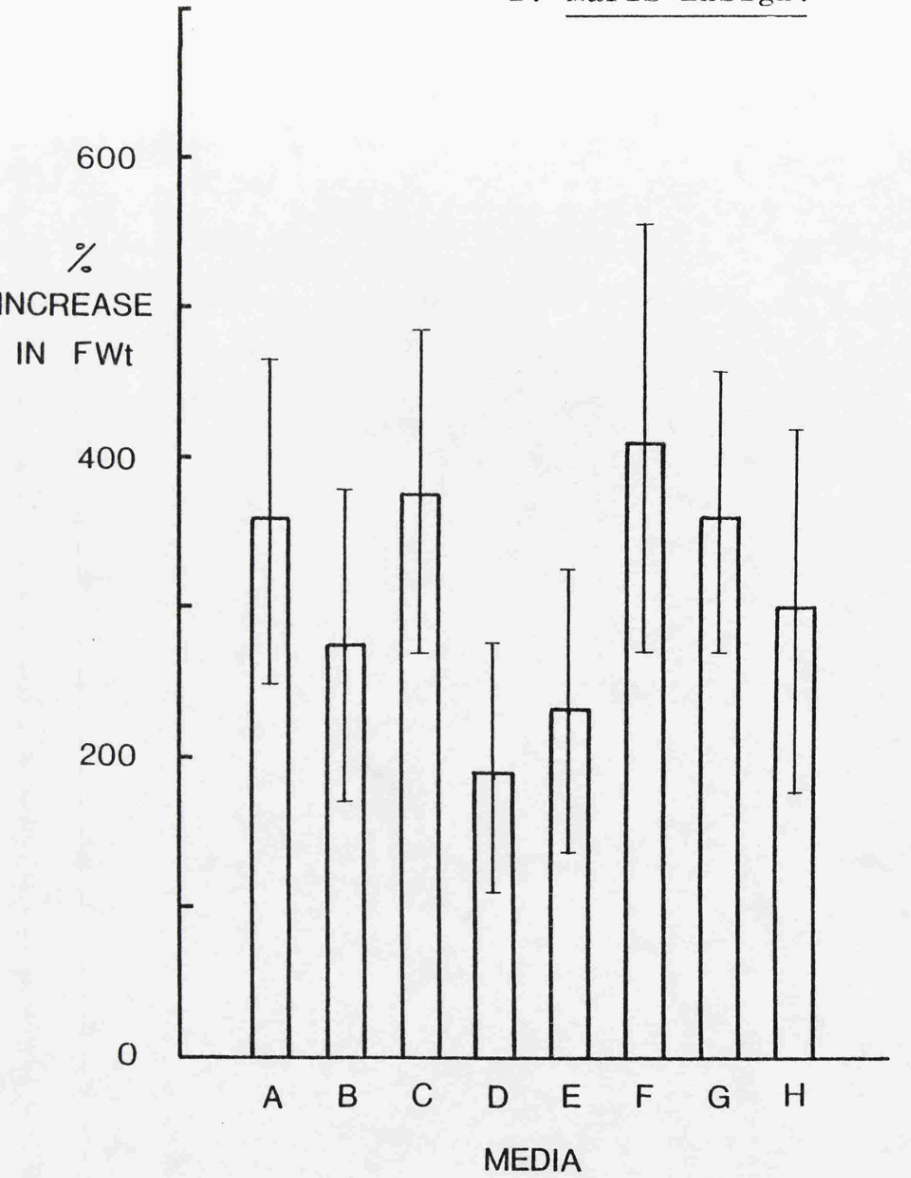
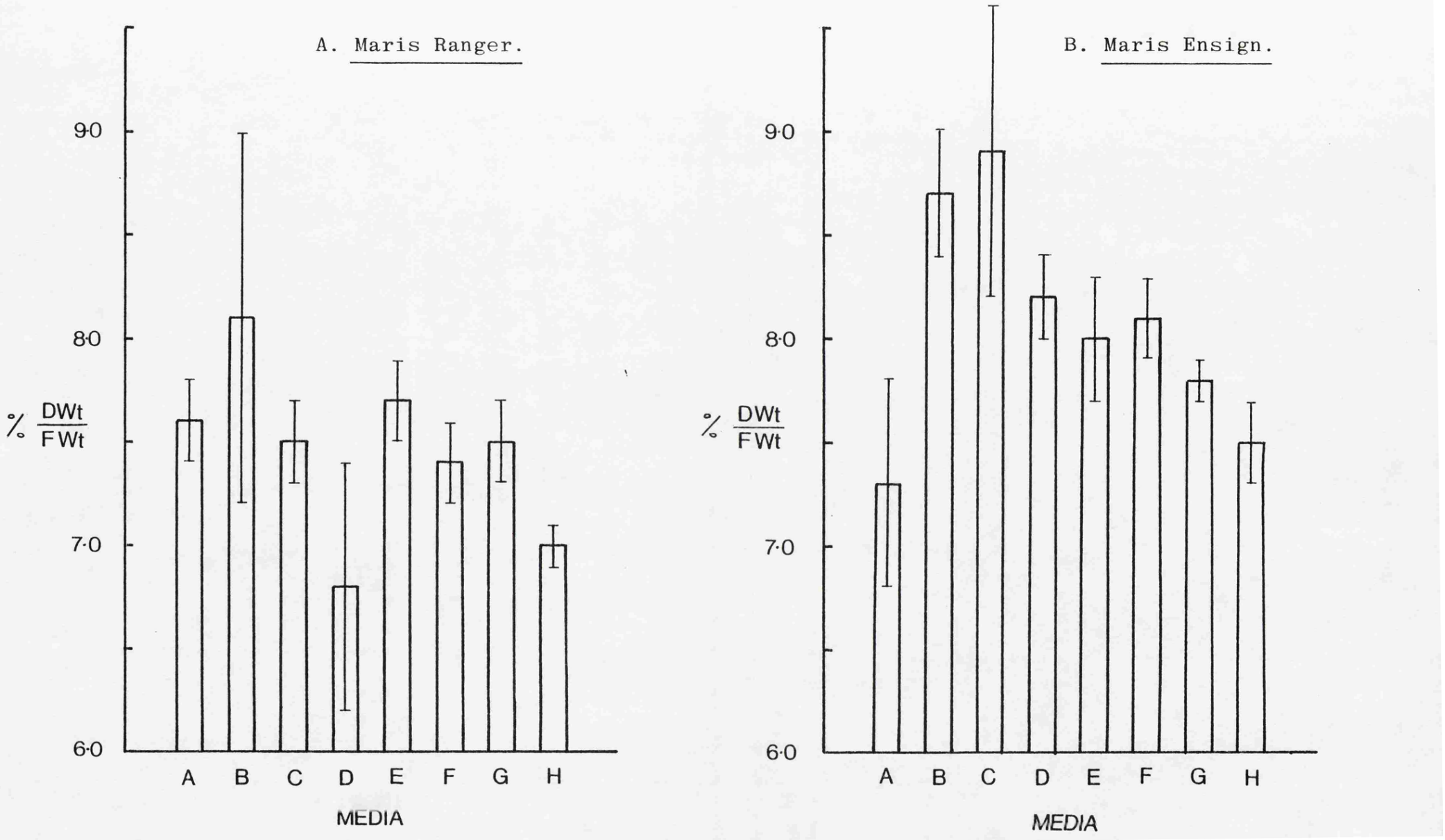


Fig.2.18 : Dry Weight as a Precentage of Fresh Weight after 4 weeks incubation of 2 varieties of Wheat Callus on several Media (A - H). ($\bar{x} \pm SE$)



end of this section (P.70).

Some differences between the media can be seen from the D.wt./F.wt. ratios (Fig. 2.18). The two varieties of wheat appeared to respond differently to Medium A and Medium D. Medium D (plus casein) had a lower D.wt./F.wt.% in Maris Ranger and a higher D.wt./F.wt.% in Maris Ensign, so that the addition of casein appeared to have a different effect on the two varieties. Comparing Medium A ("M" + sucrose) and B ("M" + glucose), there was a clear difference in D.wt. ratio in Maris Ensign but not Maris Ranger; this was also found between Medium A and F ("B5" + sucrose). Some of the media showed much more variation within replicates than others (not the same media for the two varieties).

The experiment showed that the two varieties of wheat responded differently to the range of media tested. However, no significant differences were established as far as % increase in F.wt. was concerned, and the differences were confined to the D.wt. ratios, especially in the case of Maris Ensign. Variability among replicates was such that callus appeared to grow equally well on "M" and "B5", and with either sucrose or glucose as carbon source. Addition of casein, coconut milk or yeast extract to the medium did not significantly increase productivity of the cultures. Although the variables tested may be important for optimum growth, the results again suggest that some unidentified factor may have been limiting the growth rate.

Increase in vitamin content, or addition of casein

In this experiment, the effect of increasing the vitamin content was compared to that of adding casein hydrolysate to the medium.

Brief history of the callus used: "Maris Ranger" embryos, 1 passage on 'V', 'CV', '10V'. At the end of the first passage,

undifferentiated callus was transferred to the same media:

'V' = "M" + normal level of vitamins, no casein

'IOV' = "M" + x10 normal level of vitamins, no casein

'CV' = "M" + normal level of vitamins, + 100 mg/l casein hydrolysate

The cultures were incubated in the low light of Room II. After 5 weeks, the total fresh weight and the amount of undifferentiated tissue were measured (Fig. 2.19). In passage one (discussed earlier, P 34), we found that increasing the vitamin content tended to increase the fresh weight, while addition of casein had no effect. Also, cultures on medium with either casein or increased vitamins, tended to be more differentiated than the control, 'V'. In this, the second passage on these media, the 'IOV' and 'CV' treatments produced a significantly higher % increase in F.wt. compared to the control. There was less difference between the levels of differentiation, but 'V' had the highest mean %U. So it seems that the trends started in PI, continued with more emphasis in PII. Increasing the vitamin supply seemed to be more important now the callus was growing independently, suggesting that the optimum conditions required during the first passage need not necessarily be the same for subsequent passages.

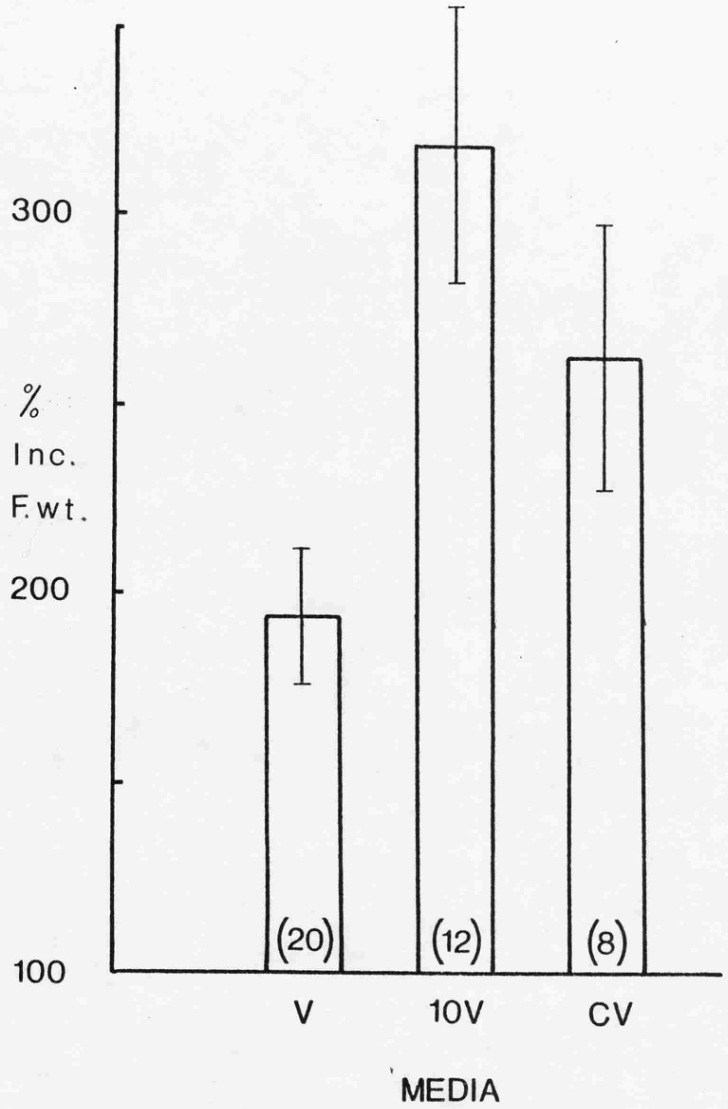
(iii) Growth substances

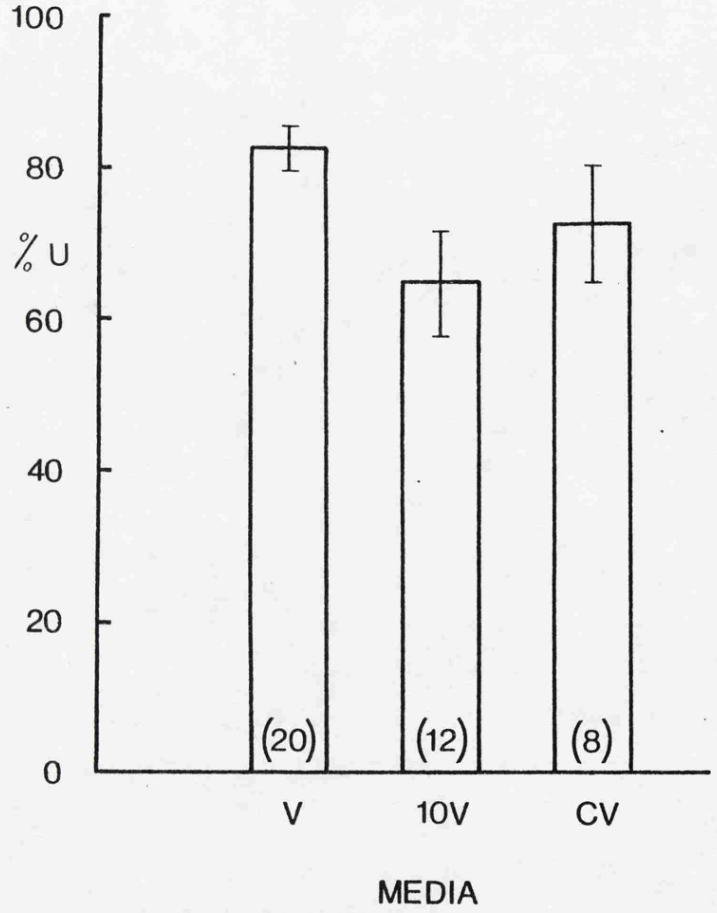
The immediately following experiments investigated the effects of several auxins and cytokinins on growth of the callus.

2,4-D, CPA & L-Tryptophan

Growth and differentiation of wheat callus beyond the first passage, was investigated using 2 auxins (2,4-D and CPA) and an auxin precursor (L-Tryptophan) either singly or in combination.

Fig. 2.19: % Increase in Fresh Weight and % Undifferentiated Tissue of Wheat Cultures, Incubated for 5 weeks on 3 Media : 'V' (normal level vitamins), '10V' (x10 vitamins) and 'CV' (normal level vitamins plus 100mg/l casein hydrolysate). ($\bar{x} \pm$ SE; number of replicates in brackets)





Brief history of the callus used :"Maris Ranger, embryo, 1 passage on "M + 2,4-D, CPA or L-Tryptophan". Weighed pieces (200 - 300 mg fresh weight) of undifferentiated callus were transferred to fresh medium of the same composition (shown below) and incubated in the low light of Room II for 14 weeks.

Medium	Casein Hydrolysate (100mg/l)	2,4-D (1 mg/l)	CPA (5 mg/l)	Tryptophan (4 mg/l)
M	+	+	-	-
A	-	+	-	-
B	-	-	+	-
D	-	+	+	+
E	-	+	+	-
F	-	+	-	+
G	-	-	+	+

Results from PI (see P.35) indicated that the presence of CPA greatly increased the percentage of undifferentiated callus produced, on the excised embryo, but there was little effect of the different media on fresh weight (except medium F which produced the highest F.wt.). At the end of PII (14 weeks) the total fresh weight and percentage of undifferentiated tissue were measured (Figs. 2.20 & 2.21). The results showed a clear difference between cultures on medium containing CPA (Media B,D,E,G) and those lacking this auxin (Media A,F,M). Addition of 5 mg/l CPA significantly suppressed the growth rate, the % increase in F.wt. for Media B,D,E & G being at least half that of Media A,F & M. No significant differences could be seen in growth between A, F & M. The effect of CPA on differentiation was also clear-cut; all cultures on media containing CPA were 100% undifferentiated even after 14 weeks incubation, by which time cultures on Media A,F & M contained many roots. Clearly 5 mg/l CPA has a very strong effect on

Fig.2.20 : Percentage Increase in Fresh Weight after 14 weeks on 7 Media. (number of replicate cultures in brackets). $\bar{x} \pm SE$.

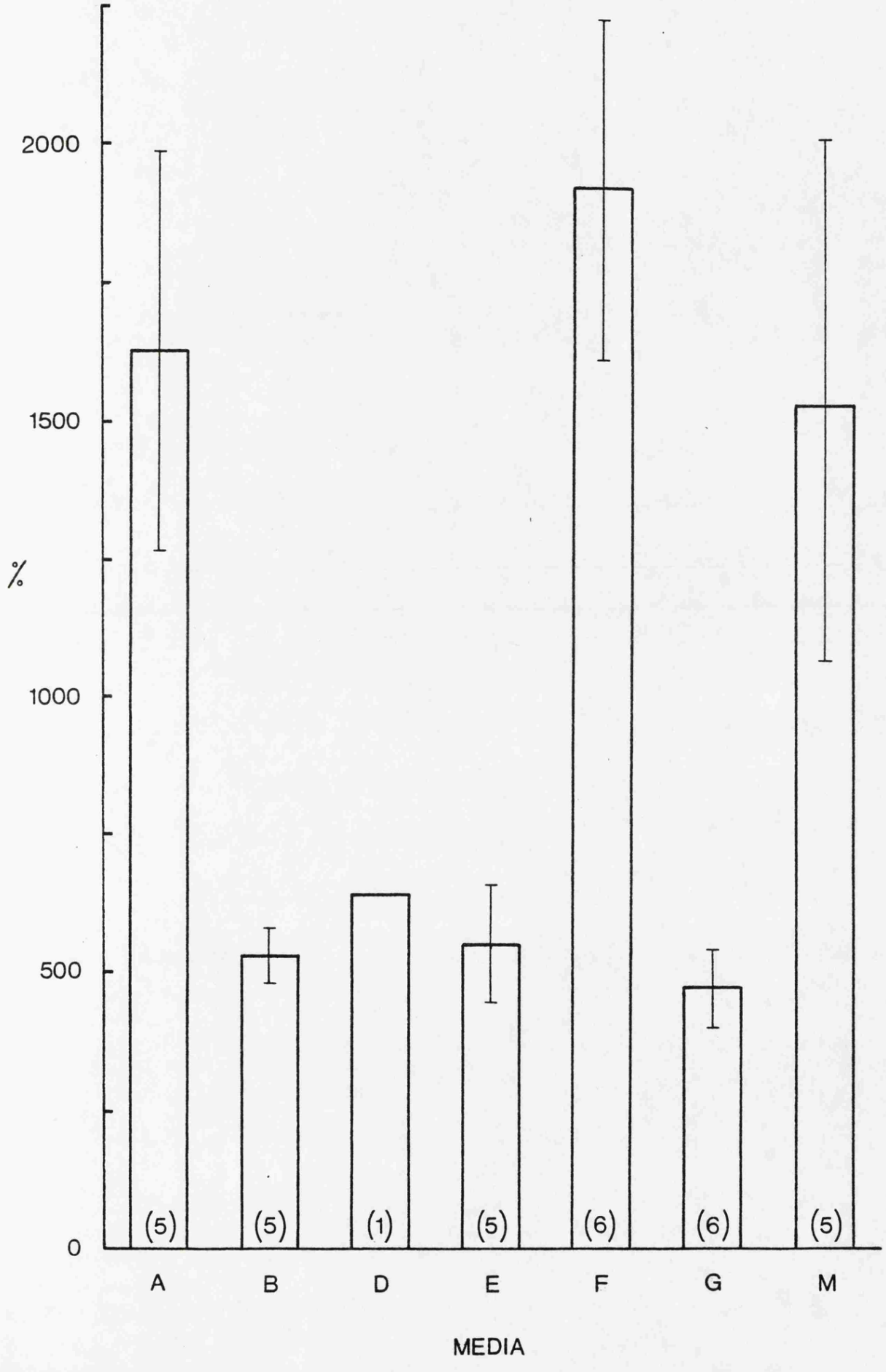
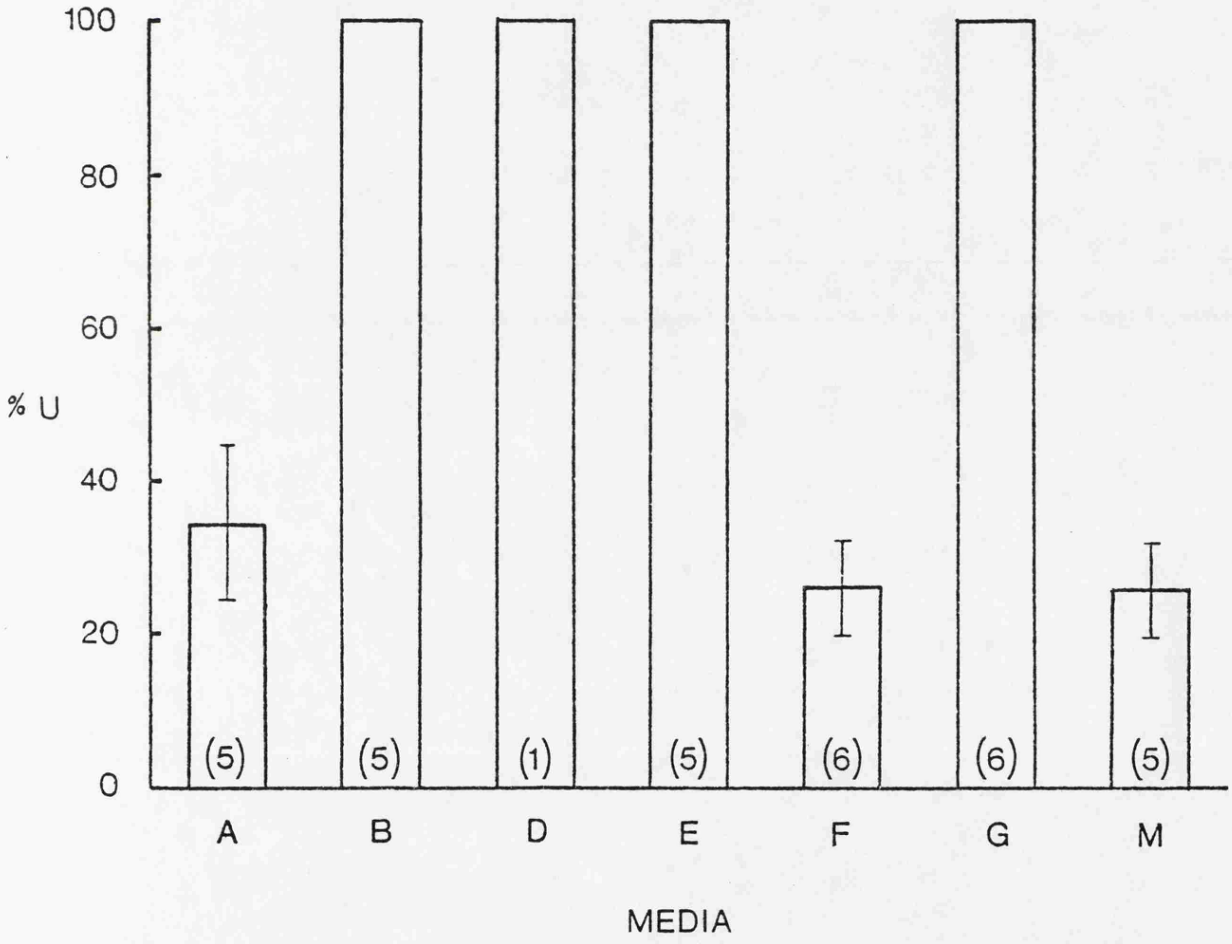


Fig.2.21 : Percentage of Undifferentiated Tissue after 14 weeks on 7 Media. (number of replicate cultures in brackets)

$\bar{x} \pm SE$



suppression of growth and differentiation. Cultures on media containing Tryptophan in the presence of a high level of auxin (D, G) showed low growth rates and were completely undifferentiated. However, in the presence of a low level of auxin (F), there is some indication that Tryptophan may have been promoting root growth, since cultures on Medium F had the highest mean % increase in F.wt, due to the presence of very many roots. It should be noted that it was the rhizogenic pieces of callus which showed enormous increases in biomass (1500% and more), having extremely rapid growth rates and a 'doubling time' of at most 0.9 weeks (see Table 2.19, C). Thus, 1 mg/l 2,4-D can sustain a high growth rate over 14 weeks, but unfortunately the increase in biomass is largely due to root production at the expense of callus proliferation.

2,4-D compared with CPA over several passages

The callus used originated from excised Maris Ranger embryos and had undergone 2 passages on the media described in the previous experiment: A - G (various combinations of 2,4-D, CPA, and Tryptophan). Undifferentiated callus was transferred to media where the effect of 2,4-D and CPA could be further investigated over several passages (described below). The cultures were incubated in the low light of Room II, and at the end of each passage, any undifferentiated tissue present was transferred to fresh medium, after taking the fresh weight. If the culture had grown well in the previous passage, it was subdivided during transfer, so that the number of replicate cultures in the experiment increased.

TREATMENT OVER 4 PASSAGES

PASSAGE	DURATION (DAYS)	MEDIA
III	39	'M' = "M" + 1 mg/l 2,4-D 'MC' = "M" + 1 mg/l 2,4-D + 5 mg/l CPA
IV	49	M) as P III MC)
V	63	'M' = "M" + 5 mg/l 2,4-D 'MC' = "M" + 5 mg/l CPA
VI	35	M) as P V MC)

Cultures on "MC" on one passage were transferred to "MC" for the next passage also, and similarly with those grown on "M".

Fig. 2.22 shows the growth of the cultures, in terms of % increase in fresh weight, over the 4 passages, while Fig. 2.23 shows the proportion of undifferentiated tissue present at the end of each passage. Passages III and IV compared the effect of 5 mg/l CPA in the presence of 1 mg/l 2,4-D. The response of the cultures in P III was very variable, especially on "M", and this was probably due both to the callus having been on several different media in the previous passage, and also to the low auxin level in "M". However, addition of 5 mg/l CPA to the medium, in the presence of 1 mg/l 2,4-D, did not make any statistically significant difference to the growth of the cultures, but it did clearly suppress differentiation in both the passages.

Passages V and VI compared the presence of 5 mg/l 2,4-D to that of the same level of CPA. Both auxins caused similar effects

Fig.2.22 : Growth of Callus over Several Passages on Media varying in 2,4-D and CPA content. (number of replicate cultures in brackets). $\bar{x} \pm SE$.

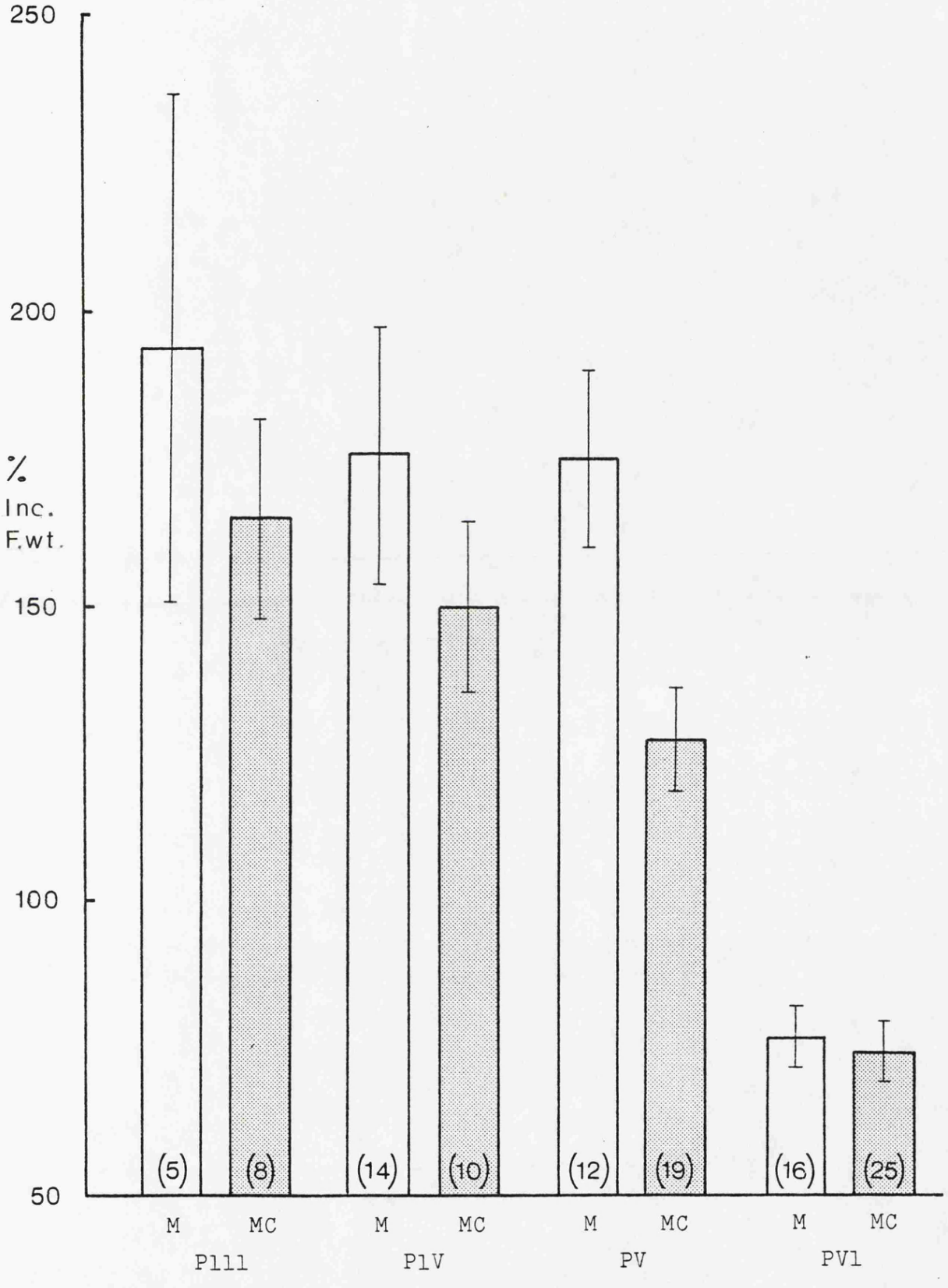
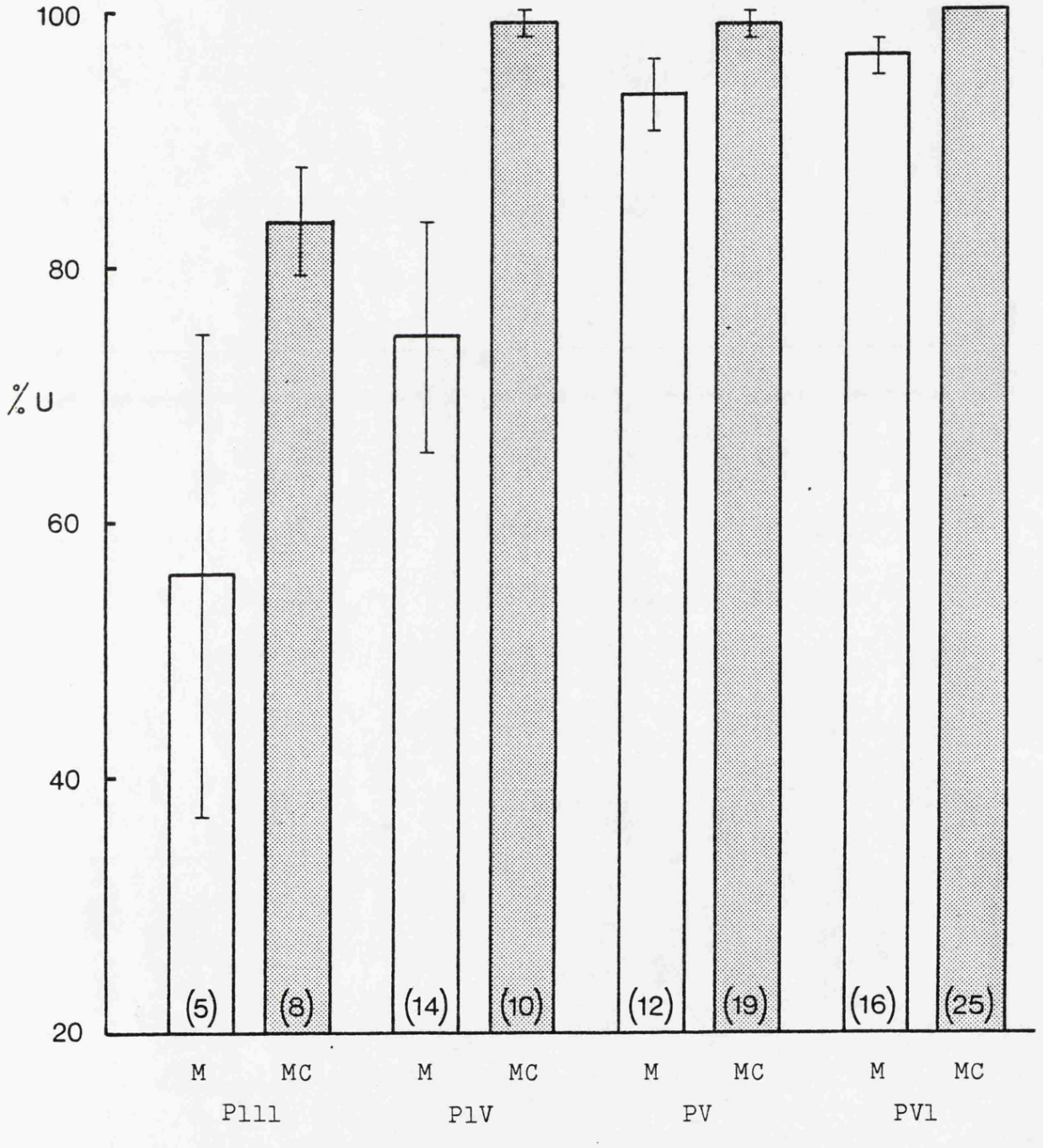


Fig.2.23 : Percentage of Undifferentiated Tissue after Several Passages on 2 Media varying in 2,4-D and CPA content. (number of replicate cultures in brackets). $\bar{x} \pm SE$.



by inhibiting growth and suppressing differentiation of the cultures. However, there was a difference, significant at P 0.01, between the % increase in F.wt. of "M" and "MC" in P V and this could be due to the change in auxin content from P IV to P V, where the auxin level was increased for "M" and slightly decreased for "MC" cultures.

In general, addition to the medium of 5 mg/l of either 2,4-D or CPA made the cultures behave more uniformly, mainly by suppressing rhizogenesis, and this was aided by selection at each passage for undifferentiated tissue.

The following table shows the "doubling-time" (see Materials & Methods) for each of the treatments:

PASSAGE	PASSAGE LENGTH	MEDIUM	"DOUBLING-TIME" (WEEKS)
III	5½ weeks	M MC	2.8 3.3
IV	7 weeks	M MC	4.0 4.7
V	9 weeks	M MC	5.1 6.7
VI	5 weeks	M MC	6.6 6.6

The time taken for the cultures to double their fresh weight increased with the number of passages, for both media. Again, the greatest difference between the two was at P V where the "doubling-time" for "MC" increased much more than that for "M". The effect of passage length and number on the "doubling-time" will be discussed at the end of this section.

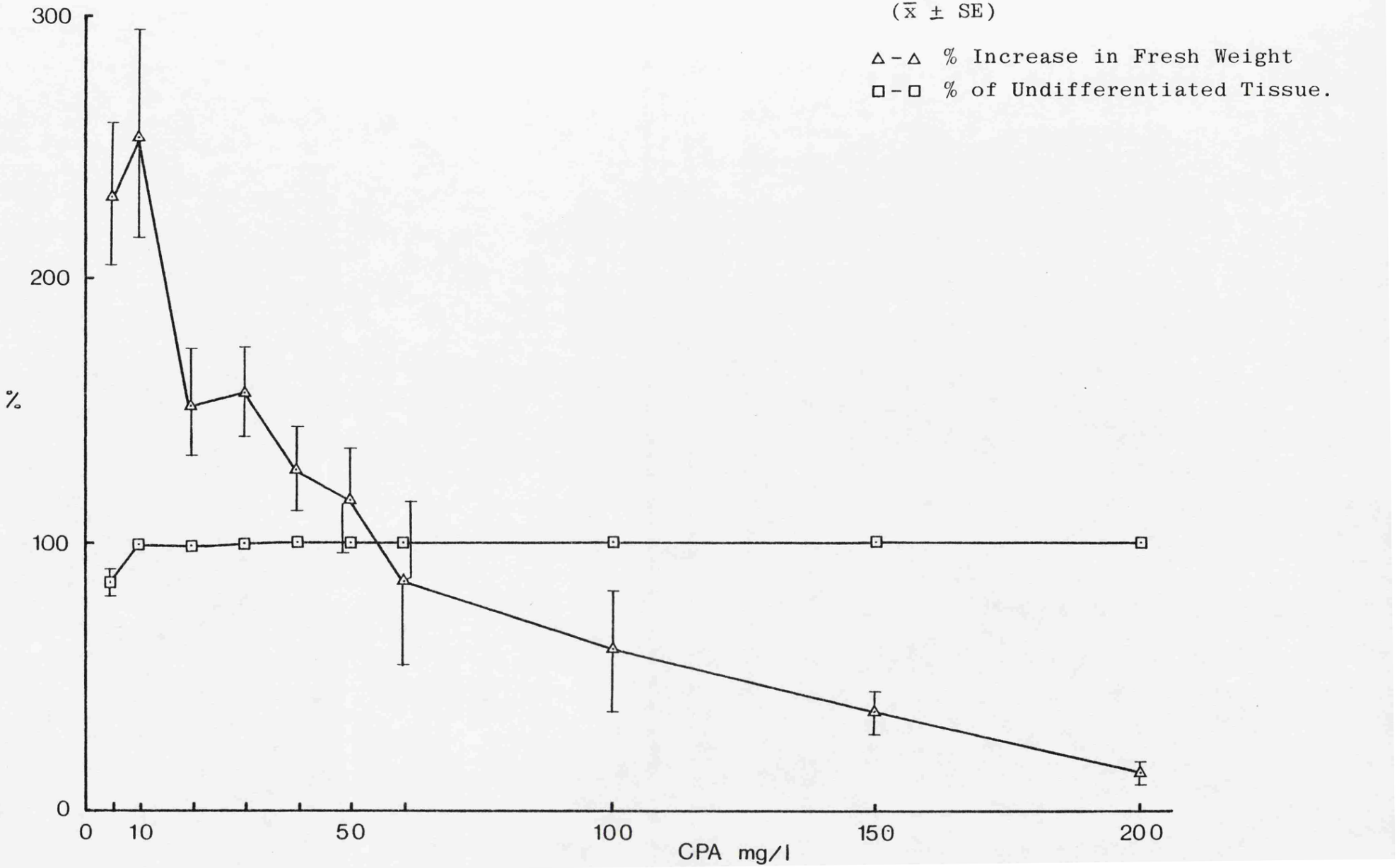
Increasing concentrations of CPA

Brief history of callus used: "Maris Ranger, embryos, one passage in the presence of a range of CPA levels". Undifferentiated callus was transferred to fresh "M" containing the same range of levels of CPA as used in Passage I (0.5 - 20.0 mg/l). Cultures were incubated in the low light of Room II for 5½ weeks, after which time they were weighed to obtain the % increase in fresh weight and the % undifferentiated tissue (Fig. 2.24). In Passage I (discussed on P.38) it was found that levels of CPA up to 6.0 mg/l caused a progressive decline in fresh weight; from 6.0 to 15.0 mg/l the fresh weight was constant, but decreased still further at 20.0 mg/l. The percentage of undifferentiated tissue which formed on the excised embryo rose quickly to near 100% and stayed there for all concentrations greater than 3.0 mg/l CPA. During this, the second passage, on these media, the % increase in F.wt. declined with increasing concentrations of CPA, the strongest fall being between 1.0 and 6.0 mg/l. Meanwhile, the tissue was almost totally undifferentiated at all concentrations above 1.0 mg/l. The results confirmed the effects of inhibition of growth and suppression of differentiation found during P I, but showed that a lower concentration of CPA (1.0 mg/l) compared to that required in P I, corresponded with the optimum level for maintaining callus in a disorganised state while permitting a reasonable increase in fresh weight.

Increasing levels of Kinetin, in the presence of CPA

This experiment investigated the effect of cytokinin on the growth of callus over one passage. Brief history of the callus used: "6 wuk 4, mature plant stem segments, 5 passages, the last one on "M" + 1 mg/l, 2,4-D". Weighed pieces (1 - 2 g fresh weight) of

Fig.2.24 : Passage II of Wheat Cultures on Medium containing increasing levels of CPA, after 5½ weeks incubation.



undifferentiated callus were transferred to "M" containing a range of Kinetin levels (0 - 5.0 mg/l); all media contained 2.0 mg/l CPA as auxin source, instead of 2,4-D. Cultures were incubated in the low light of Room II for 5 weeks, after which time they were sacrificed to obtain fresh and dry weights (about 20 replicates). No statistically significant differences were found between themeans of % increase in F.wt. for the different concentrations of Kinetin (Fig. 2.25), nor was there a difference in the dry weight ratios. Since the mean values obtained on media containing Kinetin do not differ significantly from the control medium (+ CPA but lacking Kinetin), this suggests that the presence of 2 mg/l CPA in all media may have masked any effect on growth which Kinetin would have caused alone. Unfortunately, it was not possible to repeat the experiment omitting CPA from the media. Very few cultures formed roots on any of the media, again suggesting the differentiation suppression effect of CPA, although Kinetin was able to induce a small amount of rhizogenesis, since we have seen in the previous experiment, that 2.0 mg/l CPA is sufficient to completely suppress differentiation.

Benzyl adenine (BA) or Zeatin, in the presence of CPA

This experiment investigated the effects of two more cytokinins, again in the presence of CPA. Callus used: "Maris Ranger, embryos, 10 passages, the last one on "M" + 1 mg/l 2,4-D." Weighed pieces (1.5 - 2.0 g fresh weight) of undifferentiated callus were placed on "M" containing either one of 3 levels of Zeatin (0.1 - 2.0 mg/l), or of BA (0.5 - 5.0 mg/l); all 6 media contained 2.0 mg/l CPA as sole auxin source. 20 replicates were used for each treatment, and the cultures were incubated in the low light of Room II. After 7 weeks, the cultures were weighed, and their appearance noted (Table 2.18). While

Fig.2.25,A : Percentage Increase in Fresh Weight of Wheat Callus Tissue after 5 weeks on Media containing increasing levels of Kinetin. ($\bar{x} \pm SE$).

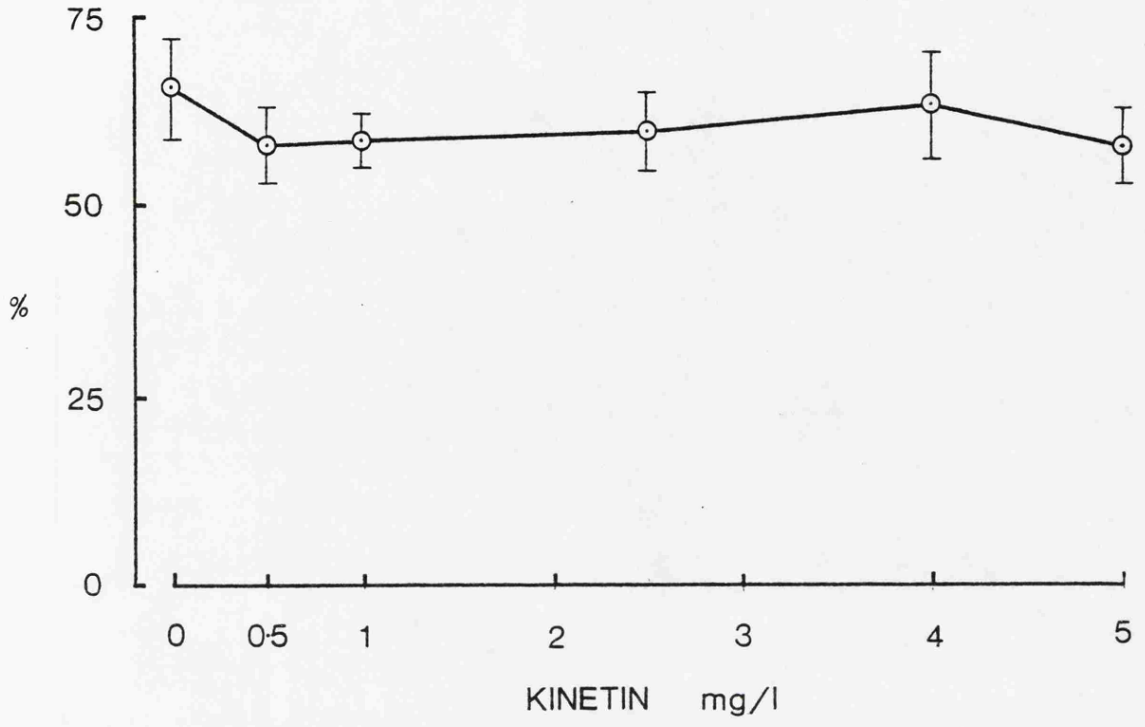


Fig.2.25,B : Dry Weight as a Percentage of Fresh Weight of Cultures after 5 weeks on Media containing increasing levels of Kinetin. ($\bar{x} \pm SE$).

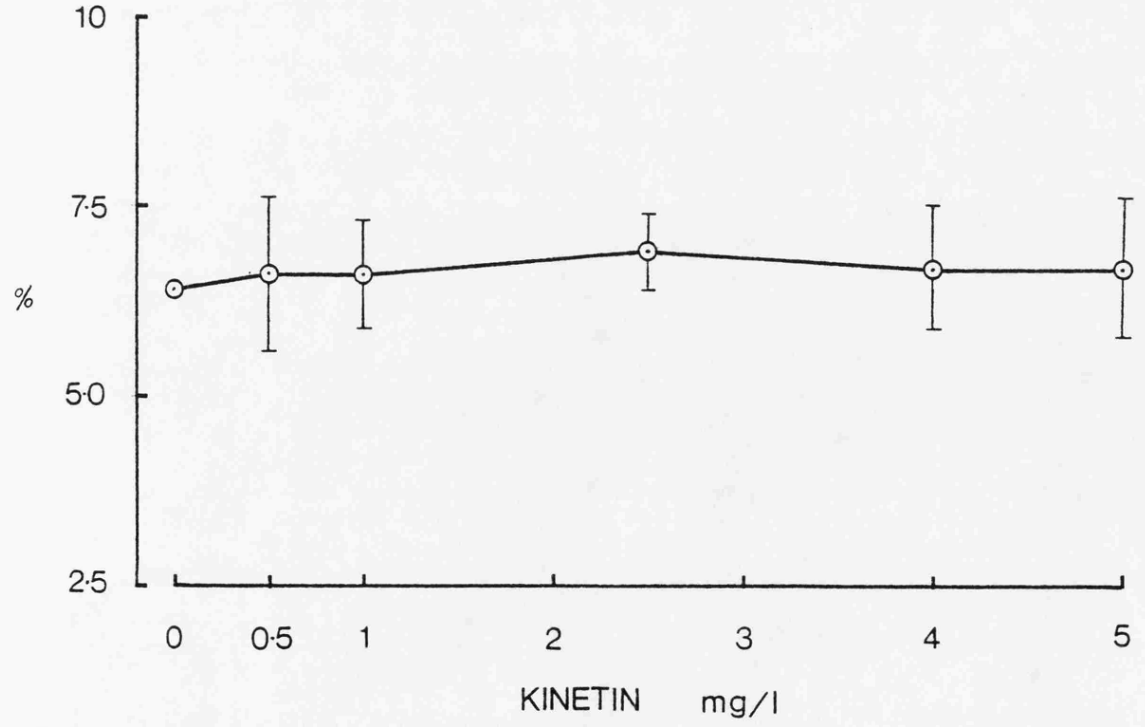


Table 2.18: Characterisation of cultures after 7 weeks on media containing Zeatin or Benzyl Adenine, in the presence of 2 mg/l CPA. (Percentages).

Medium	Total Replicates	% of cultures showing:			
		browning	greening	roots	undifferentiated
Zeatin(mg/l)					
0.1	17	0	6	29	71
0.5	19	21	5	68	32
2.0	20	25	10	85	15
Benzyl-Adenine (mg/l)					
0.5	19	21	21	74	26
2.0	20	40	10	65	35
5.0	19	79	21	95	5

the amount of root production increased with the concentration of Zeatin, this was not the case with BA, where the highest level tested did not seem to influence rhizogenesis much more than the lowest level. Similarly, more cultures showed greening on the highest level of Zeatin, but for BA, the amount of greening did not appear to be related to concentration. With respect to differentiation, 2.0 mg/l Zeatin behaved similarly to the same level of BA. However, it was observed that increasing concentrations of BA caused more of the cultures to have necrotic areas, indicating that the higher concentrations of BA tested were becoming toxic to the callus. The observation of the previous experiment, that little root production occurred on media containing Kinetin in the presence of CPA, showed that Kinetin was behaving as a weaker cytokinin than either Zeatin or BA in the presence of the same amount of CPA.

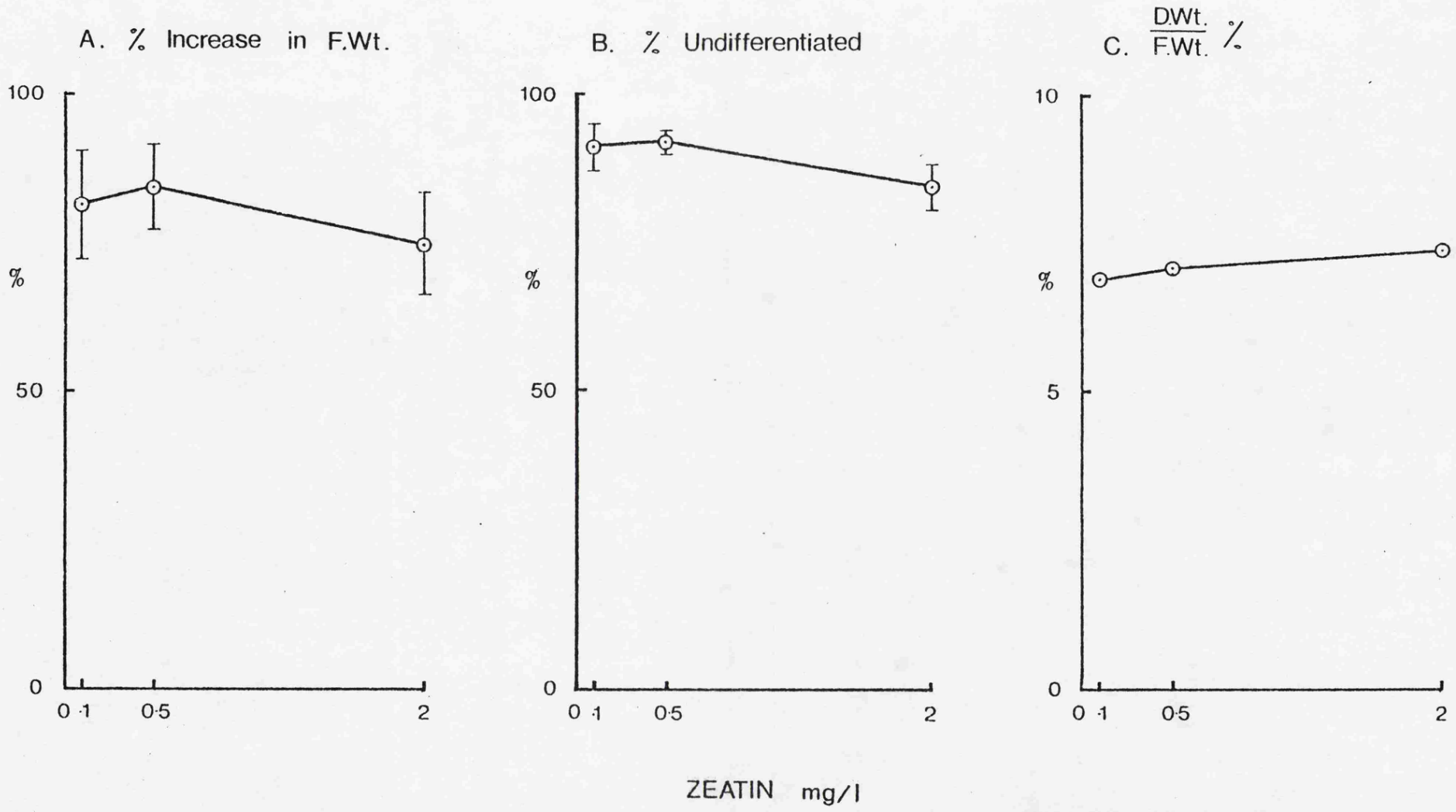
For both Zeatin and BA, no significant differences were found between the 3 concentrations tested for either % increase in F.wt.

or % undifferentiated tissue (Figs. 2.26 and 2.27). (It should be noted that while Table 2.18 showed the number of cultures in each treatment having certain characteristics, Figs. 2.26 amd 2.27 show the %U calculated as a mean from the F.wt. of each culture, so that the two sets of data are not comparable.) Analysis of variance tests did show a statistically significant difference (at P 0.05) between the D.wt./F.wt. ratios of Zeatin; 2.0 mg/l Zeatin produced the highest D.wt. ratio. If the effect of BA is compared to that of Zeatin, then media containing BA produced a greater % increase in F.wt. than did Zeatin, and both of these cytokinins produced a % increase in F.wt. which was more than twice as high as that found in the previous experiment with Kinetin. Although comparisons between the two experiments cannot be made too rigidly, since both the original callus source and also the 'age' (Passage number) of the cultures were different, it could be argued that Kinetin had much less effect on the wheat callus than either Zeatin or Benzyl Adenine. The fact that no significant differences were found between the concentrations tested for each cytokinin, suggests that the range of concentrations was not wide enough and lower levels, especially of BA, would have been informative.

(iv) Factors affecting the "doubling-time" of cultures

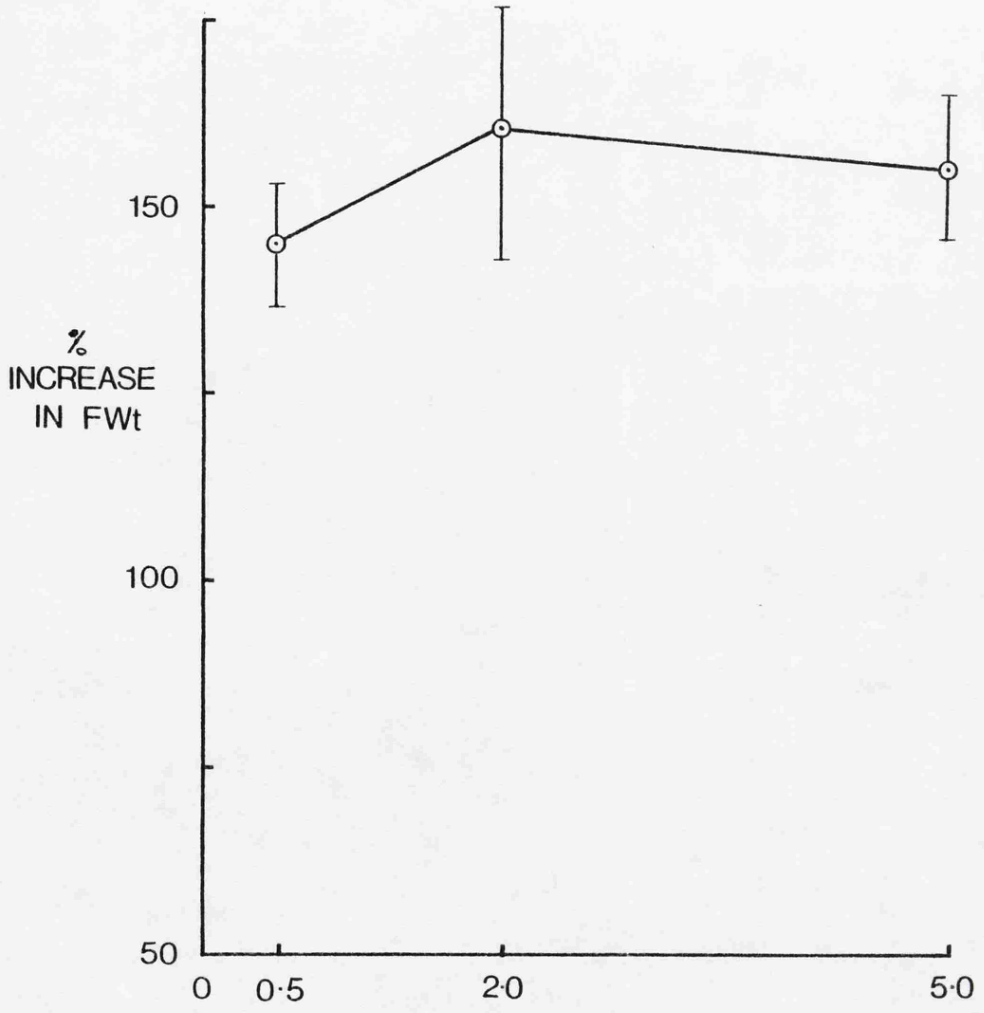
As defined in Materials & Methods, the "doubling-time" is a measure of the average time taken (in weeks), for the cultures of a particular treatment, to double their biomass (100% increase in fresh weight). As such, the estimation of the "doubling-time" assumes a linear rate of growth, whereas in actual fact, the growth of a mass of callus is more likely to be logarithmic. However, the situation is complicated, since not all of the cells in a piece of callus are engaged

Fig.2.26 : Growth and Amount of Undifferentiated Tissue present after incubation for 7 weeks on Medium containing increasing levels of Zeatin. ($\bar{x} \pm SE$)

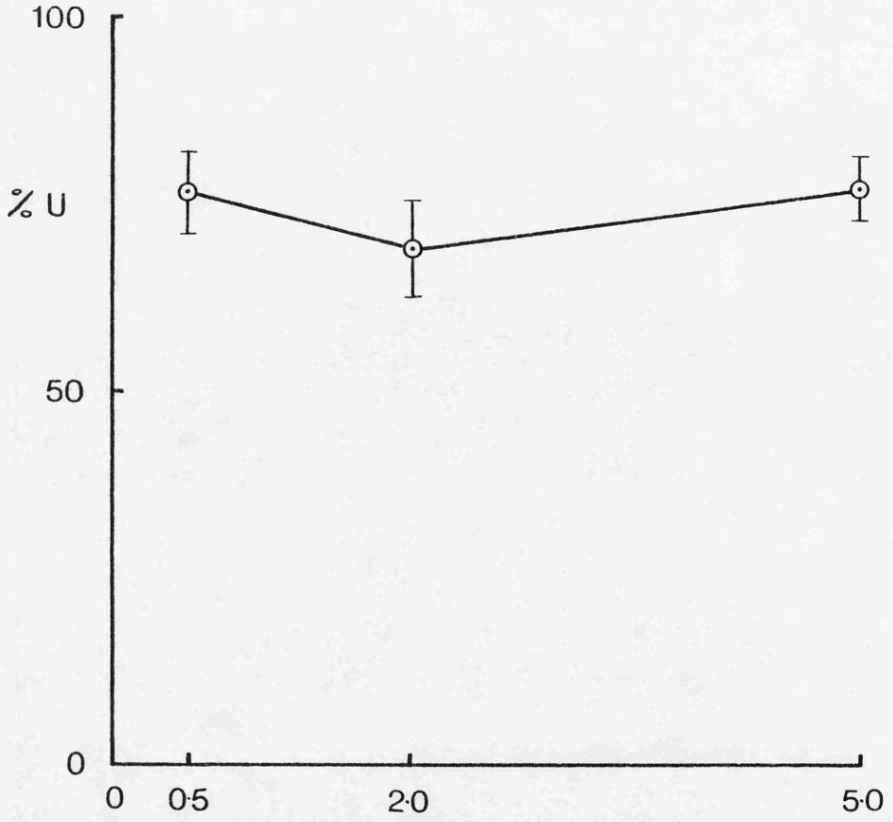


containing increasing levels of Benzyl Adenine. ($\bar{x} \pm SE$)

A. % Increase in Fresh Weight.



B. % of Undifferentiated Tissue.



BENZYL-ADENINE mg/l

in division; there tends to be a variable number of areas of activity. Thus, the growth rate of the cultures most probably lies somewhere between a linear and a logarithmic increase. Bearing these limitations in mind, the "doubling-time" can be used to compare the growth rate of cultures from different experiments. In the following discussion, an attempt will be made to determine the factors affecting the "doubling-time" using data from the 9 experiments described previously, which were concerned with the growth of callus under different conditions (Table 2.19).

In the experiment where the passage length was varied for serially-subcultured callus on "M" (Table 2.19,A), the "doubling-time" increased with passage number (number of transfers) for each régime. Also, cultures transferred every 8 weeks had a shorter "doubling-time" than those transferred every 5 or 3 weeks, at the equivalent passage number. Thus, cultures left undisturbed on the medium had a higher growth rate, but it was observed that this was associated with increasing rhizogenesis. Another point to emerge is that "M" is not the optimum medium for maintaining callus growth, since the growth rate was declining with each transfer. The "doubling-times" of several of the other experiments listed agree with these growth rates. In Experiment B, medium 'V' is the same as "M" (but lacking casein) and the "doubling-time" for Passage II (5 weeks) is 2.6 weeks which fits with that for callus transferred every 5 weeks in Experiment A. Similarly with Experiment E, where the two lowest levels of CPA have produced "doubling-times" which agree with Experiment A. In Experiment E it is clear that increasing the CPA level had progressively lengthened the "doubling-time". Experiments C and D also illustrate that altering the organic content of the medium

Table 2.19 : 'Doubling-times for Cultures of 9 Experiments, differing in Passage length and Medium.

Passage length	Passage No.	Doubling-time	Experiment.
3 weeks	II	3.6	**A** Maris Ranger, Callus from excised Embryos, cultured for several Passages on "M" + 1mg/l 2,4-D + 100mg/l casein hydrolysate; Passage length 3, 5, or 8 weeks.
	III	6.8	
	IV	5.4	
	V	8.6	
	VI	9.7	
	VII	8.3	
	VIII	9.1	
5 weeks	II	2.4	
	III	4.2	
	IV	4.3	
	V	5,9	
8 weeks	II	1.0	
	III	2.8	
5 weeks	II	2.6 (V)	**B** Maris Ranger, Callus from Embryos; 3 Media: V, CV, 10V.
		1.6 (CV)	
		1.9 (10V)	
14 weeks	II	0.9 (M)	**C** Maris Ranger, Callus from Embryos; 7 Media: M, A, B, D, E, F and G.
		0.9 (A)	
		2.6 (B)	
		2.2 (D)	
		2.5 (E)	
		0.7 (F)	
		3.0 (G)	
4 weeks	II	1.4 (A)	**D** Maris Ranger, Callus from Embryos; 8 Media: A, B, C, D, E, F, G and H.
		1.0 (B)	
		1.7 (C)	
		1.0 (D)	
		1.0 (E)	
		1.0 (F)	
		1.2 (G)	
		0.8 (H)	

(contd.)

Table 2.19 : continued.

Passage length	Passage No.	Doubling-time		Experiment.
$5\frac{1}{2}$ weeks	II	2.4	0.5 *	**E** Maris Ranger, Callus from Embryos; "M" medium containing 0.5 - 20.0 mg/l CPA(*).
		2.2	1.0	
		3.6	2.0	
		3.5	3.0	
		4.3	4.0	
		4.8	5.0	
		6.5	6.0	
		9.1	10.0	
		15.7	15.0	
		36.7	20.0	
$5\frac{1}{2}$ weeks	III	2.8	(M)	**F** Maris Ranger, Callus from Embryos; "M" medium plus 2,4-D, or "MC" medium plus 2,4-D and/or CPA. over 4 Passages of different length.
		3.3	(MC)	
7 weeks	IV	4.0	(M)	
		4.7	(MC)	
9 weeks	V	5.1	(M)	
		6.7	(MC)	
5 weeks	VI	6.6	(M)	
		6.6	(MC)	
5 weeks	IV	3.5	(M)	**G** 6 WUK 4, Callus from Nodes; 4 Media different minerals and vitamins:M,B5,G,S.
		3.2	(B5)	
		3.6	(G)	
		3.5	(S)	
5 weeks	VI	7.7	0 **	**H** 6 WUK 4, Callus from Nodes; "M" (+2mg/l CPA) with increasing levels of Kinetin (**).
		8.6	0.5	
		8.5	1.0	
		8.3	2.5	
		7.9	4.0	
		8.6	5.0	
7 weeks	XI	4.8	0.5 ***	**I** Maris Ranger, Callus from Embryos; "M" (+ 2mg/l CPA), with different levels of Benzyl Adenine (***) or Zeatin (****).
		4.3	2.0	
		4.5	5.0	
			****	
		5.3	0.1	
		5.2	0.5	
		5.6	2.0	

caused changes in the "doubling-time": in Experiment C, media containing no CPA (M,A,F) had much shorter "doubling-time" than the other media containing this auxin. Experiment D has cultures on "H" medium ('B5' minerals + 3% glucose + 1 mg/l 2,4-D) with the shortest doubling-time (0.8 weeks) noted for a short passage length. However, all the cultures on this range of media had shorter "doubling-times" than would be expected from Experiment A so this could be a genotypic effect.

Bearing in mind the discussion of each of the experiments earlier in this section, a study of the data presented in Table 2.19 shows that the factors affecting the "doubling-time" are complex. Possibly passage length and hormonal content of the medium are the most important factors, together with the number of transfers the culture has undergone. However, some very "old" cultures, such as those in Experiment I (Passage XI) have surprisingly low "doubling-times", which is most probably due to selection of callus with a reasonably high growth rate. It should be noted that "doubling-times" have been estimated in weeks, so that even the lowest ones found were several days in length. Few authors have published data showing increase in fresh weight at the end of the passage, however, a "doubling-time" of 2.1 weeks was calculated from data reported by Dudits, Nemet & Haydu (1975) for wheat tissue on medium containing 1 mg/l 2,4-D for 5 weeks; similarly a "doubling-time" of 1.2 weeks was calculated from a paper by Hendre, Mascarenhas, Pathak & Jagannathan (1975) where wheat tissue was grown for 5 weeks on Smith's medium plus 5.0 mg/l NAA and 1.0 mg/l Diphenyl-urea. Both of these are of a similar order to the "doubling-times" found in the present study, although since different media, hormones

and varieties of wheat are involved, the comparison cannot be taken too far.

IV. Organogenesis in callus on semi-solid medium, and the regeneration of plants

In the previous section, concerned with the growth of cultures on semi-solid medium, it was noted that factors affecting the growth rate very often also influenced the amount of differentiated tissue produced by the callus. It was observed that auxin concentrations above 2.0 mg/l could almost completely suppress rhizogenesis. On media with lower auxin levels, the longer the culture remained undisturbed on the medium, the more roots differentiated. There was a tendency for media with the addition of casein hydrolysate, or increased vitamin levels to produce more differentiated tissue. In the following section, the factors affecting organogenesis are discussed in greater detail. The effect of light intensity in the absence of auxin was investigated. The influence of concentration of auxin on differentiation was studied, and also the effect of using auxins and cytokinins in different ratios.

During the course of this study of wheat tissues in culture, it was observed that while root formation occurred very readily, the production of shoots was a rarity. Following the work of Green & Phillips (1975), in which maize plants were regenerated from immature embryo tissues, two experiments are described in which plantlets were obtained from callus derived from immature wheat embryos. However, shoots were also found in single cultures of many other experiments (some of which have already been described) and an attempt was made to collate relevant information concerning the conditions of culture under which the shoots were produced in the hope that consistent factors might emerge. Since so many experimentswere involved, no attempt is made to

describe each experiment fully and only the facts relevant to the particular culture which produced shoots will be presented.

(i) The effect of light intensity on differentiation, in the absence of auxin

In this experiment, the effect of different levels of light intensity on the amount of differentiation was investigated. At the same time, the carry-over effects of 2,4-D and CPA were studied, since the cultures were transferred from media containing these to medium lacking auxin.

The callus used had originated from excised Maris Ranger embryos, and had undergone 6 passages, the last one on either "M" + 100 mg/l casein hydrolysate + 5 mg/l 2,4-D, or "MC" containing 5 mg/l CPA instead of 2,4-D. Pieces of visibly undifferentiated callus, weighing roughly 1.5 g fresh weight, were transferred to fresh "M" containing 100 mg/l casein but lacking any auxin. Cultures which had previously been on medium containing 2,4-D were referred to as "M" and those which had been on medium containing CPA, as "MC". "M" and "MC" cultures were placed under 2 levels of light intensity or in complete darkness:

"D" - complete darkness, Controlled Environment Room II
"L" - light intensity 155 - 225 lux, Controlled Environment Room II
"H" - light intensity 430 - 750 lux, Controlled Environment Room I

Twenty replicates of both "M" and "MC" were used for each treatment, but unfortunately, several became contaminated, especially among those in complete darkness. The appearance of the cultures was noted after 4 days and 11 days, without removing them from the Culture Rooms (Table 2.20, A & B). Of the "M" cultures, after 4 days 82% showed differentiation (root formation and/or green spots in the callus) under

Table 2.20 : Characteristics of Cultures incubated on medium lacking auxin, in the Dark (D), under Low Light Intensity (L), or under High Light Intensity (L).

Treatments	Total replicates	% of Cultures having :			Total % of differentiated Cultures.
		roots	G.sp.*	U**	
A) After 4 days.					
"M"					
D	17	47	0	53	47
L	17	76	12	18	82
H	15	47	60	33	67
"MC"					
D	20	5	0	95	5
L	20	10	0	90	10
H	20	0	0	100	0
B) After 11 days.					
"M"					
D	14	79	0	21	79
L	17	94	59	6	94
H	15	93	100	0	100
"MC"					
D	17	35	0	65	35
L	20	45	25	55	45
H	20	35	55	35	65

* G.sp. = Green spots in the callus.

** U = visibly Undifferentiated tissue present only.

"M", "MC" refer to medium used in the previous Passage of the Cultures - see text.

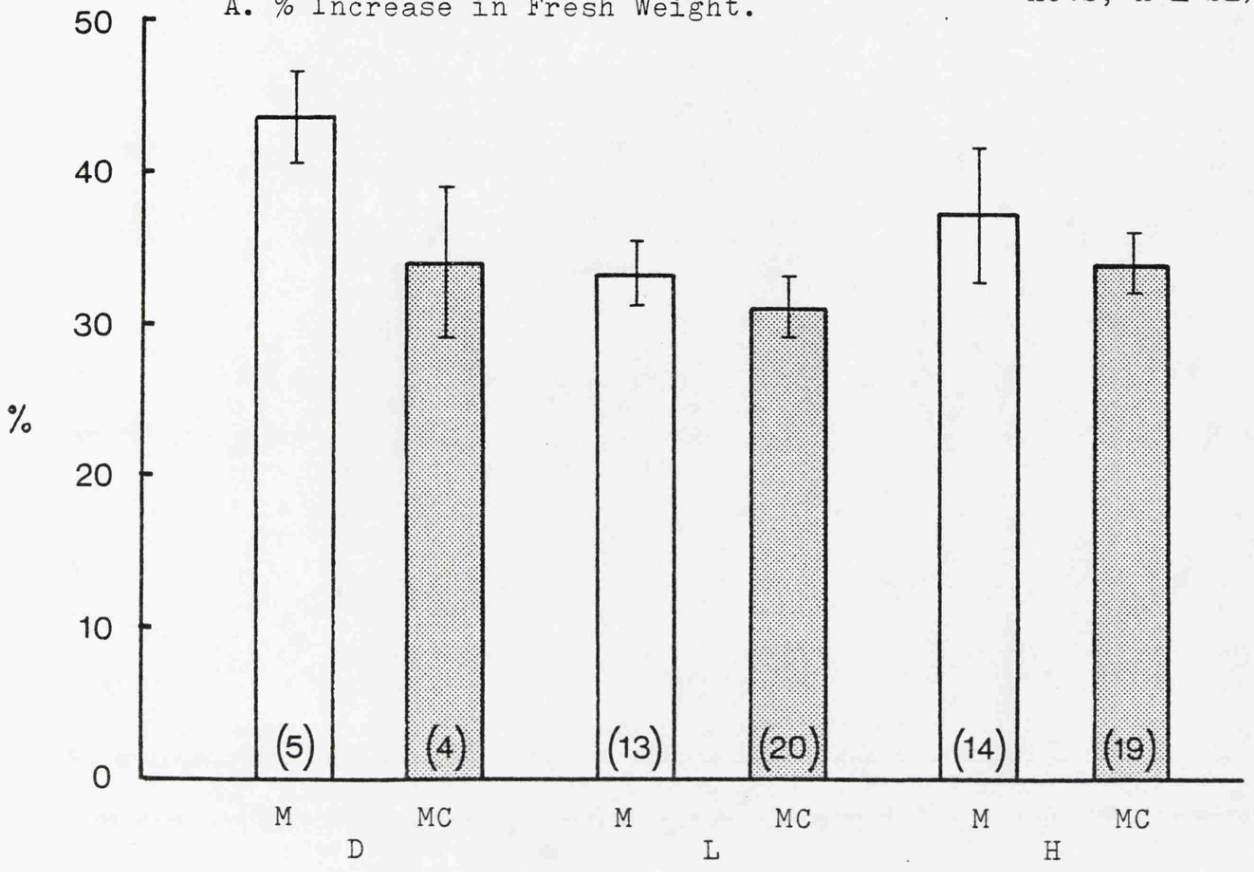
low light, "L". The highest proportion of root-forming cultures were found in "L" and the highest proportion of cultures having greening were found under high light "H". The "MC" cultures showed little differentiation; a few of the "L" and "D" cultures had roots, no greening was present. After 11 days, further differentiation had occurred: the "M" cultures under high light intensity all showed differentiation to some extent. "M" cultures still showed more differentiation than "MC". In general, increasing the light intensity reduced the number of cultures which remained completely undifferentiated. The observation that the "M" cultures began to produce organised tissue more quickly suggests that 5 mg/l 2,4-D did not have such a strong carry-over effect as did the same level of CPA. Root production and greening of areas of callus and roots were the only types of differentiation found; no shoots were produced.

After 13 days, the cultures were weighed to obtain total fresh weight and the amount of undifferentiated tissue of each culture. Fig. 2.28, A and B, shows the % increase in fresh weight and the % undifferentiated callus for all treatments. No significant differences can be seen between the % increase in F.wt. for each treatment, except for those grown in the dark, where the mean F.wt. for "M" is higher than "MC". However, as has already been mentioned, much contamination was present among the 'D' treatment, so that these means are based on only a few replicates. No significant differences can be seen for the %U between the treatments, but in each case, "MC" has a significantly higher % of undifferentiated tissue.

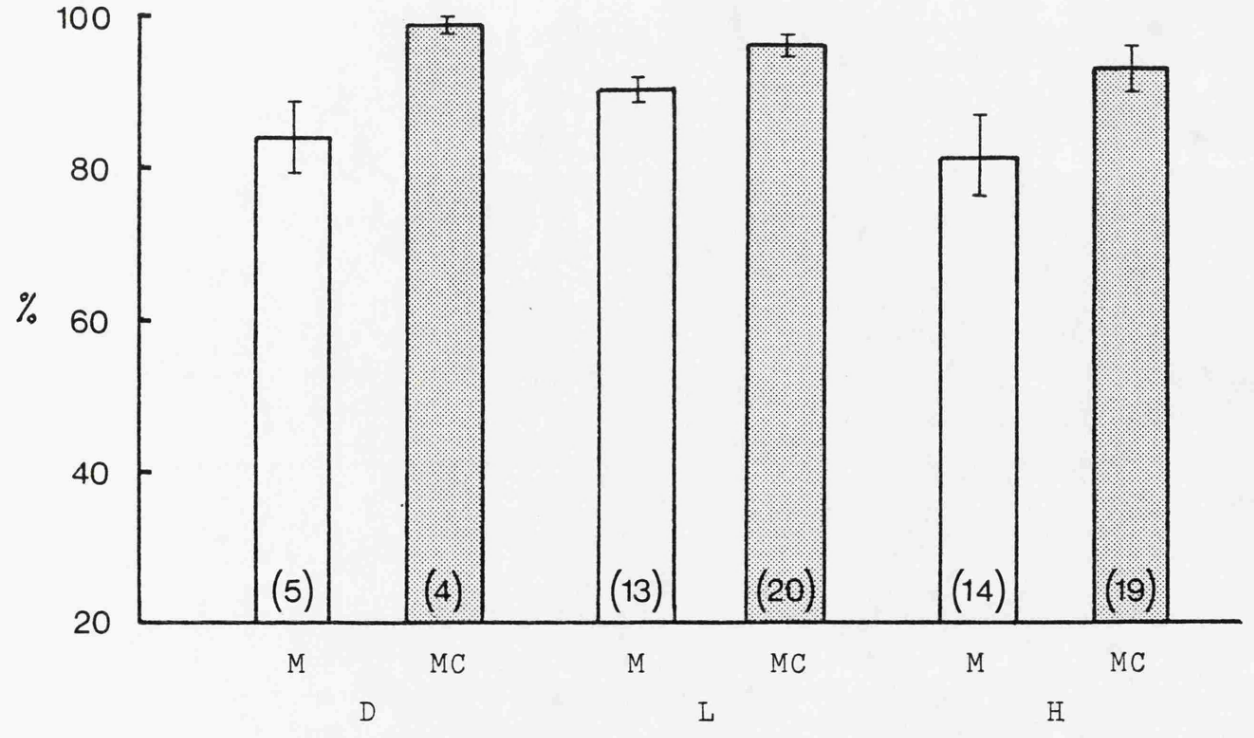
The experiment shows that light intensity does not appear to affect either the growth of the cultures, or the amount of differentiation they undergo, to any clearly significant extent. However,

Fig.2.28 : % Increase in Fresh Weight and % Undifferentiated Tissue in cultures incubated for 13 days on Medium lacking Auxin, under High Light (H), Low Light (L) or Darkness (D).('M', 'MC' = previous media - see text;no. of replicates in brackets; $\bar{x} \pm SE$)

A. % Increase in Fresh Weight.



B. % Undifferentiated Callus present.



TREATMENTS.

high light intensity enhances greening of callus and roots. The experiment shows the presence of carry-over effects from the previous passage, since cultures which had been grown on medium with CPA behaved differently to those on medium with 2,4-D, when both were in the absence of auxin.

(ii) The effect of different auxin levels on differentiation

The callus used for this experiment had originated from excised embryos of Maris Ranger, and had been maintained in culture for well over a year. It had undergone 9 passages, the last one on "M" containing 1 mg/l 2,4-D (no casein). Pieces of undifferentiated callus (fresh weight 1.5 - 2.5 g) were transferred to "M" containing selected levels of 2,4-D (see Table 2.21 below) and one medium contained 2 mg/l 2-Naphthoxyacetic acid (2-NOA). None of the media contained casein hydrolysate. The cultures were incubated in the low light of Controlled Environment Room II. After 3 weeks the appearance of the cultures was recorded (Table 2.21) and they were weighed to determine the increase in fresh weight and proportion of undifferentiated tissue present. The dry weight was taken and expressed as a percentage of the fresh weight. In each treatment, 5 of the cultures were left undisturbed to see whether further differentiation would occur. On all media, during the 3 weeks incubation, several of the cultures had produced roots, and showed greening of both areas of the callus and the roots.

Table 2.21: Characteristic of cultures incubated for 3 weeks on medium containing different levels of 2,4-D, or 2-NOA.

2,4-D mg/l	2-NOA mg/l	Total No. Replicate Cultures	% of cultures showing:		
			Roots	Greening	Undifferentiated
0	2.0	21	90	24	10
0	0	21	100	67	0
0.2	0	21	95	48	5
1.0	0	21	90	33	10
5.0	0	19	32	0	68

No shoots were found. Comparing the type of differentiation found on increasing levels of 2,4-D, the number of rhizogenic cultures was similar on all the levels tested except 5 mg/l. Raising the level of 2,4-D caused less cultures to show greening of tissue; the highest level tested, 5 mg/l 2,4-D, clearly inhibited differentiation. The presence of 2 mg/l 2-NOA had roughly the same effect on type of differentiation as did 1.0 mg/l 2,4-D, and it can therefore be suggested that it was behaving as a weak auxin.

Statistical analysis showed no significant differences between the means for % increase in F.wt. (Fig. 2.29,A) at any level of probability. The large size of the standard errors of the mean, especially at 1.0 mg/l 2,4-D, and where 2-NOA was present indicates a high variation in response. It could be that these cultures were at a threshold for a positive response to auxin, so that some cultures did and some did not respond. The D.wt./F.wt. ratio (Fig. 2.29,B) decreased for the two highest levels of 2,4-D. Highly significant differences (at P 0.05) were found between the %U for each treatment (Fig. 2.29,C). The two media lacking 2,4-D were significantly more differentiated than those containing 2,4-D, and the highest level of 2,4-D tested produced more undifferentiated tissue than the lowest. Thus, 2,4-D suppressed differentiation and this effect increased with increase in concentration. The presence of 2 mg/l 2-NOA made no significant difference to the amount of undifferentiated tissue present and behaved in a similar way to the medium lacking 2,4-D. Presumably, a much higher concentration of 2-NOA would be needed to produce the same suppression of differentiation as found with 2,4-D.

Fig.2.29,A : Percentage Increase in Fresh Weight of Wheat Callus Tissue after 3 weeks on Media varying in 2,4-D and 2-NOA content. (x + SE).

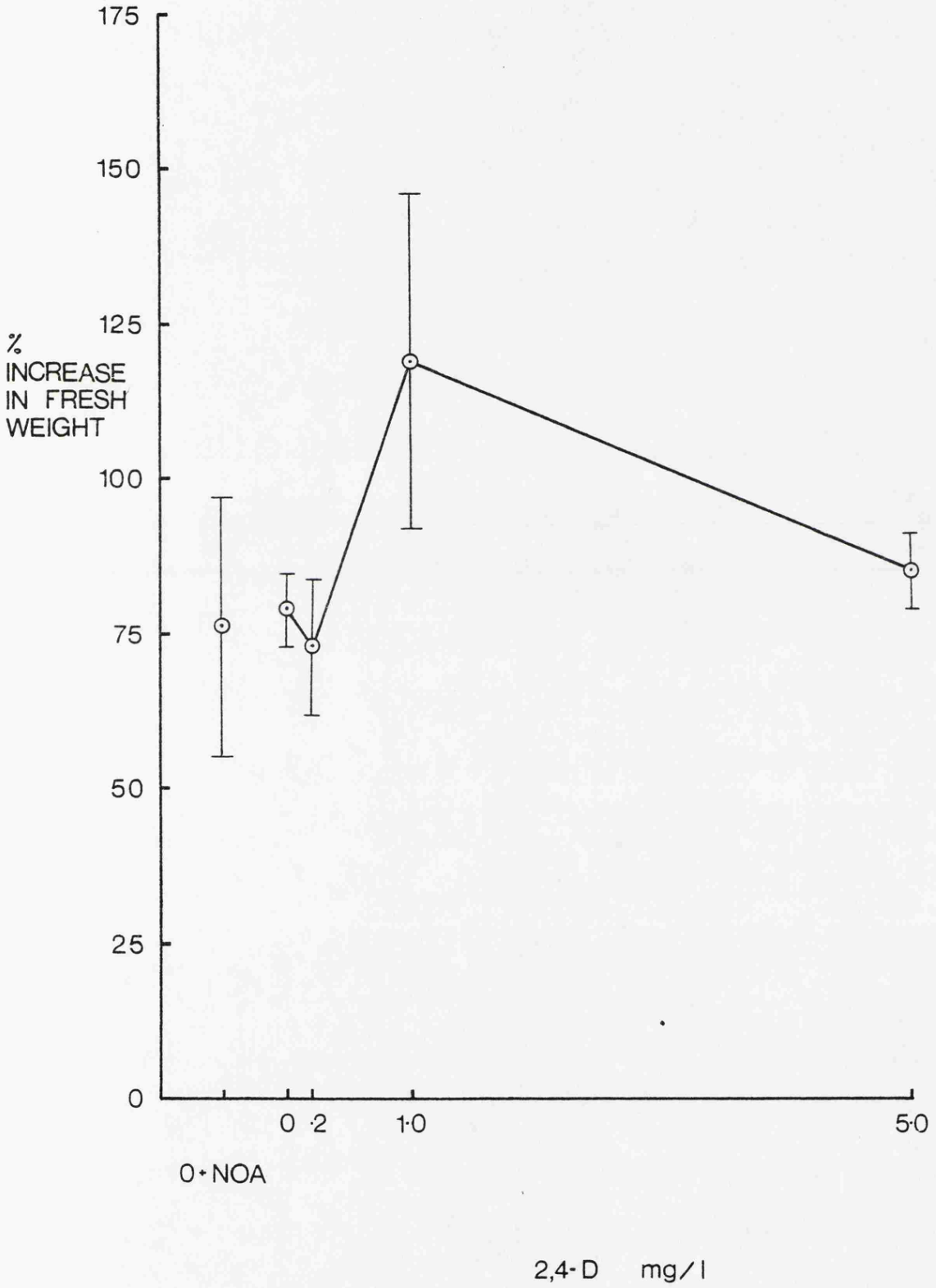


Fig.2.29,B : Dry Weight as a Percentage of Fresh Weight of Cultures after 3 weeks on Media varying in 2,4-D and 2-NOA content. (x + SE).

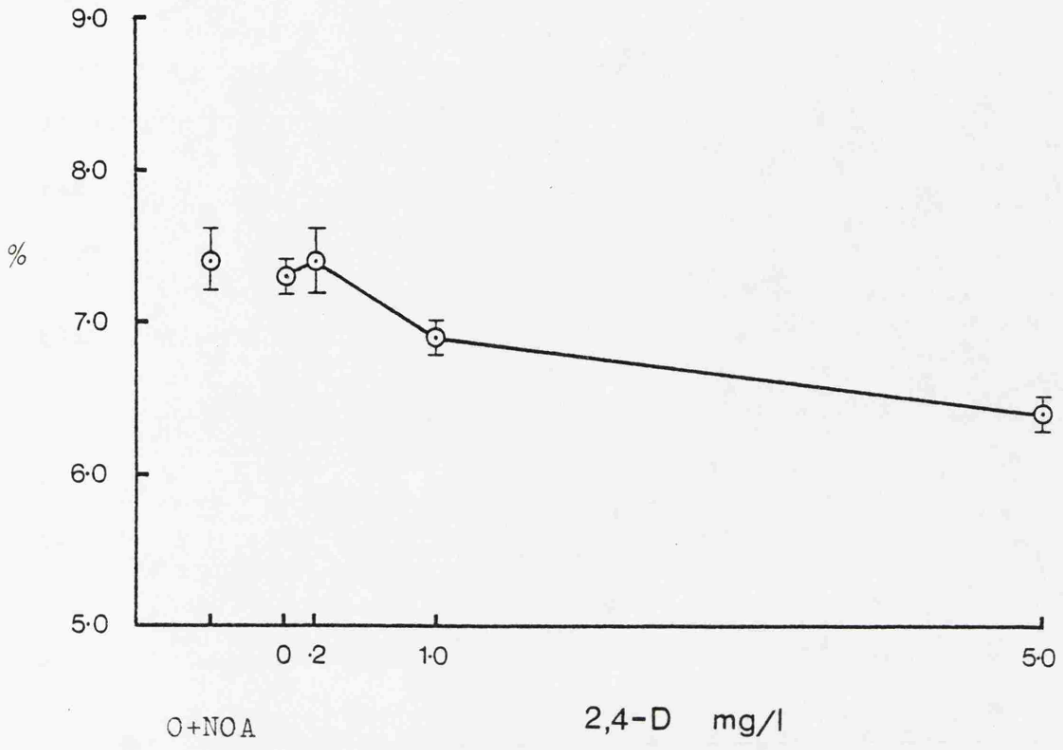
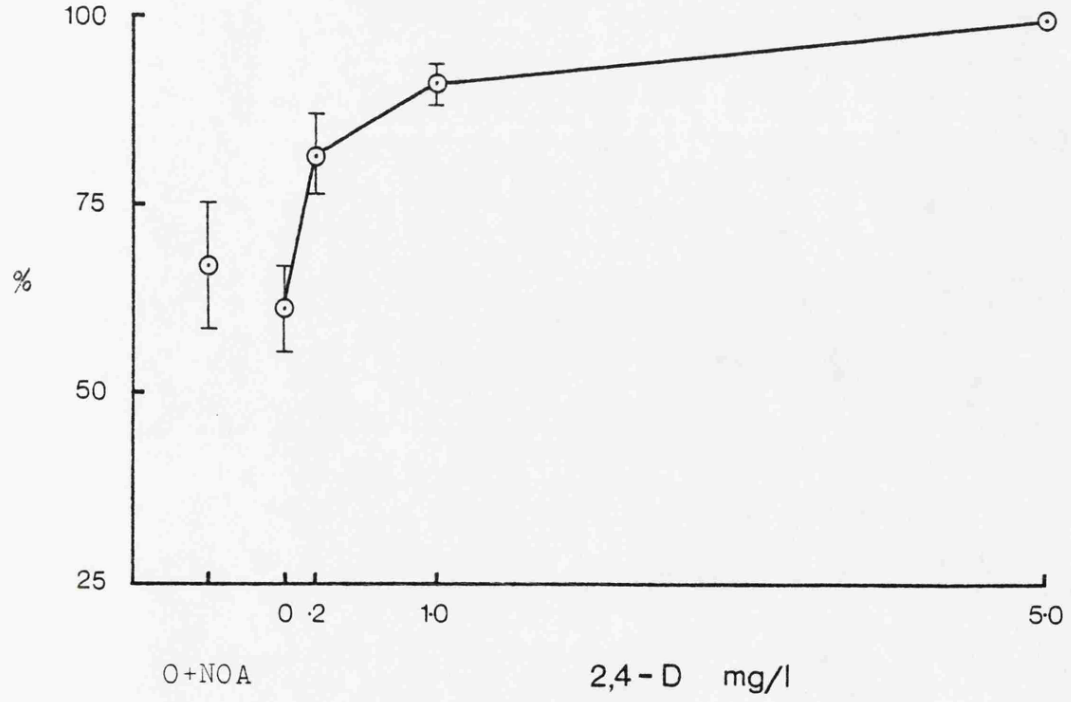


Fig.2.29,C : Percentage of Undifferentiated Tissue after 3 weeks on Media varying in 2,4-D and 2-NOA content. (x + SE).



The experiment showed that 2,4-D must be removed completely to obtain maximum differentiation. After 3 weeks in the absence of auxin, about 40% of the callus had differentiated to roots. The 5 replicates of each treatment left undisturbed continued to differentiate. After 8 weeks, most of the cultures had many green roots and the callus was beginning to dry up and die. Cultures on 5 mg/l 2,4-D showed much less differentiation and any roots present were short.

(iii) The effect of auxin and cytokinin on differentiation

In the following experiment, the effect on differentiation of auxins and cytokinins in different ratios was investigated. The callus used had come from two sources, but in both cases had originated from excised Maris Ranger embryos. Some of the cultures had undergone 5 passages and the rest had undergone 10 passages. Both types were distributed in equal proportions among the 6 media tested. For both sources of callus, the last passage was on "M" plus 1 mg/l 2,4-D (no casein hydrolysate).

Pieces of visibly undifferentiated callus, of fresh weight between 1.0 and 2.0 g, were transferred to the media R1 - R6 shown below, consisting of "M" with the addition of Kinetin and either 1-Naphthalene acetic acid (NAA) or Indole-3-acetic acid (IAA) (no casein hydrolysate).

Medium	NAA	IAA	Kinetin (mg/l)
R1	5.0	-	1.0
R2	1.0	-	5.0
R3	-	2.0	0.2
R4	-	0.2	2.0
R5	-	0.2	0.2
R6	-	2.0	2.0

The cultures were incubated under the high light intensity of Controlled Environment Room I. No attempt was made to weigh the cultures and follow their growth: the cultures were examined at intervals and morphogenetic features noted (Table 2.22, A,B). The terms "browning", "green" and "white" refer to the colour of the callus; some cultures were completely white, in others browning and greening was found in small areas of tissue. "G.sp" refers to very small dark green spots in the callus. All the roots produced were green, with most of this colouring being in the basal portion and continuing as a thin strip, just outside the vascular strands, towards the root tip (the adventitious roots arising from the nodes of wheat stems contain a similar layer of chloroplast-containing cortical cells, just outside the endodermis). "U" refers to cultures which appeared to be completely undifferentiated and showed no greening.

After 11 days under high light intensity, 90% of the cultures on all media except R1, showed some degree of differentiation. All the cultures had some white callus. There appeared to be no difference between media R2, 3, 4, 5 and 6 in the number of cultures showing greening or root formation. Fewer cultures had brown areas on media containing low (0.2 mg/l) Kinetin (R3, R5). In the whole experiment, only one culture produced shoots (R6) and these occurred as a small clump on one area of callus (roots were also present on the same culture, but no greening). Fewer cultures on R1 showed differentiation.

After 33 days, a larger number of cultures on all media showed browning. Nearly all the cultures had produced roots, except on R1 where 50% were still completely undifferentiated. No further cultures had formed shoots, and the original shoot-producing callus did not have

Table 2.22 : Characteristics of Cultures incubated under High Light Intensity on 6 Media, differing in their Auxin and Cytokinin content.

Media	Total replicates	% of Cultures having the characteristics indicated :						
		Browning	Green	White	G.sp.*	roots	shoots	U**
A) After 11 days.								
R1	20	55	40	100	0	50	0	50
R2	18	50	83	100	22	83	0	11
R3	20	10	90	95	25	85	0	10
R4	20	40	85	100	15	90	0	10
R5	18	6	89	100	22	83	0	11
R6	19	21	84	100	16	79	5***	11
B) After 33 days.								
R1	16	63	13		0	44	0	56
R2	16	50	100	not	0	94	0	0
R3	16	38	94	sco-	13	100	0	0
R4	17	47	94	red	29	100	0	0
R5	19	37	89		47	100	0	0
R6	14	86	93		7	100	7***	7

* G.sp = Green spots in callus; ** U = completely Undifferentiated callus;

*** figure represents one Culture only. R1 - R6 = 6 Media - see text.

any more areas of callus with shoots. Again, all the media had produced nearly the same response, with the exception of R1, where the high level of auxin (5 mg/l NAA) was still inhibiting differentiation in most of the cultures. After a total of 10 weeks there were still some cultures on R1 completely undifferentiated. By this time most of the other cultures were a mass of roots and any callus present was browning and dying. Green areas had mainly turned brown, though parts of the roots submerged in the agar were still green.

Although some of the original callus had been maintained in culture for well over a year, and the rest for 5 months, no difference was observed in response of these: the older callus was as capable of differentiation as the younger callus. Only one instance of regeneration occurred, and this was in a callus which had previously undergone 5 passages. Because shoot formation only occurred in one culture, and, as will be shown later, shoot formation had also been observed under many different experimental conditions, it seems unlikely that the regeneration was entirely due to the medium involved (2.0 mg/l IAA + 2.0 mg/l Kinetin). However, this would need to be tested further, using larger numbers of replicate cultures on the same medium, R6. The same 6 media were used for cultures in liquid medium, and the subsequent effect on differentiation is discussed in a later section (P. 99).

(iv) Organogenesis in cultures derived from immature wheat embryos

Recent work by Green & Phillips (1975) reported the regeneration of complete plants of Zea Mays from callus obtained by proliferation of the scutellum of immature embryos. The method involved excision of young embryos (14 - 24 days after pollination) to modified Murashige & Skoog medium, containing 2 mg/l 2,4-D. The embryos were

placed with the plumule-radicle axis in close contact with the medium, and as a result only the scutellum dedifferentiated to form callus. Plants were regenerated after subculture of scutellar callus to 0.25 mg/l 2,4-D for 30 days, followed by transfer to medium lacking 2,4-D. Small leaves first appeared on low 2,4-D. Roughly 200 plantlets were obtained from one particular line of callus, and some cultures remained capable of producing shoots for 19 months.

In view of this remarkable success of regeneration from maize callus, two attempts were made to repeat the experiments of Green & Phillips, as closely as possible, using immature wheat embryos. The embryos were placed on the same basic medium as described ("G" medium, see Appendix for complete formula) and were given the same passage length of 28 days. However, one difference in culture conditions was that the light intensity was 1200 lux (maximum) instead of 2000 lux. The major difference lies in the fact that the scutellum of the young wheat embryos did not proliferate so that the callus which did form came from embryo tissues. The initiation of callus from the immature embryos in both experiments has already been described (pp 41, 42), and passages II, III and IV are described below.

In the first experiment, immature embryos were excised from one variety of wheat, 6 wuk 4, and placed on Green & Phillips' medium* containing either 2 mg/l 2,4-D ("G") or 2 mg/l CPA ("GC"). Embryos were taken 14 - 25 days after anthesis. Cultures were incubated under high light intensity throughout the experiment. At the end of the first passage, which lasted 5 weeks, undifferentiated tissue was separated from any shoot or root extension growth from the embryo, and placed on fresh medium of the same composition. At the end of Passage II (4 weeks)

*by mistake, containing 1.98 mg/l asparagine instead of 1.98 g/l used by Green & Phillips.

again only undifferentiated tissue was transferred, this time to "G" medium with or without a low level of 2,4-D (0.25 mg/l). Much differentiation occurred during Passage III, and at the end (8 weeks) pieces with greening of callus or roots "g/r" were transferred to 'G' lacking auxin in an attempt to obtain further organogenesis. The table below summarises the events during this experiment. For comparison with the immature embryos, some mature Maris Ranger embryos were excised and underwent the same treatments.

Passage	Medium	Passage Length	Inoculum
I	G (2mg/l 2,4-D) or GC (2mg/l CPA)	5 weeks	Excised embryo
II	Same as PI	4 weeks	Undifferentiated callus
III	± 0.25mg/l 2,4-D	8 weeks	Undifferentiated callus
IV	No auxin	8 weeks	Differentiated "g/r"

In each of the passages, no marked differences were noted between immature embryos of different "ages" on the same medium, so to aid interpretation of results, all of the immature embryos were classed together (Table 2.23). The characteristics of the cultures were recorded at the end of each passage as follows:

'G.sp.' - greening of callus and/or roots

'roots' - these were generally green

'Sh.p.' - very small dark green areas in the callus which appeared to have the beginnings of organisation (possible shoot initials)

'shoots'- these occurred in clumps or alone, always green

'U' - completely undifferentiated cultures

Table 2.23 : Characteristics of Cultures derived from excised Wheat Embryos, over 3 Passages on different Media.

former medium	present medium	Total replicates	% of Cultures having : G.sp	roots	Sh.p	shoots	U
PASSAGE II (after 4 weeks)							
Immature							
G	G	74	32	3	38	0	35
GC	GC	74	4	0	12	0	85
Mature							
G	G	12	100	0	25	0	0
GC	GC	4	0	0	50	0	50
PASSAGE III (after $4\frac{1}{2}$ weeks)							
Immature	*						
G	+	27	100	85	7	0	0
G	-	32	97	91	9	3	0
GC	+	20	100	40	15	0	0
GC	-	21	95	90	24	19	0
Mature							
G	+	6	100	67	33	0	0
G	-	5	100	100	40	20	0
GC	+	1	100	100	0	0	0
GC	-	1	100	100	100	0	0
PASSAGE IV (after 10 days)							
Immature	**						
G+	-	23	96	96	30	0	0
G-	-	27	96	100	11	0	0
GC+	-	12	100	100	25	0	0
GC-	-	15	100	100	20	0	0
Mature							
G+	-	5	100	100	20	0	0
G-	-	4	100	100	75	50	0

* ='G' ± 0.25mg/l 2,4-D; ** = 'G' minus hormones.

U= visibly undifferentiated callus; G.sp, roots, Sh.p, shoots - see Materials & Methods.

At the end of Passage II, 2 mg/l CPA had inhibited differentiation to a greater extent than the same level of 2,4-D. However, the high light intensity enhanced greening in the cultures. Plate VI shows cultures at the end of Passage II. Transfer of undifferentiated callus to either low 2,4-D or no auxin led to much differentiation in P III. Cultures which had previously been on medium containing 2 mg/l CPA now showed more differentiation on medium lacking auxin: the presence of 0.25 mg/l 2,4-D was suppressing organisation in many cultures. A similar difference was not observed for cultures previously on medium with 2 mg/l 2,4-D. Regeneration of shoots was only found on medium lacking 2,4-D, and more shoot-producing cultures were found where the previous medium was "GC". The mature embryos responded in a similar way to the immature embryos. The hormone content of the medium used in the previous passage had an important influence on differentiation. By the end of P III, all the cultures were differentiated; no further shoots had formed. Pieces containing roots and greening were transferred to "G" lacking auxin to encourage further regeneration. Two of the cultures from mature embryos produced plantlets, but otherwise no further regeneration occurred. During the 8 weeks of P IV, the callus gradually turned brown and dry, and green areas also became necrotic. Roots grew well and remained green. Altogether, 8 cultures produced plantlets; 3 of these were successfully grown to maturity, and produced flowers, but only a few seeds set. An attempt was made to count the chromosomes of the root tips obtained after germinating grain from one plant and staining as described in Materials & Methods (P. 26). The mitotic figures counted had rather variable chromosome numbers, with a mean of 27. This is much lower than expected since the original callus came from a hexaploid wheat (6 wuk 4). Unfortunately, it was not possible to

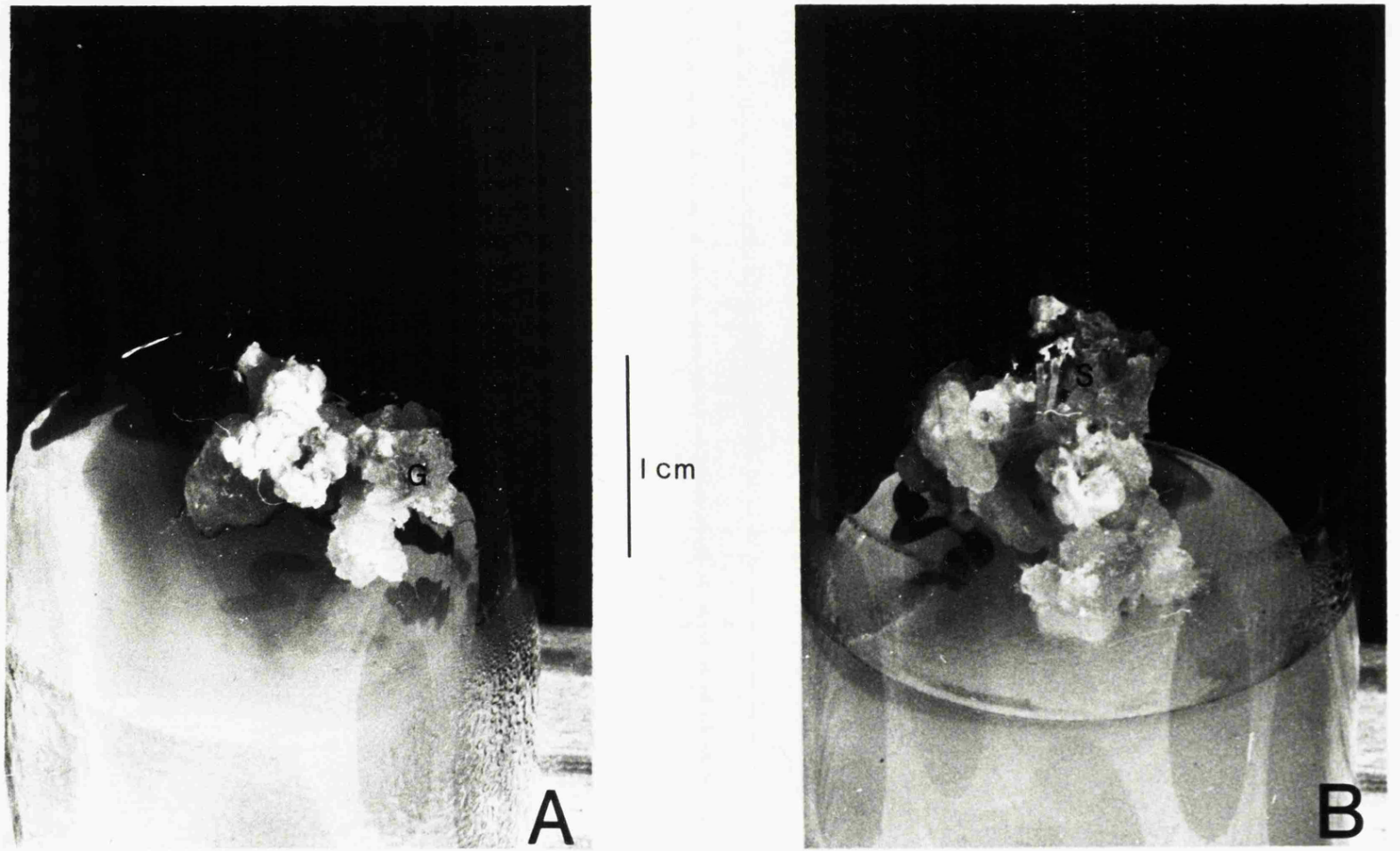
Plate VI

Cultures derived from immature wheat embryos, showing appearance after incubation for 4 weeks on medium 'G', at the end of Passage II.

A. Callus derived from an embryo excised 20 days after anthesis shows no organisation, except for an area of green (G) callus tissue.

B. Callus derived from an embryo excised 23 days after anthesis; the region marked 'S' is green and contains a possible shoot initial at the top.

Plate VI



do chromosome counts for more of the plants regenerated so it is by no means certain that such anomalous chromosome complements were present in these also.

Since the results obtained in this experiment were considered inconclusive, a further, more elaborate experiment was carried out. In this following experiment, the influence of several factors on differentiation (with reference especially to regeneration of plants) was investigated.

In Passage I, which has already been discussed in an earlier section (P.42), embryos (with the scutellum attached) were excised at different stages of development (9, 10, 12 and 14 days after anthesis) from 2 varieties of American Dwarf Wheat, 6 wuk 3, and 6 wuk 4. The initiation of callus was investigated using Green & Phillips' 1975 ("G") medium, with different additions of 2,4-D and 2iP as shown in Table 2.13. reproduced below:

Table 2.13: the 7 media used.

Medium	Asparagine	2,4-D (mg/l)	2iP (mg/l)
1	1.98 g/l	0.1	-
2	"	0.1	5.0
3	"	0.5	5.0
4	"	0.5	-
5	"	2.0	-
6	"	2.0	5.0
7	1.98 mg/l	2.0	-

Medium 7 is the same as "G" of the previous experiment. At the end of Passage I, it was noted that the highest level of 2,4-D tested, 2.0 mg/l, was the most suitable for callus formation: the two lower levels allowed too much root and shoot extension of the original embryo at the expense of disorganised cell division. Embryos older than 10 days after anthesis seemed to form callus more quickly. For the next passage,

only 2.0 mg/l 2,4-D was used, with or without 2iP (Media 5,6,7).

A certain number of the excised embryos only formed a very light covering of callus during Passage I, too small to be separated from the explant. These cultures, "A", also showed no visible development of the roots or plumule from the embryo, and so they were transferred whole to fresh medium. "A" type cultures were most common on low 2,4-D and among the 'younger' embryos. For the rest of the cultures, "U" transferred to Passage II, visibly undifferentiated callus was separated from the rest of the explant and placed on fresh medium. The treatments for the cultures over the 4 passages are shown in the table below.

Treatment over several passages:

Passage	Medium	Type of Inoculum	Passage Length
I	Media 1 - 7	Excised embryos + scutellum	5 weeks
II	Media 5,6,7	1) "A"; 2) "U"	4 weeks
III	Minus 2,4-D &/or 2iP	1) "A"; 2) "U"; 3) "D"	4 weeks
IV	Minus 2,4-D &/or 2iP	1) "U"; 2) "D"	4+weeks

At the end of P II, a certain amount of differentiation had begun, so, as well as "A" and "U" type pieces being transferred, pieces of callus showing greening were also transferred ("D"), to medium lacking 2,4-D and 2iP. Most differentiation occurred during P III, and again at the end of this passage "U" and "D" (this time consisting of greening and roots) pieces of callus were transferred to more medium lacking hormones. Table 2.24 shows the appearance of the cultures at the end of each passage. By keeping a record of the appearance of the culture

Table 2.24A : Characteristics of Undifferentiated (U) and Differentiated (D) inocula at the end of each of 3 Passages.

Variety		Total replicates	% of Cultures with appearance shown:					
			U	roots	shoots	brown	white	green
PASSAGE II	medium							
U 6 WUK 3	5	98	98	1	1	5	99	22
	6	33	91	5	0	55	58	27
U 6 WUK 4	5	92	93	7	0	2	99	20
	6	67	94	3	0	49	51	9
	7	19	89	11	0	21	89	16
PASSAGE III	former medium		U	roots	shoots	G.sp	green	
U 6 WUK 3	5	83	87	7	1	4	11	
	6	27	85	7	7	0	7	
U 6 WUK 4	5	86	81	13	1	1	15	
	6	42	93	7	2	0	2	
	7	21	38	62	0	0	57	
D 6 WUK 3	5	36	53	36	17	14	17	
	6	8	13	88	13	13	63	
D 6 WUK 4	5	31	52	16	0	0	35	
	6	11	27	27	0	9	73	
	7	5	0	100	20	0	80	
PASSAGE IV								
U 6 WUK 3	-2,4-D	92	86	13	1	1	8	
U 6 WUK 4	-2,4-D	105	93	6	0	0	3	
(7)	-2,4-D	4	25	75	0	0	75	
D 6 WUK 3	-2,4-D	35	11	80	9	3	74	
D 6 WUK 4	-2,4-D	30	10	90	7	0	33	
(7)	-2,4-D	19	0	100	0	5	68	

5, 6, 7 = media; see text.

U = visibly undifferentiated callus.

Table 2.24B : Characteristics of Cultures transferred whole at the end of each Passage (A); observations made at the end of 2 Passages.

Variety		Total replicates	% of Cultures with appearance shown:					
			callus	roots*	shoots*	brown	white	green
PASSAGE II	medium							
6 WUK 3	5	32	78	9	19	6	94	13
	6	26	65	0	0	42	62	12
6 WUK 4	5	33	97	0	3	9	91	6
	6	44	70	0	0	48	57	2
PASSAGE III	former medium							
6 WUK 3	5	34	65	47	18	not	not	29
	6	19	63	37	5	sco-	sco-	11
6 WUK 4	5	32	72	38	16	red	red	28
	6	30	57	23	3			10

* roots and shoots here assumed to be extension growth from the original embryo tissue still present in the cultures.

5, 6 = media; see text.

on transfer to fresh medium it can be seen whether "U" or "D" cultures will further differentiate most.

At the end of Passage II, only a few of the "U" cultures transferred had produced roots and one had formed a shoot. There was little difference between Media 5,6 & 7 in amount of differentiation but more browning of cultures was observed on Medium 6 (+ 2iP). At the end of P III (on medium lacking 2,4-D and 2iP), much more differentiation had occurred and several cultures (12) showed shoot formation (see Plate VII). Shoot and root formation occurred on a higher proportion of cultures which had shown greening on transfer ("D") compared to those completely undifferentiated. Medium 7 (with low level of asparagine) seemed to favour rhizogenesis and greening. There was little evidence that the previous medium (containing 2,4-D and 2iP) had influenced the response. More of the 6 wuk 3 cultures tended to show differentiation than those of 6 wuk 4. After 3 weeks on P IV, again on medium lacking hormones, little further differentiation occurred. A few (5) more cultures produced shoots, and several formed roots. Most of the "U" cultures remained undifferentiated.

In the case of the "A" cultures, transferred whole at each passage, Table 2.24,B shows that at the end of P II, root and shoot elongation from the original embryo explant, occurred in both varieties on Medium 5 only. Again, cultures on Medium 6 showed more browning, and more of the 6 wuk 3 cultures showed differentiation. At the end of P III more cultures showed root and shoot extension on both media. Removal of 2,4-D and 2iP from the medium in P III led to extension of the original embryo axis which had been 'dormant' for two passages.

Plate VII

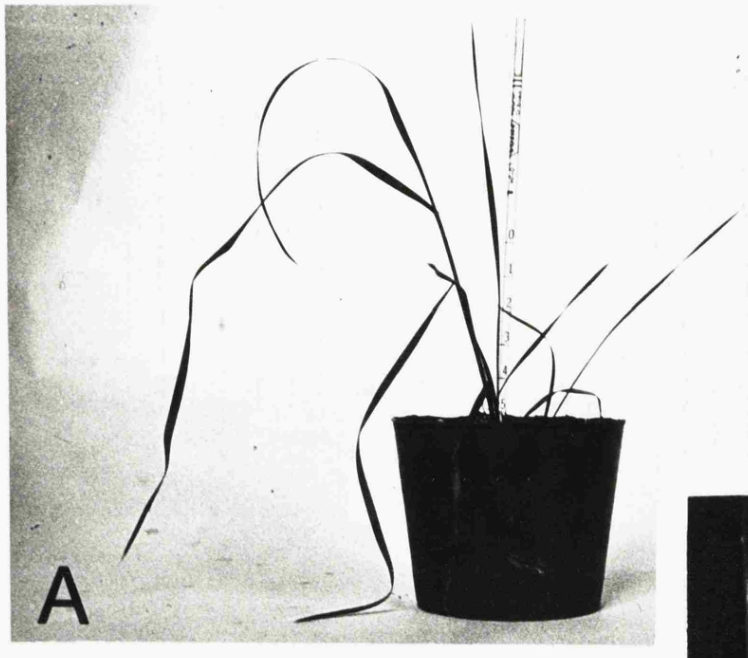
Plants regenerated from cultures derived from immature wheat embryos.

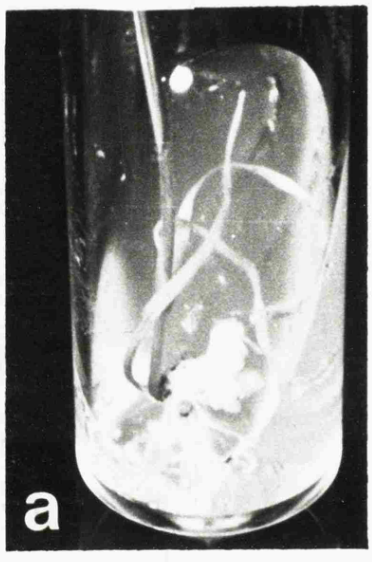
A,a. 6 WUK 3 wheat; callus originated from embryo excised 12 days after anthesis.

B,b. 6 WUK 3 wheat; callus originated from embryo excised 10 days after anthesis.

The shoots appeared during Passage III. In 'a', a single shoot and several roots formed from a culture which was visibly undifferentiated (U) on subculture. In 'b', the inoculum contained a green spot (g.sp.), and it was from this that several small green shoots and roots formed. The plantlets were potted straight into peat when larger; 'A' and 'B' show the appearance of the plants 3 weeks after potting (about 2 months after they first appeared).

Plate VII





15cm

To summarise the findings of this experiment:

a) 5.0 mg/l 2iP in the medium caused browning.

b) Slightly more cultures on Medium 5 (+ 2,4-D only) showed differentiation than on Medium 6 (2,4-D + 2iP).

c) Cultures of 6 wuk 3 variety tended to differentiate more readily than 6 wuk 4.

d) More root formation occurred than shoot formation.

e) Shoot formation was found on more cultures showing some differentiation of roots and greening when transferred ("D"), but completely undifferentiated cultures were also capable of producing roots and shoots.

f) Transfer of whole "A" cultures showed how the shoot and root primordia present in the original excised embryo may remain 'dormant' for two passages. Shoot and root extension could occur either in the presence of 2 mg/l 2,4-D or (more readily) in the absence of growth hormones.

Although several plantlets were obtained from this experiment (Plate VII), again, the success rate was small compared to that with maize in Green & Phillips' experiment. However, it has already been noted that although similar conditions of high light intensity, passage length and medium were used, the callus which did proliferate originated from the embryo tissues rather than the scutellum (as with maize). It may be that if the scutellum tissue could be induced to proliferate, then the amount of regeneration would be much improved.

(v) Plantlet formation in many different experiments

Shoot formation, as well as the more common root formation, occurred in small numbers of cultures in many different experiments. Table 2.25 contains the relevant data from 12 of these experiments, some of which have already been partly discussed in previous sections

Table 2.25 : Summary of the Experiments where Shoot Formation occurred.

Original Source of Callus	Passage when Shoots appeared and duration	Previous Medium; Passage length	Type of inoculum : "U" or "D"	Medium on which Shoots developed	Number of Cultures having Shoots*	Passage where further Shoots developed
Exp. A. Maris Ranger Embryos	II 5½ weeks	i)"M" (no Inositol, vitamins) ii)"M" (no Inositol, Vitamins or FeEDTA) i) and ii) + 1mg/l 2,4-D (10½ weeks)	U U	"M" + 1mg/l 2,4-D "M" + 1mg/l 2,4-D	1 (8) 1 (8)	PIII (below)
	III 19 weeks	"M" + 1mg/l 2,4-D (5½ weeks)	U†	"M" + 1mg/l 2,4-D (no Inositol or Vitamins)	2 (21)	-
Exp. B Maris Ranger Embryos	II 5 weeks	i)"M"+ 1mg/l 2,4-D ii)"M"+ 1mg/l 2,4-D + x10 Vitamins (9 weeks)	U† U	"M" + 1mg/l 2,4-D "M" + 1mg/l 2,4-D	2 (21) 1 (12)	PIII (below)
	III 5 weeks	"M" + 1mg/l 2,4-D (5 weeks)	D‡	"M" - 2,4-D	1 (32)	-
Exp. C Maris Ranger Embryos	II 9 weeks	i)"M" + 1mg/l 2,4-D ii)"M"+ 1mg/l 2,4-D + 5mg/l Kin. iii)"M"+1mg/l 2,4-D + 2.5mg/l 2iP (6 weeks)	U† U† U†	as PI as PI as PI	2 (8) 1 (6) 3 (11)	-
Exp. D Maris Ranger Embryos	II 5 weeks	"M" + 0.5mg/l CPA (10 weeks)	U†	as PI	1 (15)	PIII (below)
	III 5 weeks	i) "M"+0.5mg/l CPA ii)"M"+1.0mg/l CPA iii)M"+6.0mg/l CPA (5 weeks)	U† U† U†	as PII as PII as PII	1 (11) 3 (13) 1 (3)	PIV (below)
	IV 4 weeks	i)"M"+1.0mg/l CPA ii)"M"+3.0mg/l CPA iii)M"+4.0mg/l CPA (5 weeks)	D‡ D‡ D‡	"M" minus auxin "M" minus auxin "M" minus auxin	2 (2) 2 (2) 1 (1)	PVI (below)
	VI 5 weeks	"M" + 1mg/l 2,4-D (5 weeks)	U	"M" + 2.0mg/l IAA + 2.0mg/l Kin.	1 (8)	-
Exp. E 6 WUR 4 Immature Embryos	III 8 weeks	"G" + 2mg/l 2,4-D or 2mg/l CPA (4 weeks)	U‡	"G" minus auxin	6 (59)	PIV (below)
	IV 4 weeks	"G" minus auxin (8 weeks)	D†	"G" minus auxin	2 (86)	-

Exp. F 6 WUK 3, 6 WUK 4 Immature Embryos	II 4 weeks	"G" + 0.5mg/l 2,4-D (5 weeks)	U	"G" + 2mg/l 2,4-D	1 (164)	PIII (below)
	III 4 weeks	i)"G"+ 2mg/l 2,4-D; ± 2iP ii) as i)	U ‡ D ‡	"G" minus hormones "G" minus hormones	5 (259) 8 (91)	PIV (below)
	IV 7 weeks	i)"G" minus hormones ii)"G" minus hormones	U † D †	as PIII as PIII	1 (197) 5 (65)	-
Exp. G Maris Ranger Stem segments	II 10½ weeks	i)"M"+ 1mg/l 2,4-D ii) "T" iii) "N" (5 weeks)	U † U † U †	as PI as PI as PI	3 (12) 1 (14) 2 (18)	PIII (below)
	III 4 weeks	i) "T" ii) "N" (10½ weeks)	D † D †	as PII "T" as PII "N"	1 (14) 1 (14)	PIV (below)
	IV 10 days	"C" (4 weeks)	D ‡	"M" minus hormones	3 (13)	PV (below)
	V 3 weeks	"M" minus hormones (10 days)	D	"M" + 1mg/l 2,4-D	1 (3)	PVI (below)
	VI 3 weeks	"M" + 1mg/l 2,4-D (3 weeks)	D ‡	"M" minus hormones	1 (3)	-
Exp. H Maris Ranger Embryos	II 8 weeks	"M" + 1mg/l 2,4-D (8 weeks)	U †	as PI	1 (8)	-
	III 5 weeks	"M" + 1mg/l 2,4-D (5 weeks)	U †	as PI I	1 (9)	-
	IV 3 weeks	"M" + 1mg/l 2,4-D (3 weeks)	U †	as PI II	1 (9)	-
Exp. I 6 WUK 3 (haploid) Floret seg.	II 8½ weeks	"M" + 1mg/l 2,4-D + 5mg/l CPA (7 weeks)	U †	as PI	1 (2)	PIII (below)
	III 10 weeks	"M" + 1mg/l 2,4- D + 5mg/l CPA (8½ weeks)	U ‡	"M" + 5mg/l CPA	1 (3)	PV (below)
	V 7½ weeks	"M" + 1mg/l 2,4-D (7 weeks)	D ‡	"M" minus hormones	1 (3)	-
Exp. J Maris Ranger Embryos	II 6 Weeks	i)"M" + 1mg/l 2,4-D ii)"MK" (9 weeks)	U † U †	as PI as PI	5 (10) 1 (5)	-
Exp. K M Ranger, Emb	II 10 weeks	"M" + 1.5mg/l 2,4-D (30 weeks)	U	**Caramiello + 0.5 mg/l 2,4-D	1 (2)	-
Exp. L *** "MSxR", Emb.	III 13 weeks	"M"+ 1mg/l 2,4-D + 5mg/l CPA (13 weeks)	D ‡	"M" + 0.1mg/l 2,4-D	1 (5)	-

* total cultures in brackets. "T" = "M"+1mg/l 2,4-D+5mg/l Triacanthine;

"N" = "M"+1mg/l 2,4-D+5mg/l 1-NAA; "C" = "M"+1mg/l 2,4-D+5mg/l CPA; "MK"="M"+1mg/l 2,4-D+5mg/l Kin.

see Appendix for Caramiello medium ; *"MSxR"=Male Sterile x Maris Ranger hybrid wheat.

of this thesis dealing with initiation and growth of callus. The table summarises the factors considered most likely to have influenced shoot production. These include details of the passage previous to the one in which shoots first developed since it was possibly the change in hormonal conditions which led to shoot formation.

In about two-thirds of the cases cited, the inoculum which later developed shoots was visibly undifferentiated ("U") at the start of the passage. In other cases, the pieces of callus transferred were already partially differentiated ("D"), having some root formation and greening of the callus and roots. However, the number of cultures having shoots (with the total number of cultures in brackets) is at a similar proportion of 10% or less for both "U" and "D". Thus "U" cultures are as likely to develop shoots as are "D" cultures, and this raises the possibility that although visibly undifferentiated, the "U" cultures could nevertheless contain organised meristems within the callus mass. Further to this point, in all but 2 of the 12 experiments, shoots first appeared in the second passage. At the end of the first passage, it was routine practice to remove any extension growth of the plumule or radical from the embryo explant, and transfer only visibly undifferentiated tissue. However, this callus quite possibly would contain meristemmatic material from the original embryo (see later section dealing with the formation of callus on the embryo, P. 101). Also, wheat is a tillering plant, so it is highly likely that many small shoot primordia are present at the base of the plumule and these could be transferred within the bulk of the callus. These factors may have an important bearing on the several instances where a single vigorous shoot developed in the culture.

Of the 12 experiments, 7 showed shoot formation in more than one passage. Sometimes the shoots arose from the same individual culture, but more often, different cultures produced shoots in different passages. A total of 42 'sets of conditions' is listed over the 12 experiments; in 12 of these 'sets of conditions' (marked ‡) shoot formation followed transfer from a high auxin level to either a lower level or absence of auxin. In another 23 cases (marked †), however, shoot formation occurred after transfer to the same medium as previously. This shoot formation could occur in the presence of auxin and cytokinins; indeed, in Experiment D (P III), Experiment G (P II & III), and Experiment I (PII & III), the shoots developed in the presence of over 5 mg/l of auxin. This is in direct contrast to rhizogenesis which was only observed to occur where the level of auxin was low or absent. In these 3 experiments mentioned above, shoot formation occurred without the formation of roots if CPA was present in the medium.

Plate VIII shows the appearance of several regenerating cultures (details of which are described in the legend accompanying the Plate), and illustrates the variety in the proportion of roots, shoots and callus present.

In the majority of cases cited in the 12 experiments above, the shoots arose as a clump from deep within the callus mass and were already quite vigorous by the time they had pushed their way to the surface and were visible. It is this type of shoot formation which is suggestive of origination from a primordium within the callus mass. We have already observed in a previous experiment (P.86) using immature embryos, that the primordia of the embryo may remain dormant for several passages before developing into shoots. This type of shoot

Plate VIII

The appearance of several cultures of wheat in the process of regenerating plantlets, under many different conditions. See Table 2.25 for details of experiments.

A. After 4 weeks on "M" + 1mg/l 2,4-D (PII, Exp.J), a few roots have formed, and a small, curled green shoot can be seen near the surface of the agar.

B. After 5 weeks on "M" + 1mg/l 2,4-D, lacking inositol and vitamins, (PIII, Exp.A), 3 clumps of small green shoots are visible, and a few white roots have formed at the top of the culture.

C. After 8 weeks on "M" + 1mg/l 2,4-D + 2.5mg/l 2iP (PII, Exp.C), a vigorous clump of shoots and roots have formed.

D. After 3 weeks on "M" lacking auxin (PIII, Exp.B), the culture, which had roots and greening when transferred, has produced long green shoots and several roots.

E. After 10 weeks on "M" + 1mg/l 2,4-D (PII, Exp.G), the culture has vigorous green shoots. Note the long root hairs covering the roots.

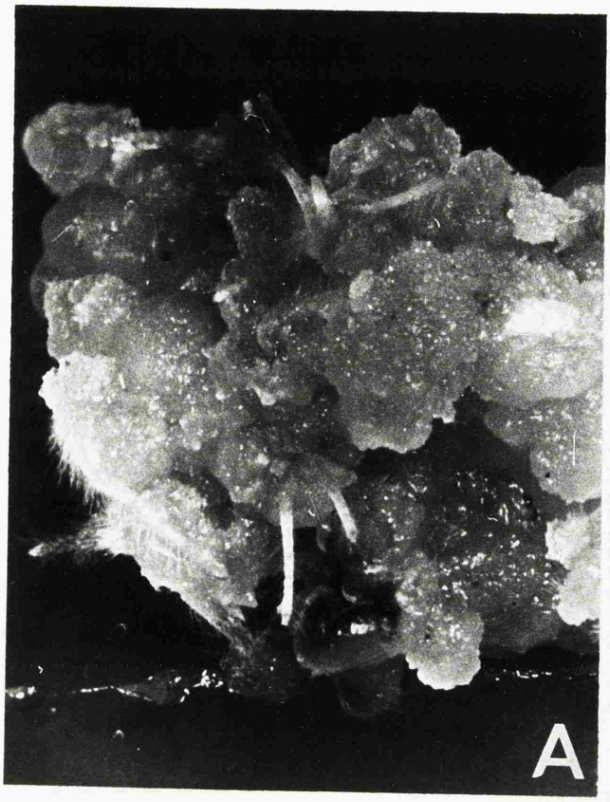
F. After 3 weeks on "M" lacking auxin (PV, Exp.I), the inoculum, which contained several green spots (g.sp.) when transferred, now has developed tiny leaves (S) from these.

G. After 10 days on "M" lacking auxin (PIV, Exp.D), the g.sp. transferred in the inoculum has developed into a small, sturdy shoot; a root has also formed and little callus is left.

H. After 10days on "M" lacking auxin (PIV, Exp.D), the g.sp. transferred in the inoculum now has a green, leaf-like appearance. A patch of hairs (H) marks the beginning of a root.

'G' and 'H' were subcultured at the same time to the same medium, but while 'G' had formerly been on medium containing 1mg/l CPA, 'H' was on medium with 3mg/l CPA and shows some inhibition of organogenesis.

Plate VIII



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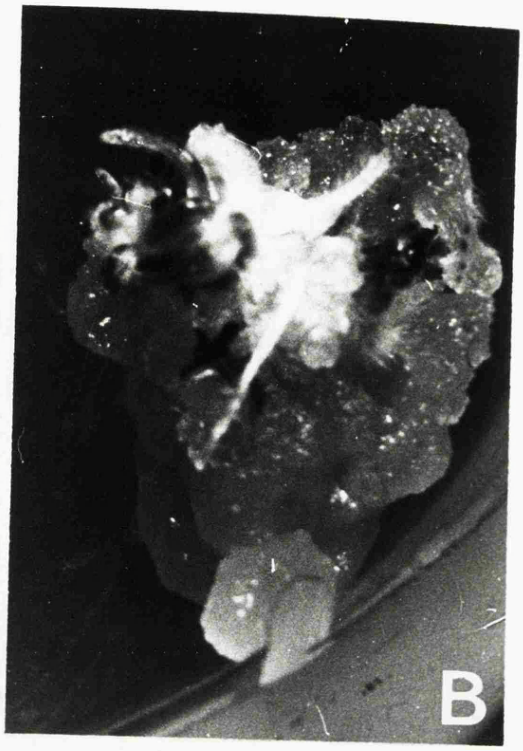

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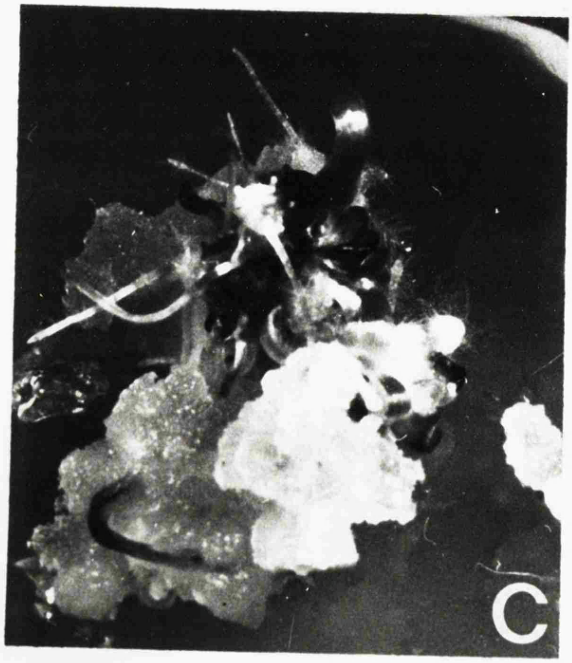

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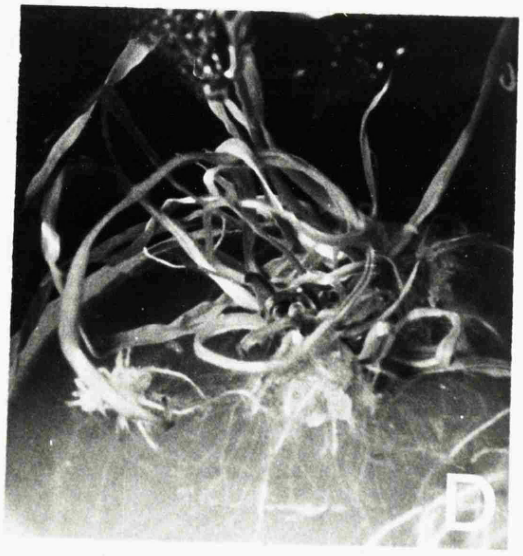

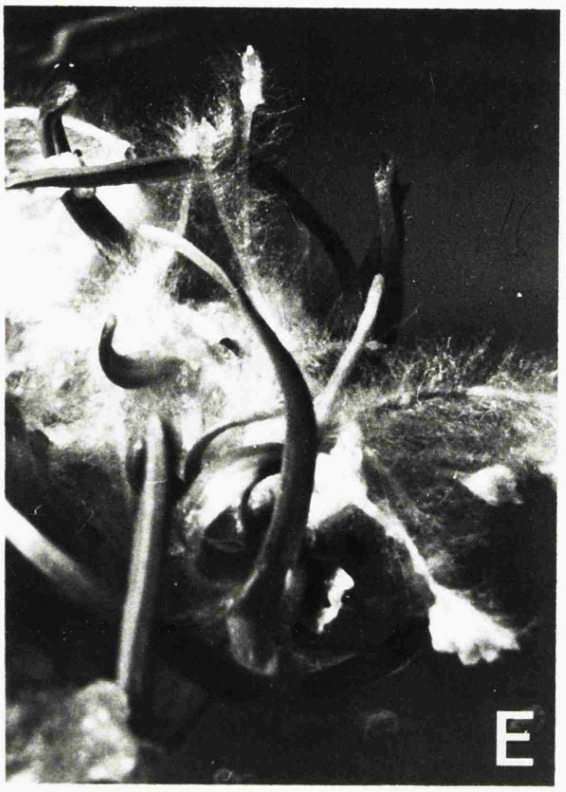

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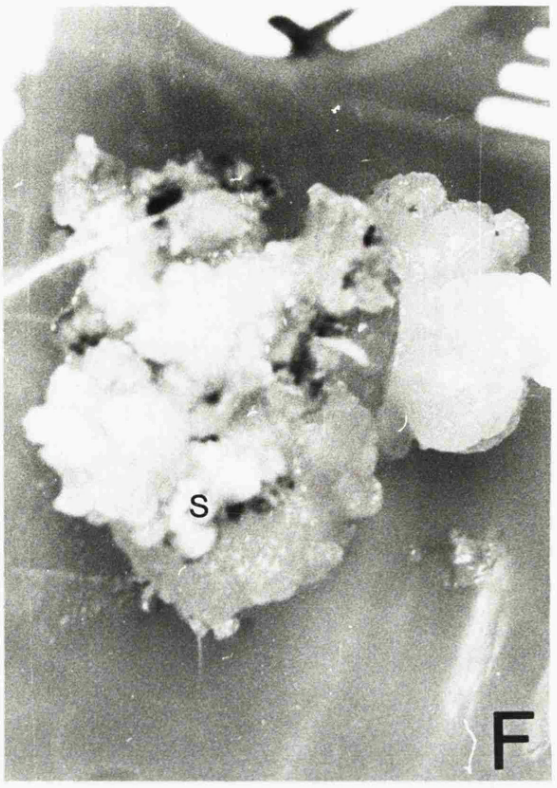

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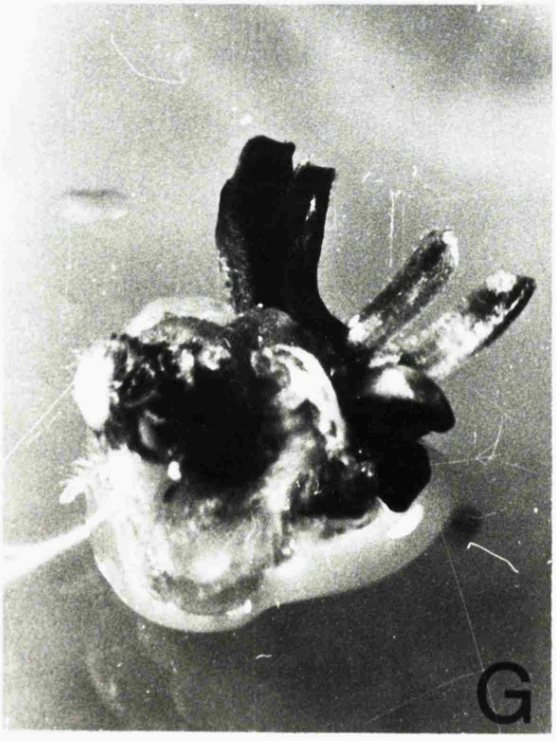
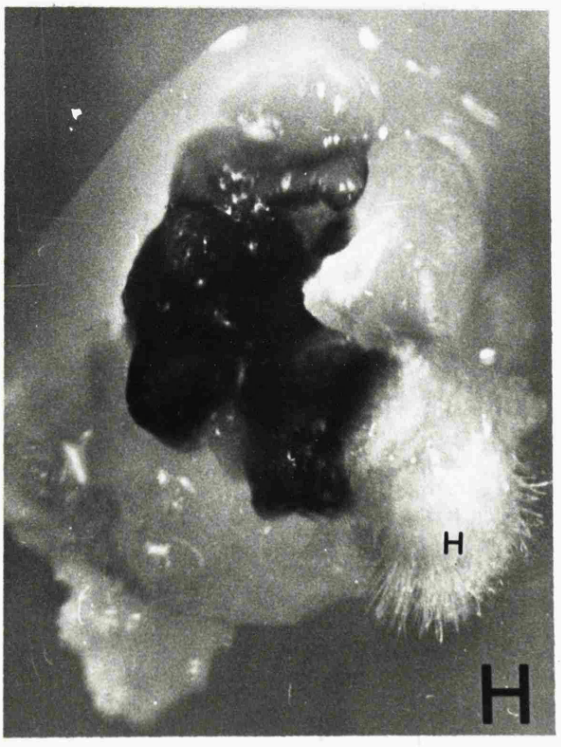




development was found mainly in Passages II and III, but was also the type found in P VI of Experiment D. If these cultures were left undisturbed, then in some cases, further patches of shoots would occur elsewhere in the culture; however, more often, no further shoots formed. Shoot formation seemed independent of root formation, although it was noted that shoots did not occur on cultures where many roots were present. Roots would form whether or not shoots were present, as long as the auxin level was low enough.

The second type of shoot formation was more accurately leaf-formation or phyllogenesis and is further described in a later section dealing with the anatomy of shoot-bearing callus. In this type of shoot formation, small leaves were visible on the surface of the callus, (see Plate VIII 'F' & 'H'), often in several areas of the cultures. In some cases, these had developed from "green spots" transferred in the inoculum. However, the problem was that there were different types of 'green spot' and these were not distinguishable at an early stage. This is one reason why the "D" pieces of callus transferred did not produce a higher percentage of shoots. The greening which occurred at the base of the roots was not observed to lead to shoot formation. It was the greening of small areas of callus away from the roots which in some cases did give rise to shoots, and this was more likely if the green spots were very dark green. However, in Experiment B, where several "D" inocula containing these dark green spots were placed under high light intensity, only one culture developed shoots. In the other cultures, the dark green spots gave rise to fat dark green roots, of a type not previously found in culture. These became paler green as they elongated.

To summarise, the nature of the factors responsible for shoot formation remains unclear following this survey of the conditions under which shoots developed. However, there are strong indications that many of the instances of shoot formation could be due to expression of previously-suppressed primordia already present within the callus mass. In cases where shoot formation occurred from callus originating from stem segments on the inflorescence of the mature plant (Experiment G & Experiment I), this explanation seems less probable, especially since in these experiments some of the shoots arose first as leaf-like outgrowths from the callus surface.

Much more investigation needs to be carried out into the problem of shoot production from callus. The findings of other workers in this field will be described in the Discussion, but from the present study of regeneration, it seems that actual induction of shoot development may only have occurred in a few cases and cannot be attributed to a particular set of conditions. In order to eliminate the confusing effects of the presence of 'dormant' primordia, we can conclude that regeneration studies would be better carried out using tissue from parts of the plant unlikely to contain such primordia.

(vi) Stages in plantlet development

Plate IX shows a series of possible steps in the development of shoots and roots leading to plant regeneration, starting from "green spots".

The small pieces of callus in A - D were taken from a highly-differentiated culture which was a mixture of small shoots, callus and roots, similar to the culture shown in F. The callus was

Plate IX

A series of possible stages in the development of shoots and plants. (see text)

A. Green ridges on a small piece of callus also having 3 roots.

B. The other side of 'A': no organised structures are present.

('A' and 'B' are a culture previously on medium containing 4mg/l CPA, and transferred with green areas to medium lacking auxin).

C. Small piece of callus having shoots only.

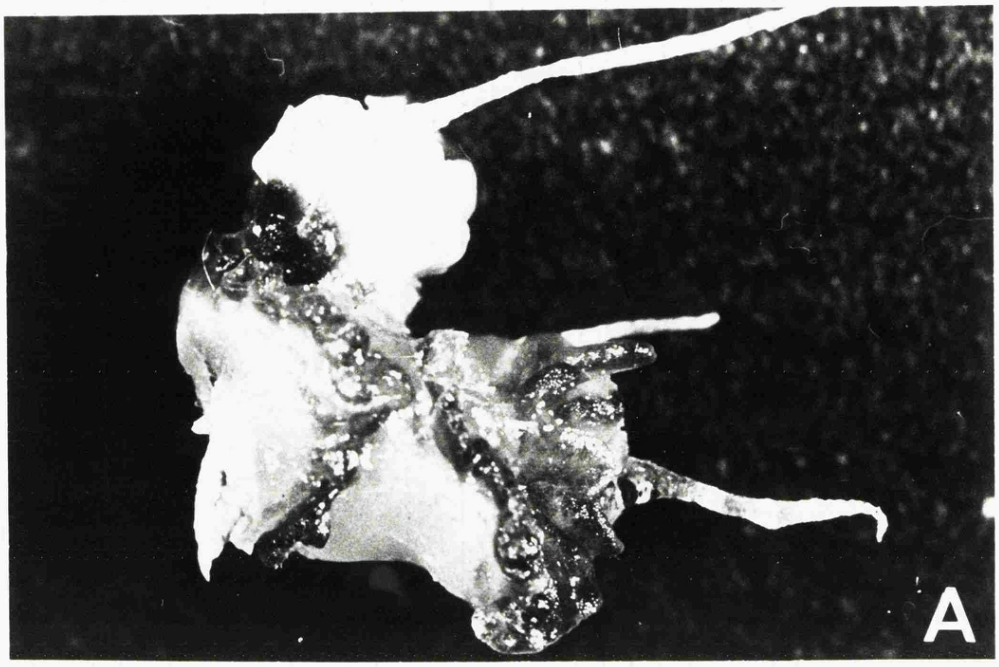
D. Small piece of callus having both shoots and partly-formed roots.

('C' and 'D' are from cultures incubated on medium lacking auxin, having been previously on medium containing 1mg/l 2,4-D plus 5mg/l CPA)

E. and F are well-differentiated cultures, being a mixture of small shoots, roots and a few pieces of callus. Both were transferred to medium lacking auxin in a partially-differentiated state, 'E' had small shoots and 'F' had green spots, and further organogenesis has occurred in the absence of auxin.

When the shoots which have developed in culture look vigorous and have some roots associated with them, they can be transferred to either a large tube (G) with a filter paper bridge, or to vermiculite in a 250ml flask (H) in both cases with liquid medium to encourage roots and retain humidity. The plantlets are gradually introduced to less humid conditions by removing the bung (I) several days before transferring to well-watered peat (J).

Plate IX



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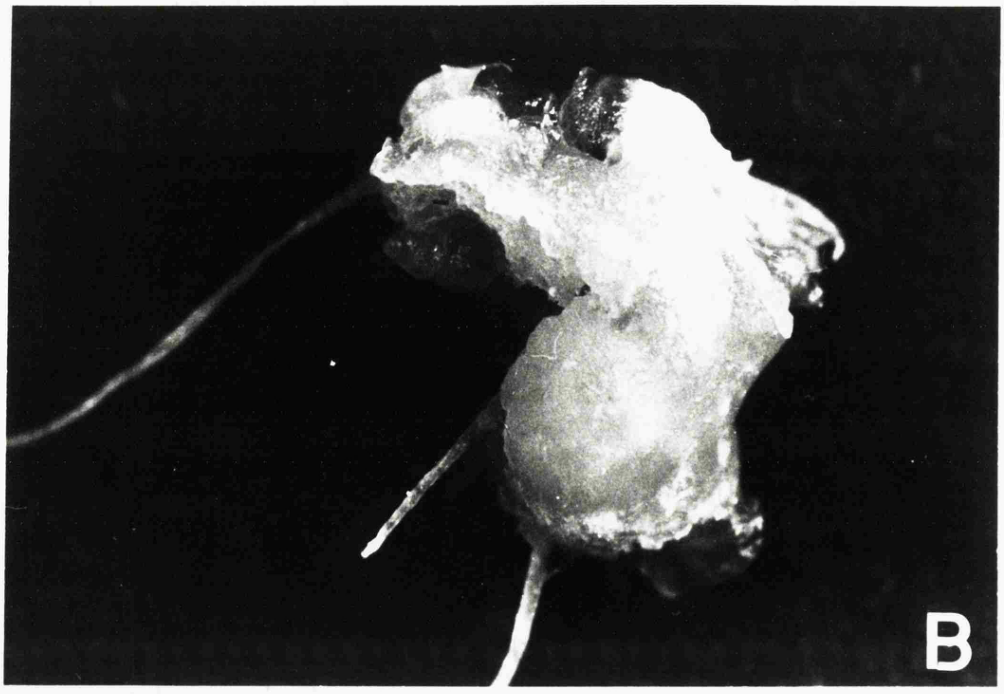

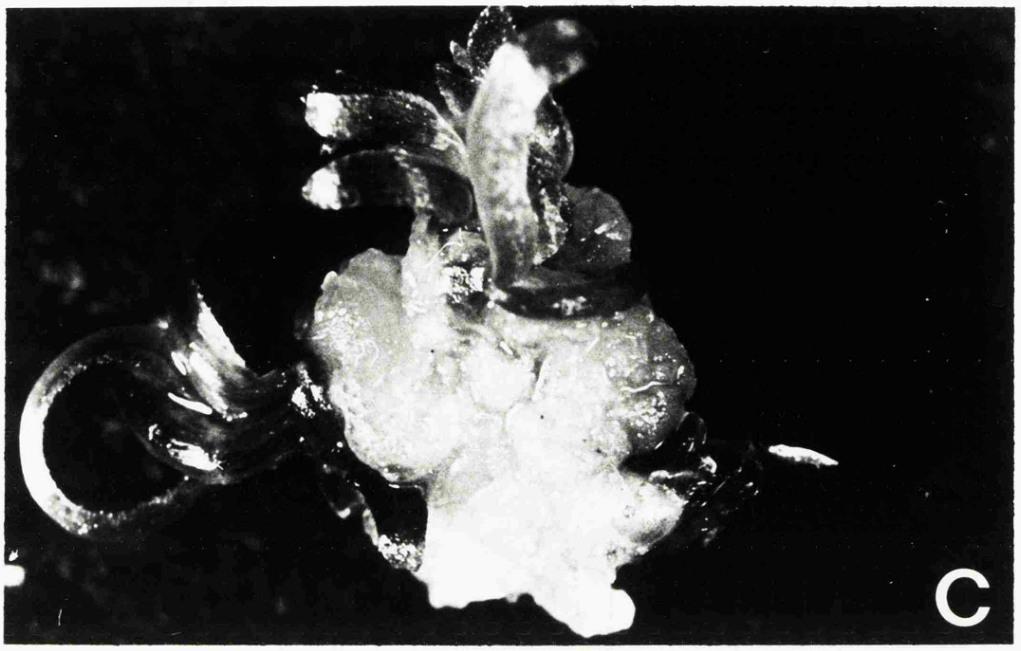

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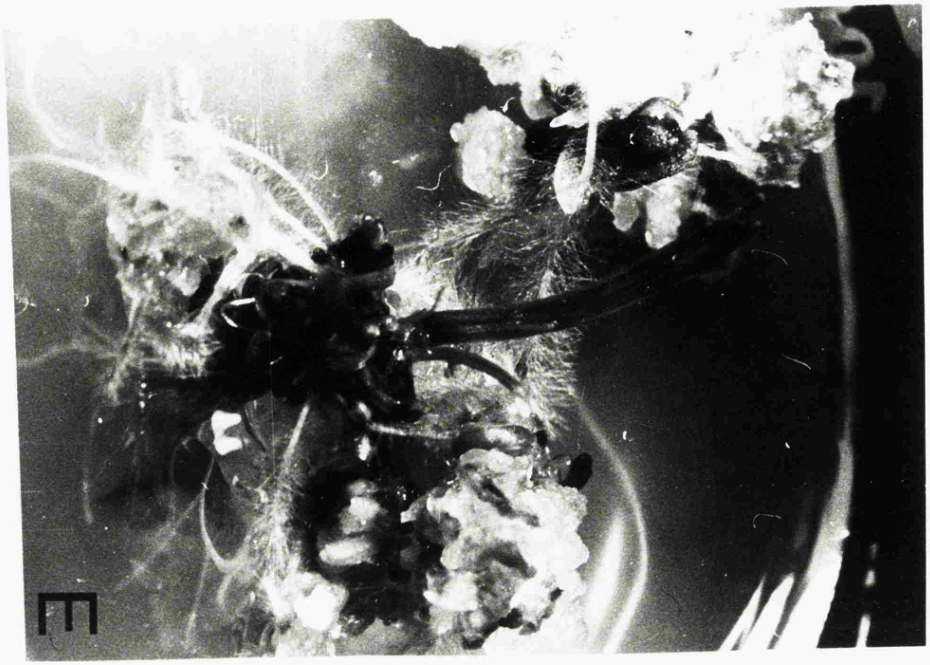
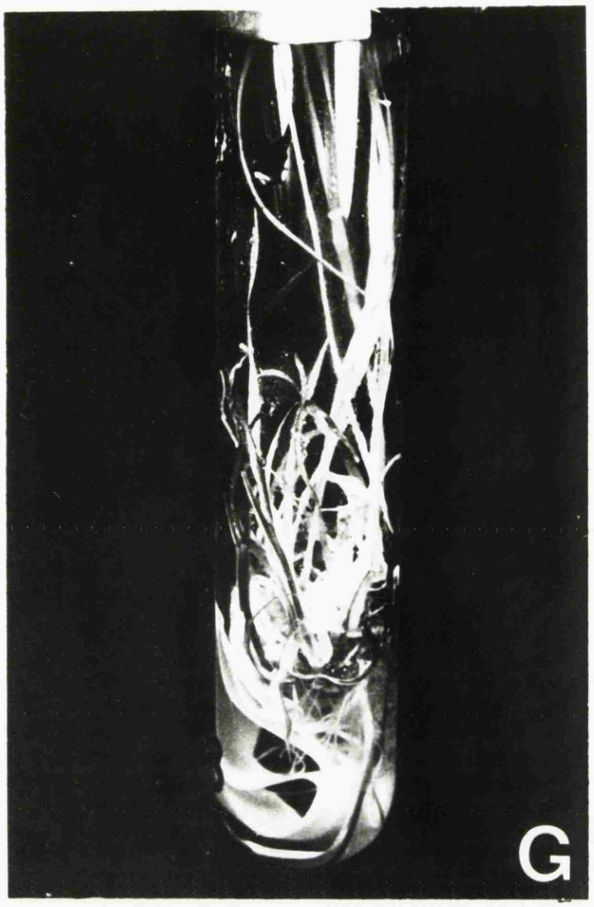
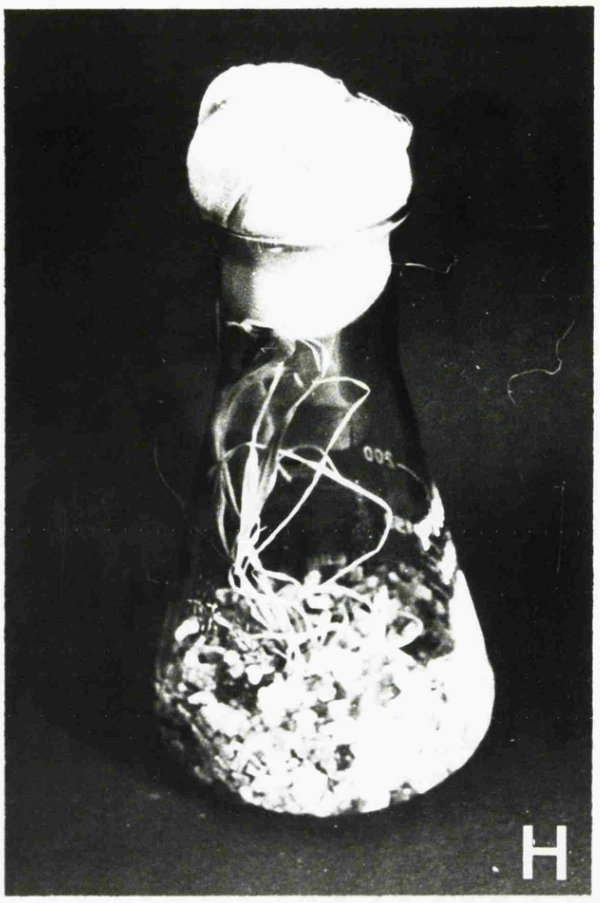

E


F


G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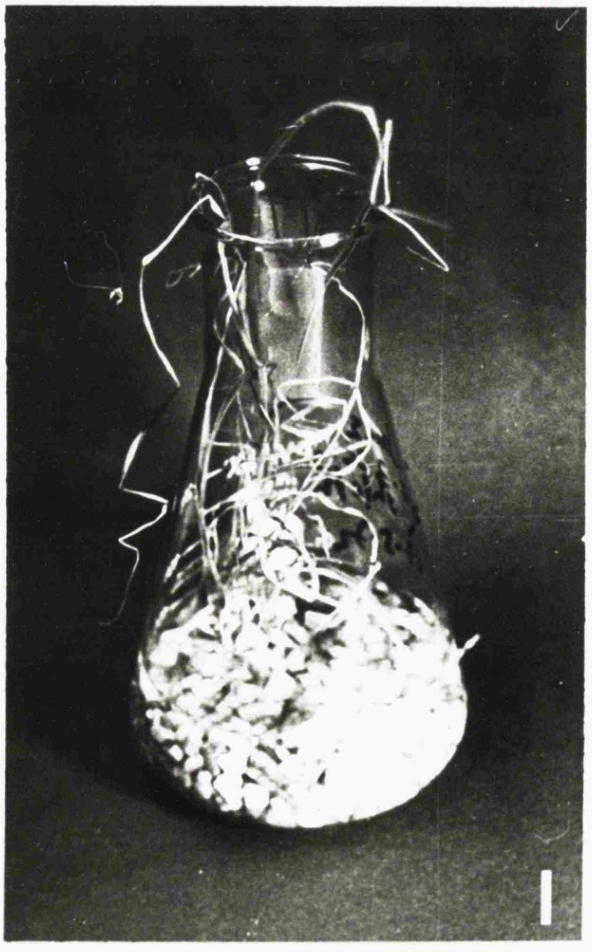

I


J

very firm in texture and had green 'ridges' of leaf-like structures (A,B). Other pieces had small clumps of shoots (C) arising from the callus, with or without nearby roots (D). Often, the culture would have one shoot more vigorous than the rest (E) and this was generally associated with many roots. No attempt was made to separate out as many plantlets as possible to grow up further: usually only the most vigorous shoot was transferred. It was noted that many of the small shoots did not develop further, if left undisturbed. The vigorous shoots (together with any associated roots) could be transferred to large test tubes and supported on filter paper over dilute liquid medium (G). Alternatively, the shoots were placed in 250 ml flasks containing Vermiculite soaked with dilute liquid medium (H). In both cases, the plantlets were kept under sterile conditions until a good rooting system had developed. A few days before potting, the covering of the culture vessel was removed, to gradually 'harden' the plantlet. When first transferred to peat in a plant pot, the whole was covered with cellophane, to retain high humidity. The covering could be removed after a week, but the plants were kept well-watered. As will be seen later, these precautions were essential to prevent drying-out, since the plantlets had very permeable leaf cuticles. After potting, the first few leaves usually died off and were replaced by vigorous new shoots (J). The success rate for transferring to peat and growing further was very high. Of the one or two plants which did die, this was due to contamination of agar left clinging to the roots.

B. Tissues cultured in liquid media

I. Inoculation into liquid medium

In liquid medium, all the pieces of tissue have equal access to available nutrients; this type of culture is thus very suitable for investigations concerning the effect of alterations to the medium on growth and morphogenesis. Several sources of inoculum for liquid cultures were tested, but the most effective proved to be pieces of callus.

In a preliminary experiment, small, (pea-size) pieces of visibly undifferentiated callus from two varieties of wheat: Triticum aestivum cv Maris Ensign, and T. durum cv Rosetta, were placed in 20 ml of liquid "B5" medium (one piece of callus per flask). Variations were made in the conditions as follows:

i. the medium contained 0.2 mg/l 2,4-D or 2.0 mg/l 2,4-D
ii. the medium contained 2% sucrose or 3% sucrose
iii. rate of agitation on the horizontal rotary shaker was 58, 120, or 135 r.p.m.
iv. cultures were incubated in the dark or low light
v. a few cultures were put into torpedo-shaped flasks fixed to a slowly rotating drum (low light).

Quantitative data was not collected: the cultures were observed at intervals and their appearance noted. A difference emerged in the response of the two varieties of wheat to a low 2,4-D level. While cultures of Maris Ensign developed many roots after 3 weeks on 0.2 mg/l 2,4-D, those of Rosetta showed no rhizogenesis even after 6 weeks. This difference was only observed at the slower shaker speeds;

at 135 rpm none of the cultures formed roots on any level of 2,4-D.

After 3½ weeks on liquid medium, several cells had been sloughed off the original piece of callus, causing the liquid to appear cloudy. Otherwise, no break up of the callus was observed. These cells free in the liquid were generally large and irregularly shaped. They often contained little cytoplasm and thus had the appearance of senescent cells. By 5½ weeks, several small clumps were present, as well as one or two layer aggregates, and this suggested that the original callus had grown and dispersed. More aggregates were present in the cultures agitated at 135 rpm than at slower speeds. Cultures placed in torpedo flasks at a slow speed of rotation, showed very little or no breakup of the original piece of callus, even after 6½ weeks.

Apart from the rhizogenesis observed with Maris Ensign cultures at 0.2 mg/l 2,4-D, no other differences could be ascribed to the level of 2,4-D or sucrose. Also, whether the cultures were incubated in low light or complete darkness had no apparent effect on growth or dispersion. Thus, the most important factor seemed to be rate of agitation: a fast rate aided dispersion of the original piece of callus. The cultures were transferred (by pouring) to fresh "B5" containing 2 mg/l 2,4-D in 250 ml flasks (60 ml) and agitated at 120 rpm under low light. Several of the cultures grew and could be further subcultured, using a perforated spoon (as described in Materials & Methods) since the aggregates remained larger than 1 mm. Often, cultures which initially had slow growth, grew very well during subsequent passages, possibly due either to habituation or selection effects.

Attempts were made to form suspension cultures by other means. One of these involved macerating segments of young seedling roots in a glass homogeniser and transferring the broken pieces to liquid

medium. The method was found to be unsuitable because of a very high contamination rate and also little growth occurred, perhaps due to excessive cell damage during maceration. In another attempt, germinating embryos from imbibed seeds of '6 wuk 3' were excised and placed in 30 ml of liquid "M" containing 2 mg/l 2,4-D. Different numbers of embryos (3, 5, 10 or 15) were placed in each flask to see whether this had an effect on dispersion. The cultures were agitated at 120 rpm in the low light of Controlled Environment Room II. After 4 weeks, little dispersion of the explants had occurred, although the liquid was beginning to appear cloudy due to cells becoming separated from the outer layers of callus which had formed over the embryos. Callus appeared to be initiated in a similar manner as when excised embryos were placed on semi-solid medium. The cultures were transferred to fresh medium about every 6 weeks, during which time growth was extremely slow. Small aggregates, originating from the explants, began to appear after 3 passages, and, a little earlier where 10 or 15 embryos were present. Thus, excised embryos formed callus much more readily on semi-solid medium than in liquid culture.

II. Attempts to obtain a fine suspension

Wheat callus grew well in liquid medium, whether "B5" or "M", containing at least 1 mg/l 2,4-D. However, as Plate X shows, the aggregate size remained large. Hand-cut sections through the aggregates showed the outer layers of cells to be actively dividing (Plate X,A) being composed of small, densely-cytoplasmic cells, while the innermost cells were large and highly vacuolated. The outermost cells were irregularly and loosely arranged and so easily washed off into the surrounding liquid (Plate X, B). If some of this 'cloudy' solution,

Plate X

The appearance of wheat cultures grown in liquid medium.

A. Part of a hand-cut section through an aggregate similar in appearance to those in photograph C. Cells in the central part are large and vacuolate, and are surrounded by a layer of small, densely cytoplasmic and actively dividing cells. (x40)

B. The same section as shown in 'A', but focussed to show the loose arrangement of the cells at the surface of the aggregate. (x40)

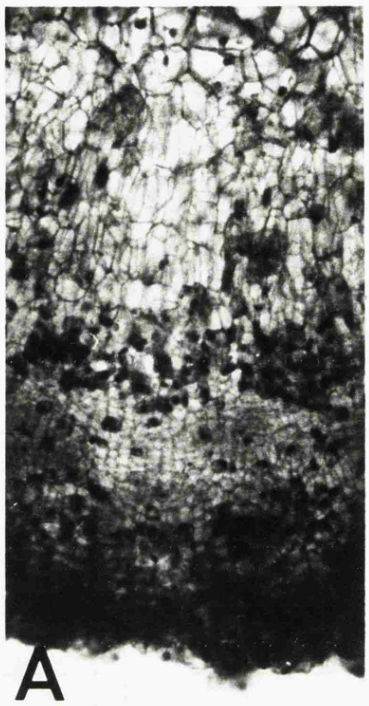
('A' and 'B' are photographs of an aggregate taken from a culture of Maris Ranger callus incubated in liquid "B5" medium for 3 weeks. The sections were stained with dilute Paragon stain.)

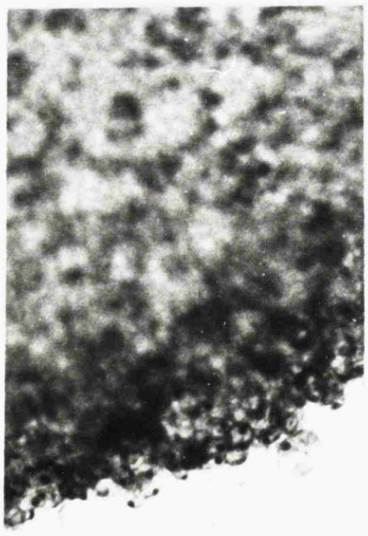
C. A typical wheat culture in liquid medium ("B5"); the aggregates are of assorted sizes, nodular in appearance, and show no visible organisation.(Rosetta wheat)

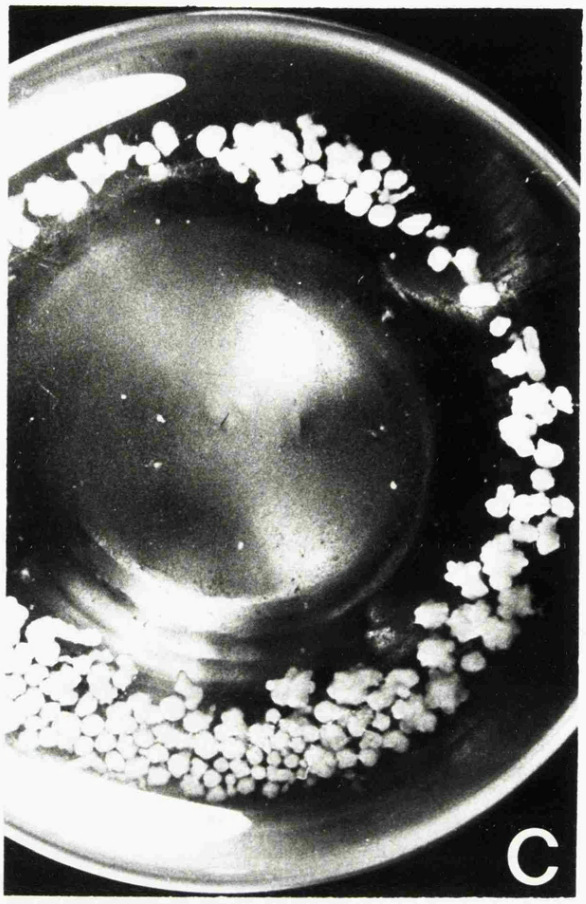
D. A culture of Koga wheat, incubated in the same medium as 'C', and containing the same level of auxin (2mg/l 2,4-D), but a few of the aggregates show root formation and they are not as regular in appearance.

('C' and 'D' cultures were photographed through the bottom of a 250ml flask (xl.6) immediately after subculture to fresh medium.)

Plate X







containing largely single cells, was pipetted off and placed in fresh medium, growth was extremely slow: larger aggregates were not evident before at least 2 months. Such cultures could be transferred by automatic pipette (see Materials & Methods) for several passages during which the growth rate improved. Unfortunately, as the growth increased, so did the aggregate size so that eventually the cultures had to be transferred using the perforated spoon. Attempts were made to obtain a finer suspension with smaller aggregates by varying firstly the concentration of 2,4-D, and later the supply of sucrose.

(i) Increasing concentrations of 2,4-D

Cultures of Maris Ensign tissue which had been grown in liquid medium for one year were transferred to "M" containing 20 g/l sucrose and from 0 - 10.0 mg/l 2,4-D. A perforated spoon was used to transfer the aggregates: one 'spoonful' (about 1 g fresh weight) per 50 ml of medium, contained in 'arm-flasks' so that growth could be followed by measurement of the settled cell volume (SCV) as described in Materials & Methods. Three replicates were used for each treatment, and the cultures were agitated at 120 rpm on a horizontal rotary shaker in Controlled Environment Room II (low light). Table 2.26 (overleaf) summarises the appearance of the cultures over one passage of 25 days. The SCV measurements are not comparable from one flask to another, so the table indicates the change in SCV for each individual flask. A few roots were present in all the inocula; in media containing no 2,4-D or 0.2 mg/l, many more roots developed, and this accounts for the huge increase in growth in these cultures by Day 8. Cultures in media containing 2.0 mg/l 2,4-D or more showed little increase in SCV by Day 8.

Table 2.26: Characteristics of cultures over one passage in liquid "M" containing a range of 2,4-D levels

2,4-D (mg/l)	Flask number	DAY 8			DAY 25		
		Growth	Roots	Green	Growth	Roots	Green
0	1	+++	+++	+++	-	Tissue gone brown	
	2	+++	+++	+++	-		
0.2	1	+++	+	+	-	+++	Brown
	2	++	+	+	-	+++	"
	3	+++	++	-	-	+++	"
2.0	1	+	+	-	++	+	+
	2	+	+	+	++	+	+
	3	-	+	+	+++	+	+
5.0	1	-	+	-	++	-	+
	2	+	+	+	-	-	+
10.0	1	+	-	-	+	-	-
	2	+	-	-	+	-	-
	3	+	-	-	+	-	-

- = no change in SCV ++ = SCV doubled
+ = slight increase in SCV +++ = SCV more than doubled
'roots', 'green' = further development of roots or greening; the number of '+' indicates relative amount of roots or green.

Cultures in 2.0 mg/l 2,4-D had grown very well by Day 25, but the large increase in SCV is due to the aggregate size also being larger than where the 2,4-D level was higher. Some of the cultures on 5.0 mg/l and 10.0 mg/l 2,4-D did seem to contain finer aggregates and could be subcultured using an automatic pipette with a 2.0 mm diameter canulus.

The experiment showed that in liquid medium containing 0.2 mg/l 2,4-D or less, the aggregates differentiated to a mass of roots and although partly green in the early stages, they soon turned brown and ceased growth. Levels of 2,4-D higher than 2.0 mg/l suppressed differentiation and seemed to keep the aggregate size small. However, if passaged in medium containing a lower 2,4-D level again, these 'finer' cultures gradually increased their aggregate size.

It would be undesirable from the point of view of chromosome stability, to maintain cultures in such a high level of 2,4-D. Further experimentation is needed to determine whether 'fine' cultures, obtained by treatment with 10.0 mg/l 2,4-D, could be passaged through a series of lower levels of auxin and yet retain their small aggregate size as they became gradually habituated.

(ii) Different sucrose levels

Callus from thesame liquid culture line as used for the previous experiment was transferred for two passages to "M" + 2 mg/l 2,4-D containing 10, 20, 30 or 40 g/l sucrose. The 3 replicates for each treatment were agitated at 120 rpm in the low light of Controlled Environment Room II.

At the end of the first, 26 day passage, the cultures on 10 g/l sucrose showed least growth (by visual comparison) and the aggregates had started to brown. During the second, 22 day passage, growth was monitored by taking the SCV at intervals. All the cultures showed some increase in SCV by day 5. However, those cultures in media containing 10 g/l sucrose grew no further and started to brown; cultures in media with 20 g/l or 30 g/l sucrose had a final SCV (at day 22) roughly double the original SCV; where 40 g/l sucrose was present growth was also good although the SCV did not increase as much. Throughout the experiment, altering the sucrose concentration was not observed to have any appreciable effect on aggregate size.

Although not quantified, the wheat tissue seemed to grow in liquid medium at least as well as tissue on semi-solid medium. The main problem, yet to be overcome, was to obtain a culture whose growth could be accurately monitored, ideally by cell-counting methods. There are indications that finer cultures can be obtained, but much more

attention needs to be directed to this problem.

III. Organogenesis in cultures in liquid medium

We have already noted that lowering the level of 2,4-D caused the aggregates to develop a mass of long roots, at the expense of callus growth. Apart from rhizogenesis and some greening of callus or roots, no other morphogenetic changes were observed upon decreasing the auxin level. The following experiment investigated the morphogenetic response of the cultures to IAA, NAA and Kinetin in several combinations.

Cultures were used which had been maintained in liquid medium for different lengths of time, and had originated from different varieties of wheat:

a. "18" - from callus derived from stem segments of 'haploid' 6 wuk 3 plants, and which had been grown in liquid medium for 10 months

b. "ME-3" - from callus of Maris Ensign which had been maintained in liquid medium for over a year

c. "6 wuk 3" - originating from excised 6 wuk 3 embryos grown in liquid medium for 5 months

All 3 cultures were capable of producing roots upon lowering the level of auxin in the medium, but while "18" and "6 wuk 3" had a tendency to show green areas on the aggregates, this had never been observed in "ME-3".

The medium used was the same as that described in a previous experiment, to study the effect of auxin and cytokinin on differentiation of cultures on semi-solid medium (R1 - R6; P. 78)

but was not solidified with agar. The medium consisted of "M" with the addition of Kinetin and either Naphthalene acetic acid, or Indole-3-acetic acid, as shown below. Using a perforated spoon, one 'spoonful' (roughly 1 g fresh weight) of each culture was transferred to 60 ml of liquid medium in 250 ml flasks, and placed on a horizontal rotary shaker (120 rpm) in the low light of Controlled Environment Room II. In the case of "6 wuk 3" there was only enough tissue for one replicate each of R5 and R6, but for "ME-3", 2 replicates were used for each of R1 - R6, and 4 replicates of each medium were used for "18".

	NAA (mg/l)	IAA (mg/l)	Kinetin (mg/l)
R1	5.0	-	1.0
R2	1.0	-	5.0
R3	-	2.0	0.2
R4	-	0.2	2.0
R5	-	0.2	0.2
R6	-	2.0	2.0

Plate XI shows some of the results for "6 wuk 3" and "18" cultures. For both of these, before treatment the aggregates were of all sizes and a few roots were present (Plate XI, A, D). After 12 days, many long roots were present in the "6 wuk 3" cultures and some greening was evident. There was little difference between R5 and R6, and in both these media, some aggregates remained completely undifferentiated. In the "18" cultures many long roots had formed in all media, with perhaps more greening on R2. As Plate XI, E and F show, little callus was evident as roots grew at the expense of the callus. On Media R2, 4, 5 and 6, the aggregates looked pale brown. All of the "ME-3" cultures had many long roots, but no greening. Again there was browning of

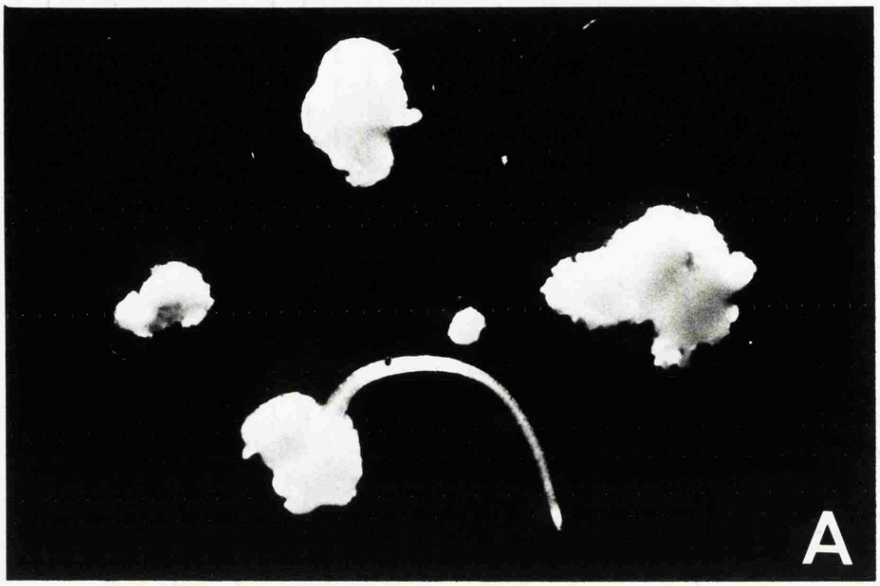
Plate XI

The appearance of wheat cultures incubated in liquid media (R1-R6) containing different ratios of auxin and cytokinin (see P 100).

A, B and C show a few typical aggregates from cultures of 6 WUK 3 before subculture (A), and after incubation for 12 days on R5 (B) and R6 (C). Little difference was noted between the appearance of the aggregates in the two media, even though R5 contained only 0.2mg/l of both IAA and Kinetin, compared with R6 which contained 2.0mg/l of both IAA and Kinetin. In the inoculum a few roots were present, and further root formation continued in both R5 and R6, together with a little greening of the callus at the base of the roots. Some aggregates in both R5 and R6 remained unorganised.

D, E and F show the appearance of representative aggregates from cultures of "18" line of wheat, before subculture (D), and after incubation for 12 days on R2 (E) and R6 (F). Again, several small roots were present in the inoculum, and much more rhizogenesis occurred on each of the media (R1-R6) tested (see text). There was perhaps more greening in cultures on R2 (1.0mg/l NAA + 5.0mg/l Kinetin). As E and F illustrate, the cultures were given over so completely to root formation that very little callus remained in most aggregates.

Plate XI



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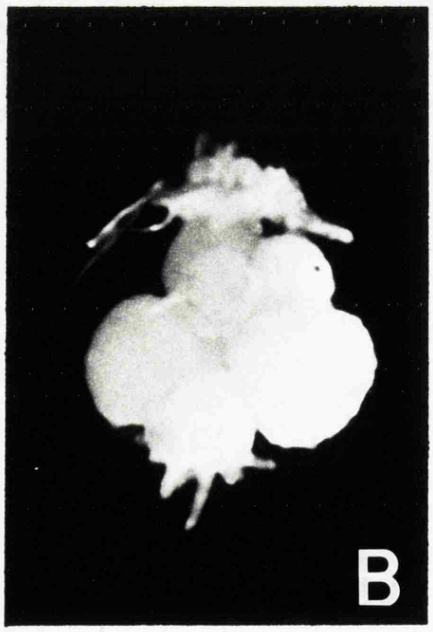

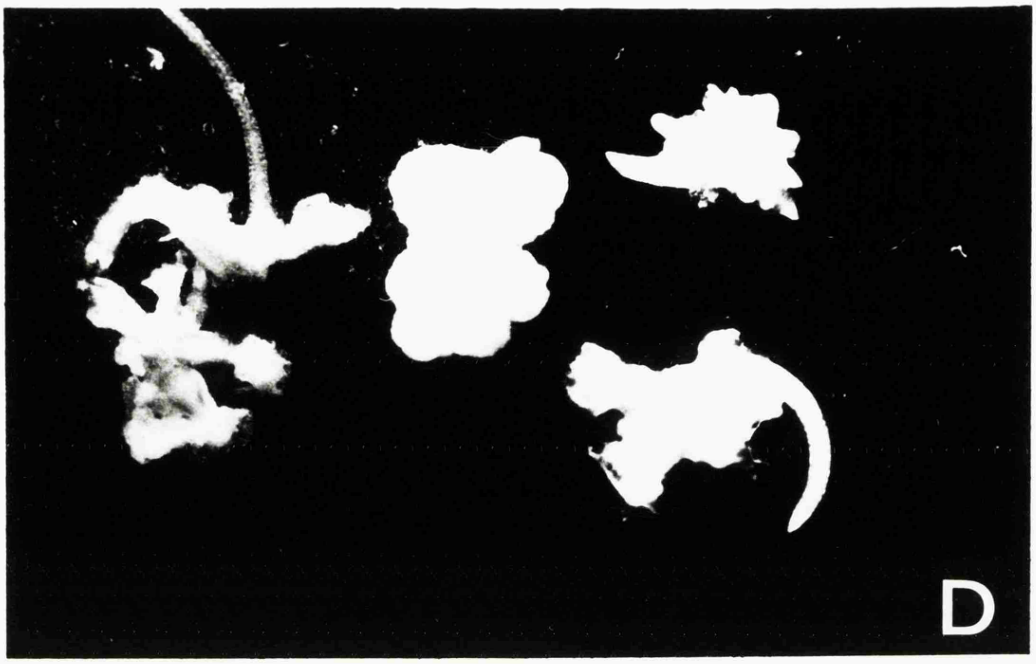

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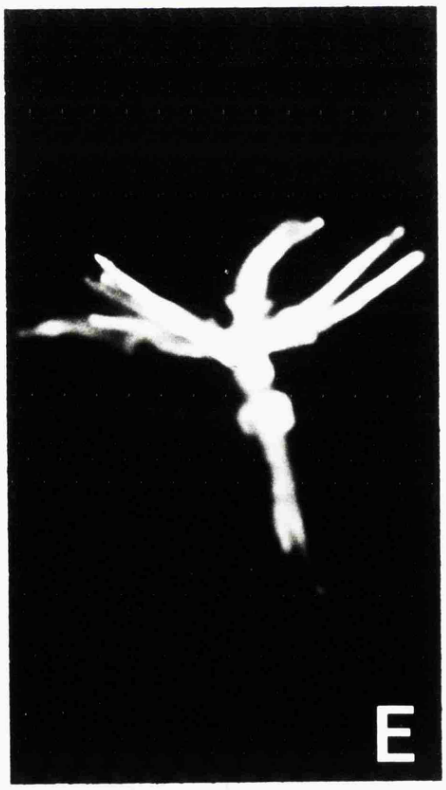


tissue on all media except R2, with most necrotic tissue occurring on R1 and R5.

When the cultures were transferred to the same media again for a further 3 weeks, root growth continued, some reaching 13 cm in length, but no further differentiation or growth of other tissues was observed.

The experiment showed a lack of differential response to the different levels of Kinetin and NAA or IAA tested; all the cultures produced long roots. Greening occurred on some aggregates, but this did not appear to be related to one particular medium. No indications of shoot or leaf formation were observed under any conditions in liquid medium. However, nearly all of the cultures in liquid were started from callus which had itself often been in culture for several months, and as we have noted earlier, the appearance of regenerated plants was more frequent in "young" callus on semi-solid medium. Thus it is probable that the cultures in liquid medium had a decreased "regenerative capacity".

C. The anatomy of cultured tissues

I. Formation of callus from the embryo

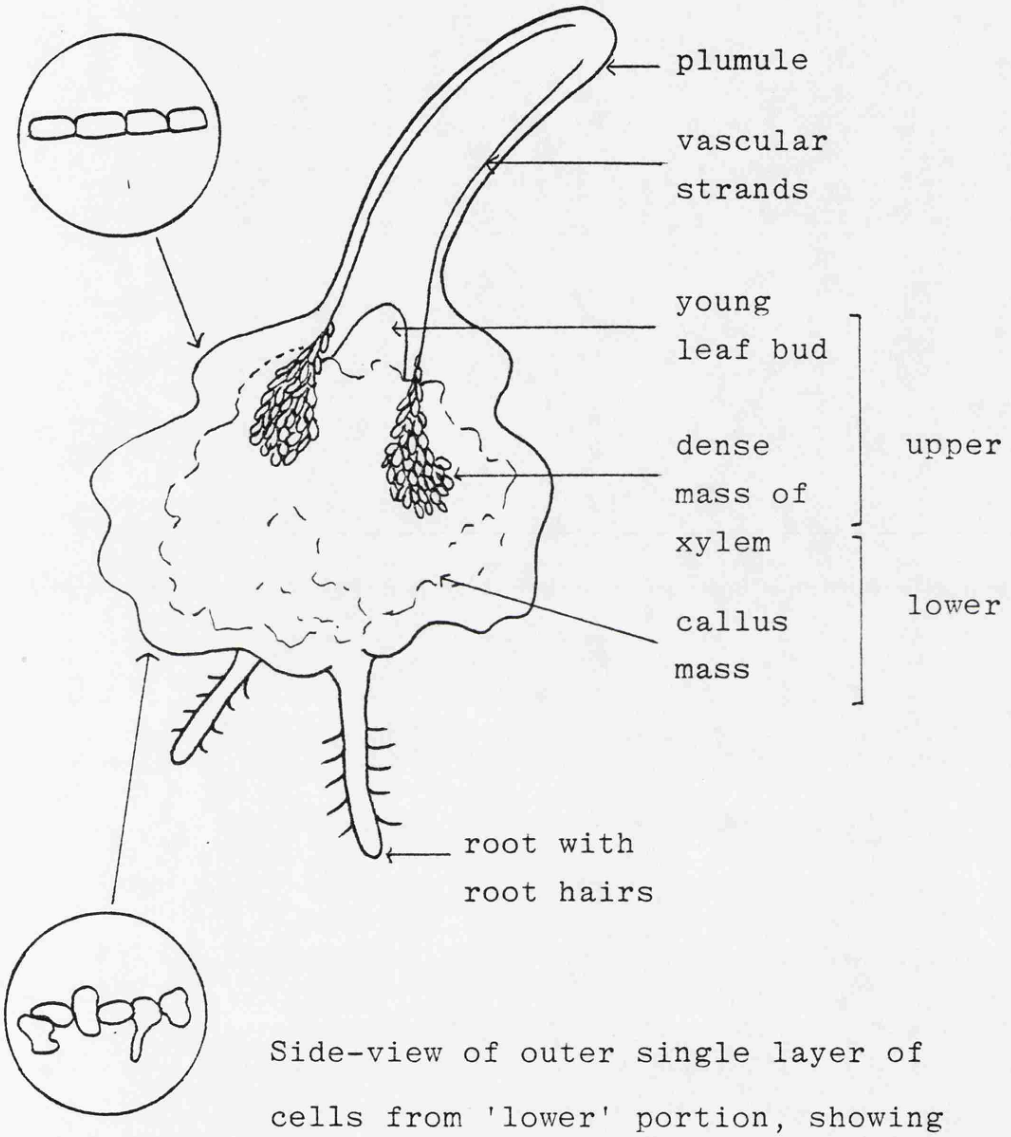
Techniques involving sectioning of fixed and embedded material and dissection of cleared whole explants (as described in Materials and Methods), were used to investigate the formation of callus from the embryo. Caramiello & Montacchini, 1970, reported callus formation at the base of the plumule of excised wheat embryo; Carter, Yamada & Takahasi, 1967, observed that callus formation from

oat seeds replaced the root system, and this same observation was made by Nishi, Yamada & Takahasi, 1968, with rice seeds; Ahloowalia, 1975, reported callus formation at the base of the plumule of rye grass seeds. There is thus a good deal of agreement as to where the callus formed, but few workers (with the exception of Caramiello & Montacchini, 1970) have made anatomical studies of callus formation on the explant.

In the present study, it was noted that when excised embryos were incubated on "M" containing 1 mg/l 2,4-D, although the plumule continued to extend in most cases, root extension was more rarely seen, and callus covered the whole embryo. We have seen in previous experiments, that raising the hormone level suppressed development of the embryo axis and conversely, lowering it allowed both root and shoot extension at the expense of callus production. The following observations relate to embryos cultured on "M" plus 1 mg/l 2,4-D. Many observations were made after clearing and dissecting embryos which had been 7 - 14 days in culture. Unfortunately, it proved difficult to obtain a good photographic record of cleared preparations, so that the following description is accompanied by simple diagrams.

The bulk of the callus mass could be seen at the base of the extended plumule. The outer layer of callus cells had retained the regular order and appearance of epidermal cells (Fig. 2.30) in the 'upper' portion of the explant, and this was continuous with the outer epidermis of the plumule. However, although the epidermis had continued to grow as a single layer of cells, it formed a sheet of cells which was much-folded and loosely attached to the underlying callus, perhaps due to having a different growth rate. Where root extension had occurred, a similar loose, outer; single layer of cells continuous with the root

Fig. 2.30 :Simplified diagram to show Callus Formation from a typical Excised Embryo after 14 days in culture.



epidermis, was present in the 'lower' region of explant (Fig. 2.30). However, here the cells had quite a different character, being irregularly shaped, loosely attached to one another, and projecting in all directions. The underlying mass of tissue was compact and firm at the inner 'core' and more loosely arranged in the outer regions. Vascular strands could easily be seen within the cleared tissue. The fine strands of xylem running along the plumule ended in a dense mass of reticulate xylem cells inside the callus. Within the cleared tissue it was also possible to see small intact domes at the base of the plumule: presumably the young shoot buds.

Plate XII shows sections through excised embryos similar to those described above. In 'A' the loose epidermal layer continuous with the plumule can be seen. Sections through the base of the plumule (B,C) show how the outer leaf tissue has begun to form callus. D, E are longitudinal sections and show callus formation below the plumule while the inner leaves retain their shape. Transverse sections through the root region (F, G) show the presence of a few roots surrounded by callus. Photographs H - K show section through an excised embryo which had been incubated on "M" + 1 mg/l 2,4-D for 8 months and had remained visibly undifferentiated. However, as the sections show, the callus contains many types of cells, and the inner core of the explant is a mass of vascular tissue.

To conclude, callus formed in the region at the base of the extended plumule. Roots were often completely surrounded by callus, and were not visible externally. The original embryo explant remained as a core of compact callus and vascular tissue. There was evidence to suggest that some of the very young shoot apices remained intact while

Plate XII

Transverse and longitudinal sections through excised wheat embryos incubated for 7 days on "M" + 1mg/l 2,4-D.

A. External appearance of the explant; callus has formed at the base of the plumule (P), covering the rest of the embryo. The dashed line indicates the position of the sections shown in B and C.

B, C. Transverse section through the base of the plumule (enlarged in C, x80), showing the regular arrangement of the inner leaves while the outer tissues are proliferating to form callus(C).

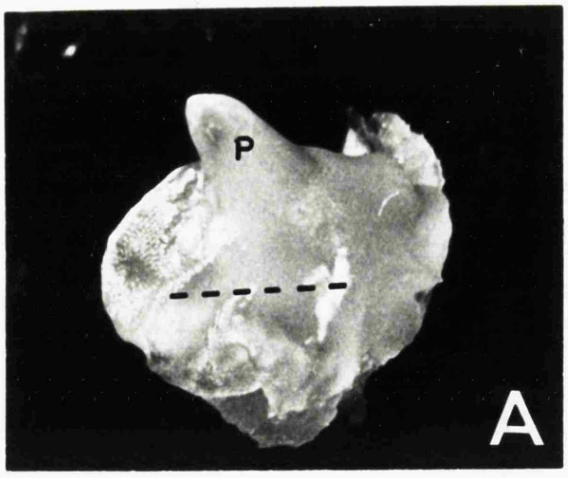
D-G show sections through a wax-embedded embryo, again incubated for 7 days on "M" + 1mg/l 2,4-D.

D, E. Longitudinal sections through the explants, showing the plumule (P) and enclosed young leaves to be unaltered in appearance, while callus (C) is forming from the rest of the embryo tissues. (x30)

F. T.S. through the root region (x30, enlargedto x70 in G) of the excised embryo, showing the presence of a few roots (R), surrounded by callus cells; part of the epidermis (E) is still intact.

H-K are transverse sections through an excised embryo of Triticum monococcum which had been incubated for 8 months on "M" + 1mg/l 2,4-D. The sections were cut using the freezing microtome, after first staining with Feulgen. The inner core of tissue, within the callus, consists of a mixture of cell types and (enlarged in J,x100) clearly-formed trachaery cells.

Plate XII



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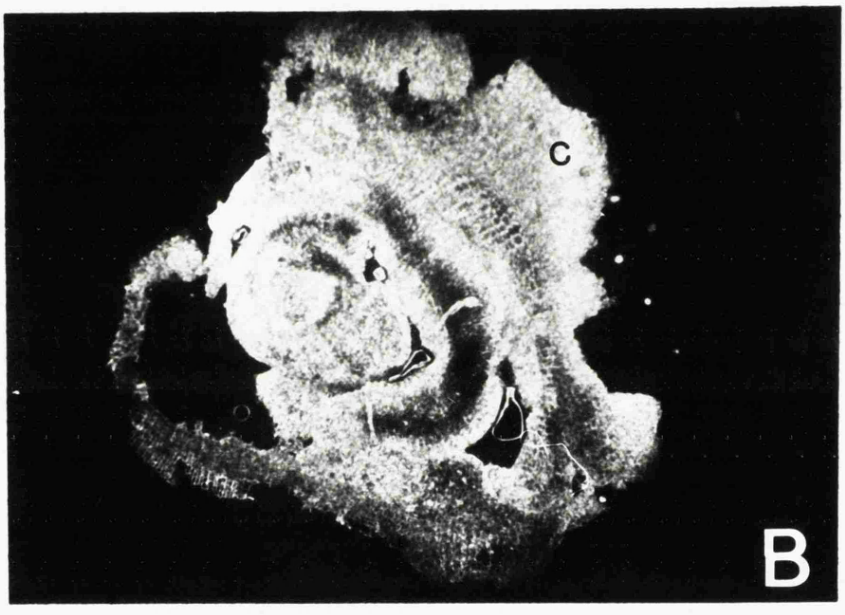
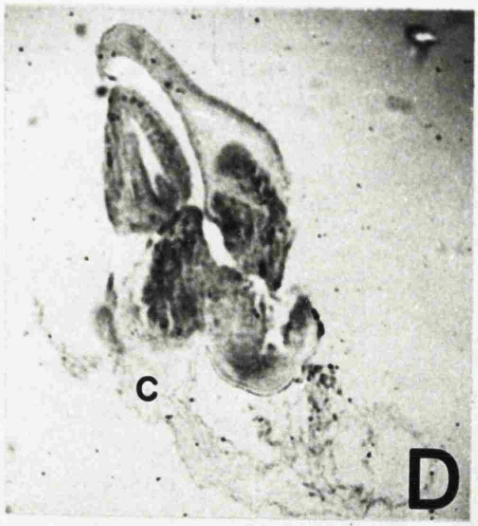
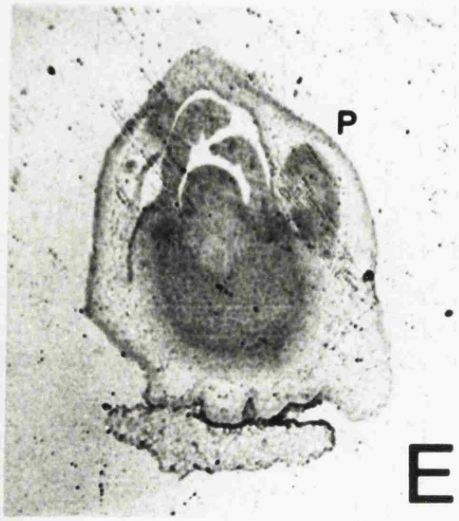
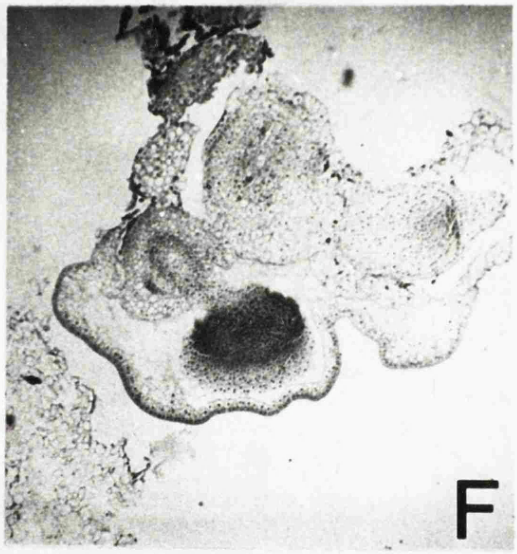
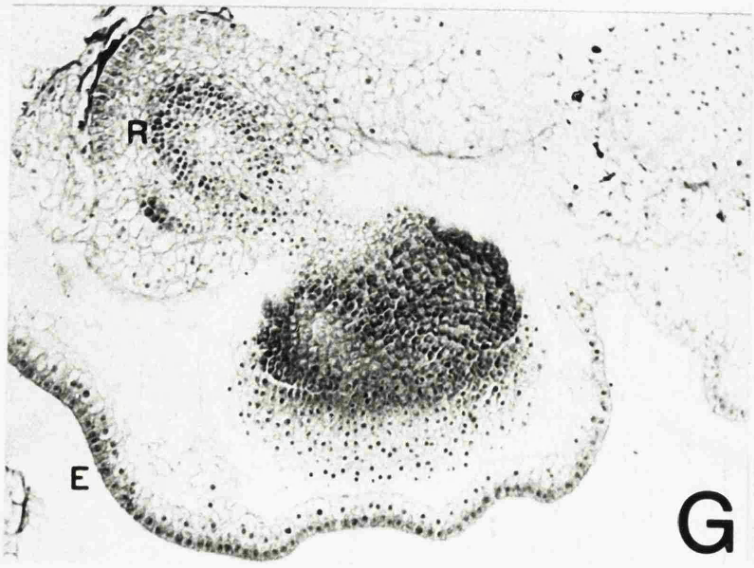
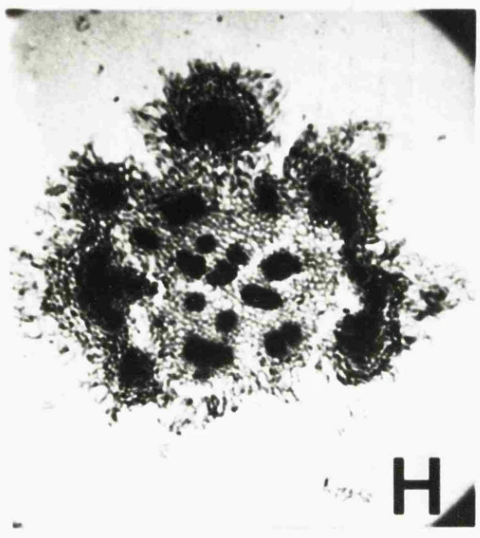


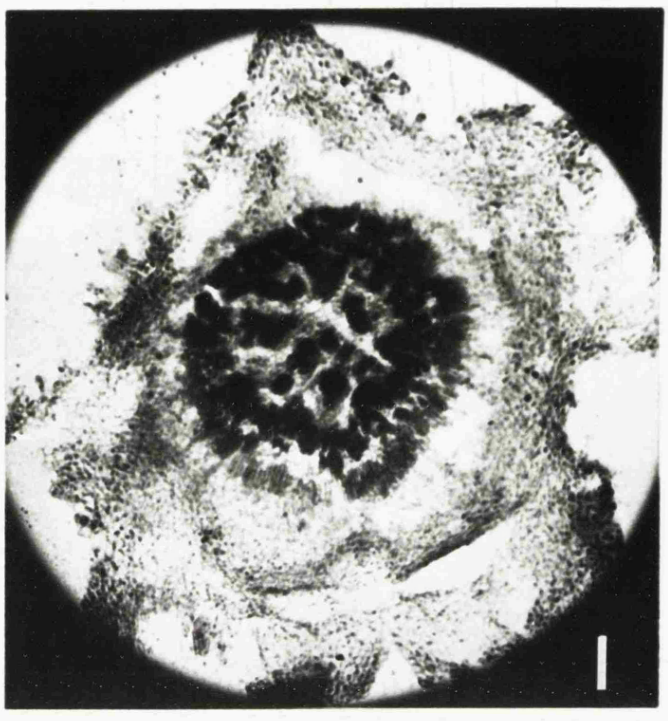

C
D


P
E


F


R
E
G





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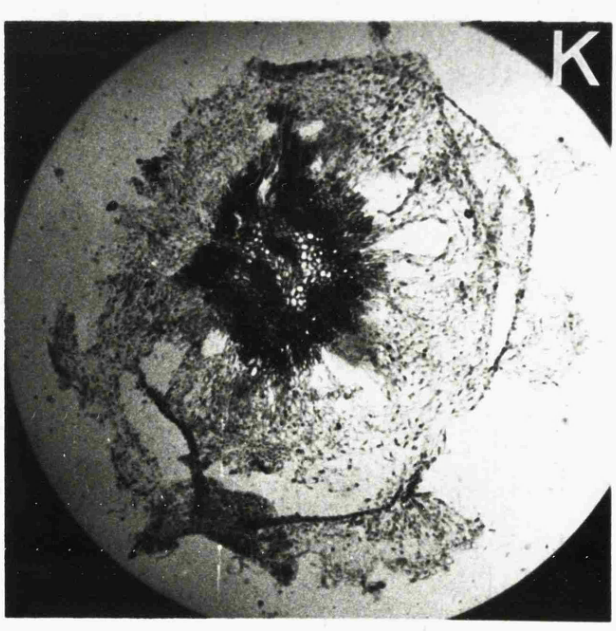


callus proliferated nearby.

Thus, when excised embryos are used as a source of callus, careful selection over several passages would be required to ensure obtaining homogeneous tissue, which should behave in a uniform manner. The variability in response which was encountered, especially during early passages may be due to the diversity of cell types present.

II. The formation of callus on root explants

When excised segments of seedling roots were placed on "M" containing at least 1 mg/l 2,4-D, proliferation of callus occurred quite readily, although its rate of increase was much slower than that of callus forming on excised embryos. Callus might completely cover the explant (Plate XIII, A) or only occur as small nodules along the root (see Plate V, C) or at the root tip. Sections cut transversely through a callussed root showed that the bulk of the callus consisted of large, highly vacuolated, disorganised cells. There were regions of smaller cells with dense cytoplasm (probably areas of active division) and these seemed to originate from cells of the central stele of the root (Plate XIII, C), which retained much of its structure. Thus, the callus may have been of pericyclic or endodermal origin. Parts of the epidermis of the root explant remained intact (Plate XIII, B), but most of the original explant cells were extremely expanded. The callus mass was not observed to contain any vascular tissue (apart from the original stele) even one month after initiation. Cultures older than this were not sectioned, but some were observed to form new roots, suggesting internal differentiation of several cell types.

Plate XIII

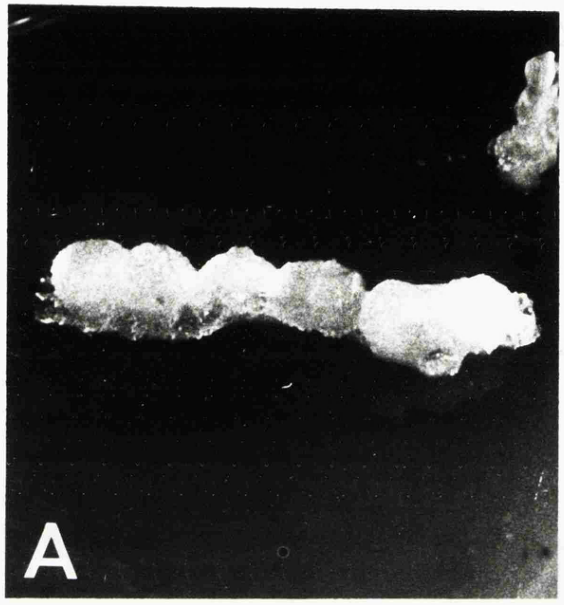
The formation of callus from root explants.

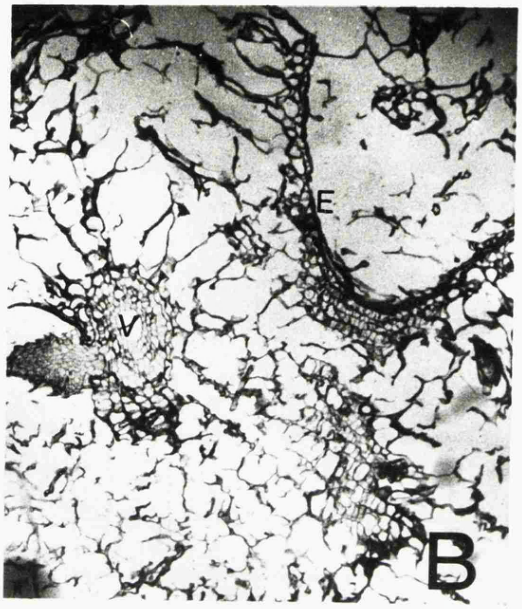
A. A 2cm segment of root, excised from a young wheat seedling and incubated for 5 weeks on medium containing 1mg/l 2,4-D. Callus covers the explant.(x4)

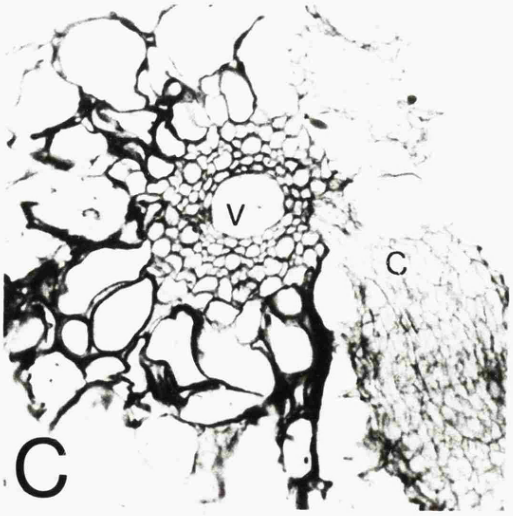
B, and D show transverse sections cut from plastic-embedded root cultures. The stele of the root(V) is recognisable in the midst of expanded cells of the cortex. Localised areas of densely-cytoplasmic and actively dividing callus cells (C) are present. In places, the epidermal cells (E) remain unchanged. (x40)

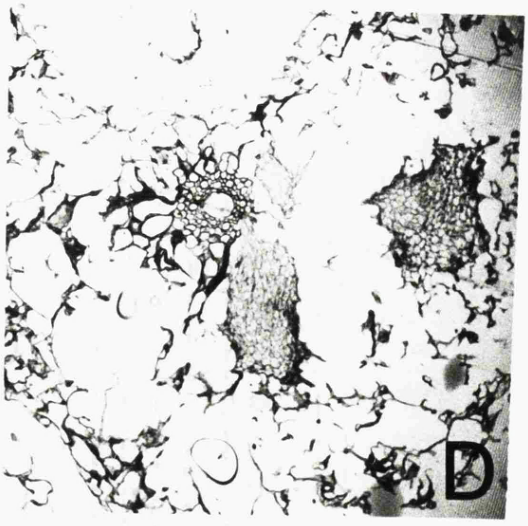
C. Enlargement to show the callus cells (C) in close association with cells of the stele (V). (x120)

Plate XIII









III. The formation of callus on shoot explants

Sections were cut through callus which had formed from the cut end of a segment of coleoptile, taken from a young seedling (Plate XIV). The original leaf material was still recognisable one month after initiation of callus, although many of the cells were distorted. Callus appeared to have formed by localised proliferation of the leaf tissue (B, C). Localised regions of densely-cytoplasmic, actively-dividing cells (D), could be distinguished. These latter areas were always associated with vascular bundles of the original leaf material (as had been observed with root explants) and this suggests either origin from living cells of the vascular strands or initiation of division in the mesophyll cells adjacent to the vascular strands. Fine hair-like cells projecting from the surface of the callus (A) were a feature of such leaf callus, but these were not always observed, and their formation was influenced by the hormonal content of the medium. The explant described was incubated on "M" + 1 mg/l 2,4-D + 5 mg/l Triacanthine, for one month.

An advantage of using either shoot or root explants as a source of callus, is that although much of the original plant material remains, this is easily separated from the newly-formed callus, and so a fairly homogeneous mass of tissue can be obtained at the first sub-culture.

IV. Types of callus observed

Variation in response to a particular set of cultural conditions has been commented upon several times in the course of the present study. Morphological differences among pieces of callus growing

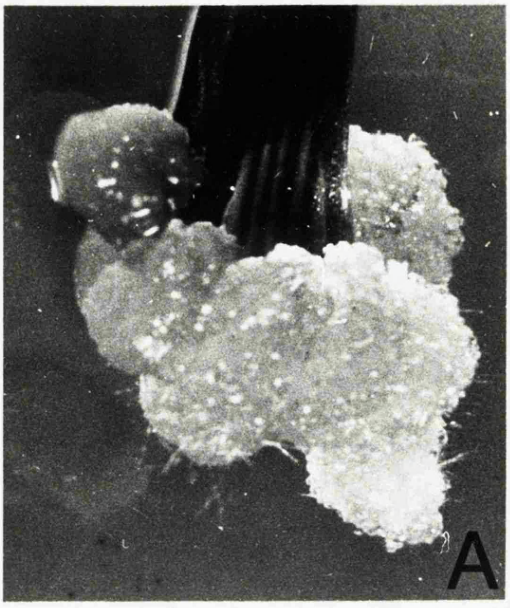
Plate XIV

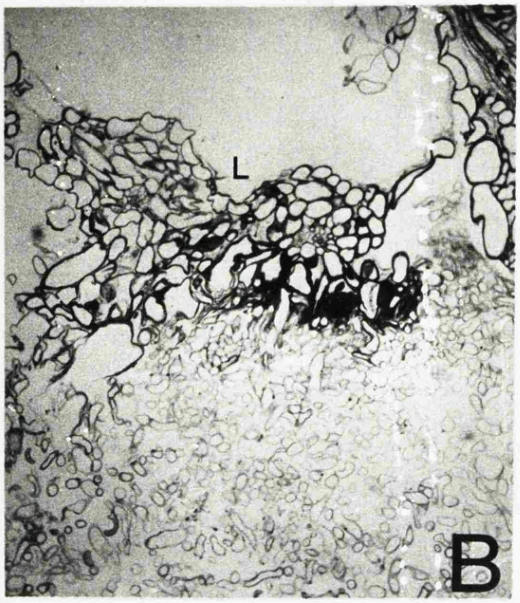
The formation of callus from shoot explants.

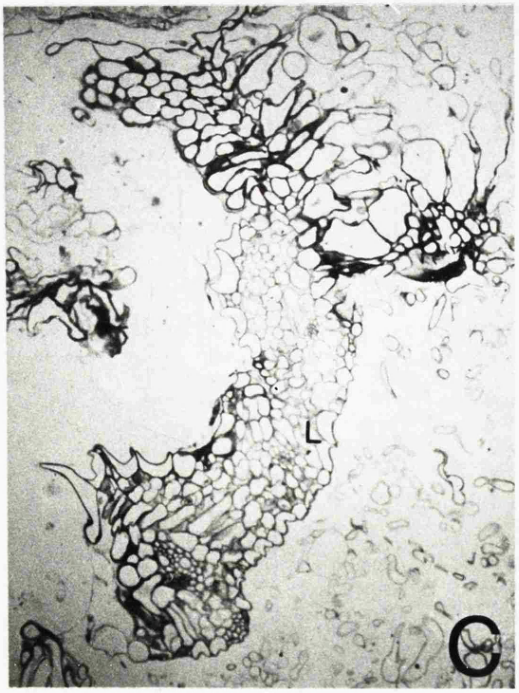
A. Callus formation from the basal, cut end of a segment of coleoptile taken from a young wheat seedling. Note the fine hairs from the surface of the callus. (x4)

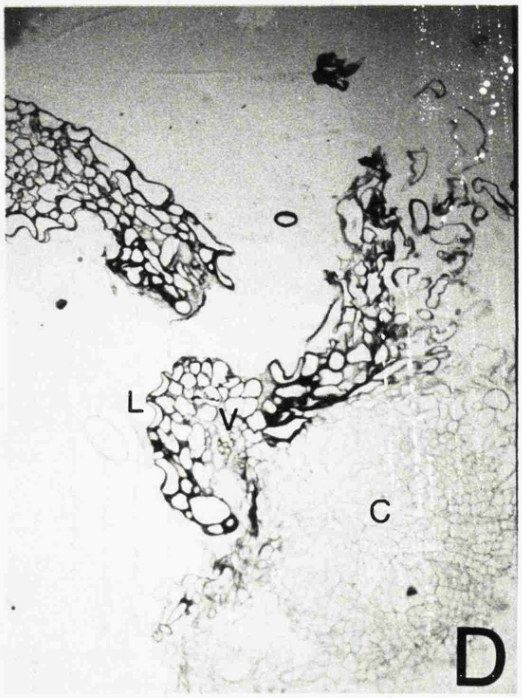
B, C and D show sections cut through material such as A, embedded in plastic. Parts of the original leaf structure (L) are still recognisable among the undifferentiated callus cells (C). Areas of active division appear to be associated with the cells of the original vascular bundles (V) (x40)

Plate XIV









on the same medium can partly be attributed to the internal complement of cell types; as we have seen, the primary cultures may be extremely heterogeneous. Together with this inherent variability, the hormonal content of the medium influenced the appearance of the callus. A low auxin level encouraged root formation. Similarly, a piece of callus left for several weeks on "M" + 1 mg/l 2,4-D would eventually become a mass of roots (Plate XV, C) and the callus would turn light brown and die as the auxin was depleted. Alternatively, we have observed that levels of 2,4-D or CPA greater than 3 mg/l would keep the callus visibly undifferentiated and smooth in texture (Plate XV, A).

Callus cultures consist of discrete regions, each growing at a slightly different rate, so that the overall appearance is nodular (if the callus was growing evenly, it would have a spherical shape). As well as differing in growth rate, these small areas may also differ in texture and colour. Roots or shoots may form more readily in one area than a neighbouring one (Plate XV, B). We have noted that rhizogenesis is controlled by auxin level, but the factors promoting shoot formation are less obvious (see P. 87). Greening of areas of callus and roots was clearly influenced by light intensity and the readiness with which it occurred suggests that the cells already contain plastids capable of being converted to chloroplasts when the light intensity is high enough. However, although in liquid cultures entire aggregates could turn green, this was not observed in cultures on semi-solid medium, where greening was confined to localised regions of callus and the base of the roots.

Sections were cut of root-bearing and shoot-bearing pieces of callus, and descriptions of these follow.

Plate XV

Photographs to illustrate the general appearance of three types of callus commonly observed among wheat tissue cultures. The appearance of the culture was not necessarily related to the content of the medium, although root formation only occurred with low auxin.

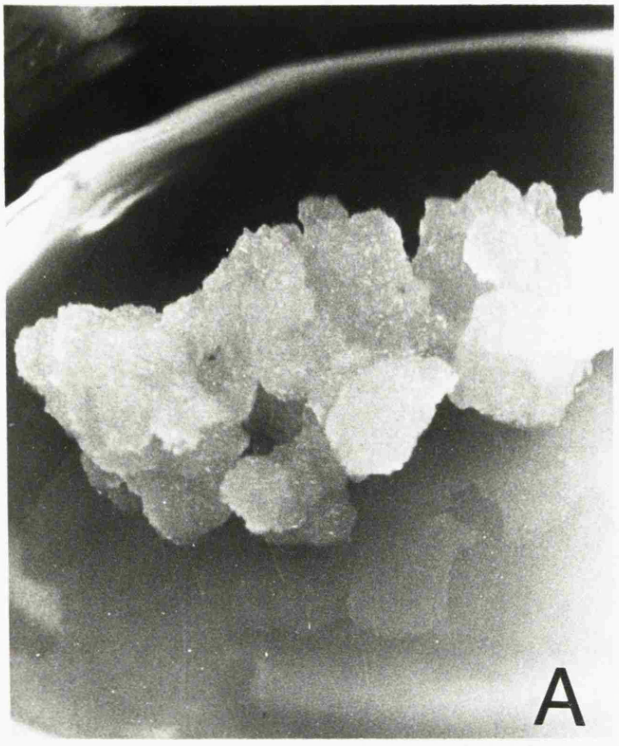
A. Visibly undifferentiated callus, white in colour and having a smooth texture.

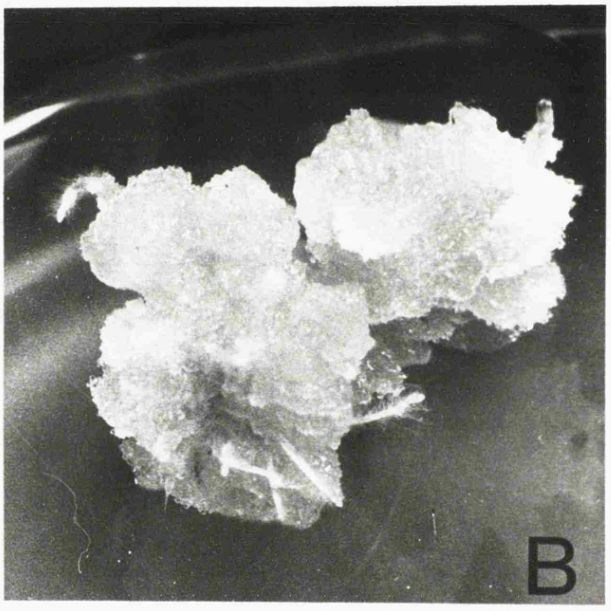
B. White callus with a rough texture where the outermost cells are clearly translucent, and project from the callus surface. Root formation has begun, but in separate parts of the culture.

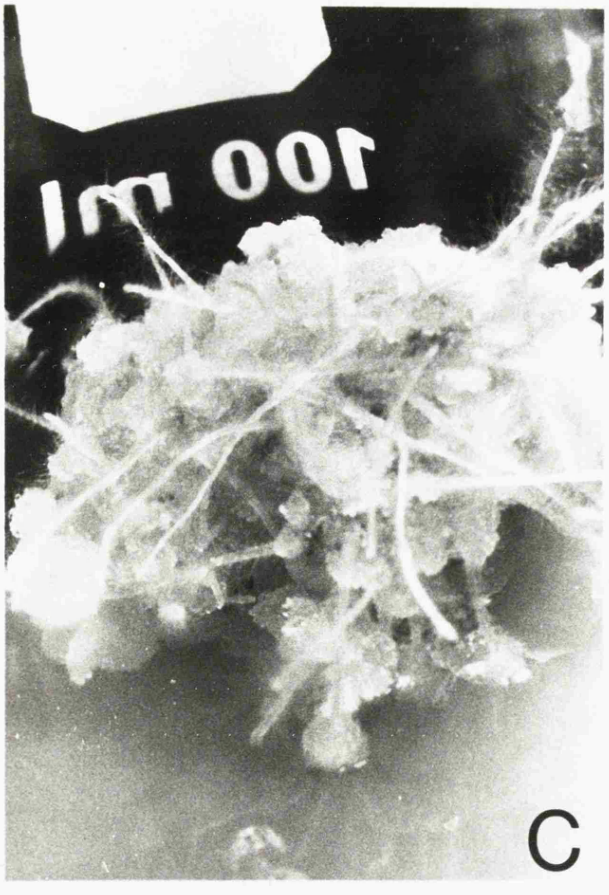
C. Culture growing as a mass of callus and long, white roots (a later stage of development from B). Under high light intensity, such a culture would show greening of the callus and roots.

(no details of conditions of culture are given since the photographs are only to demonstrate the appearance of typical wheat cultures)

Plate XV







1·0 cm

(i) Rhizogenic callus

Two types of root were observed to form from callus: thin, white roots which could grow to a great length and develop lateral branches, and fatter, green roots which had a diameter roughly three times that of the white roots. The green roots were observed to form under high light intensity, and they may not be a type distinct from the white roots, since as they elongated, their diameter decreased and the green colour remained mainly in the basal portion. All roots observed had many root hairs. Roots could arise from any point over the surface of the callus, and grew towards and into the agar.

Sections through a root-bearing piece of callus (Plate XVI) show that the roots have an internal structure very similar to seminal roots. They have a central stele with a fairly distinct endodermis. Large central xylem vessels were not always present, but this may depend upon the stage of development of the roots, or the conditions of culture. The outer piliferous layer was always present. Roots appeared to be associated with areas with a high proportion of vascular tissue, and in some cases several roots seemed to be developing from one small area in the callus (Plate XVI, C, D). Under high light intensity, roots generally were associated with a small green area of callus at their base. Upon clearing and dissection of this region, no organised structures could be found, but the cells near the vascular tissue contained many chloroplasts.

(ii) Phyllogenic callus

Shoot production from callus has already been briefly described (P. 89) and it was noted that shoots either formed as a

Plate XVI

The internal structure of rhizogenic callus.

A. A small piece of root-bearing callus, taken from a culture of Maris Ensign incubated for 4 weeks in liquid "B5" medium containing 2mg/l 2,4-D.

(x4)

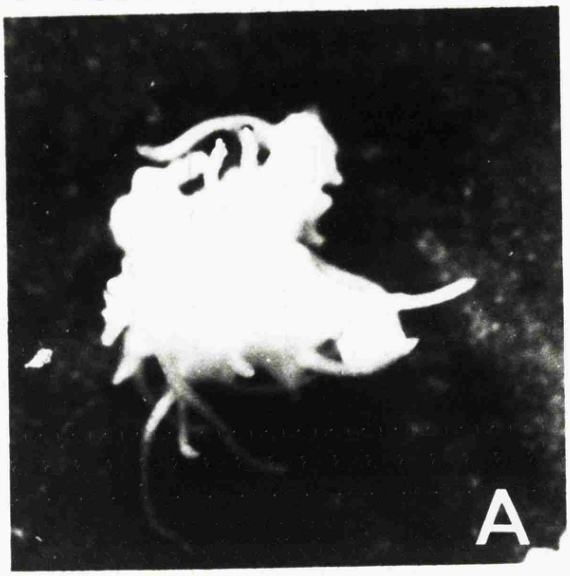
B. Transverse section through one of the roots from A, showing the internal structure to be similar to that found in seminal roots, with piliferous layer, cortex, endodermis and central stele.

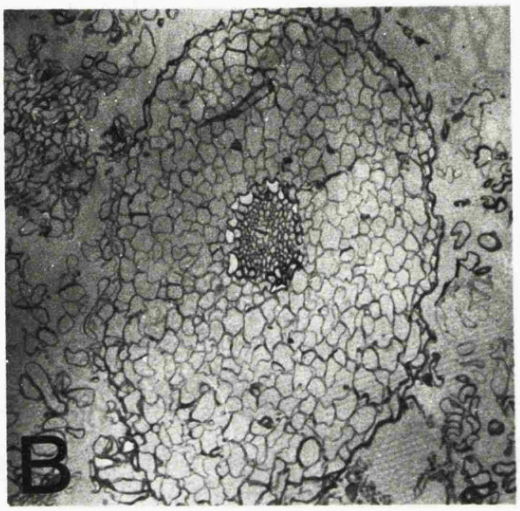
(x40)

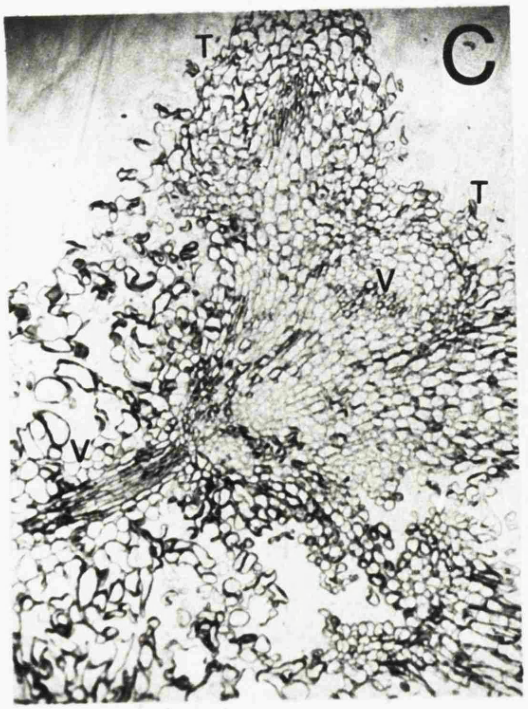
C, and D show sections cut lengthwise along the roots; where several roots are forming in the same small area, their central vascular strands appear to run together further down in the callus (V), indicating perhaps a common point of origin for these roots.(R). 'T' marks the tip of a newly-developing root.

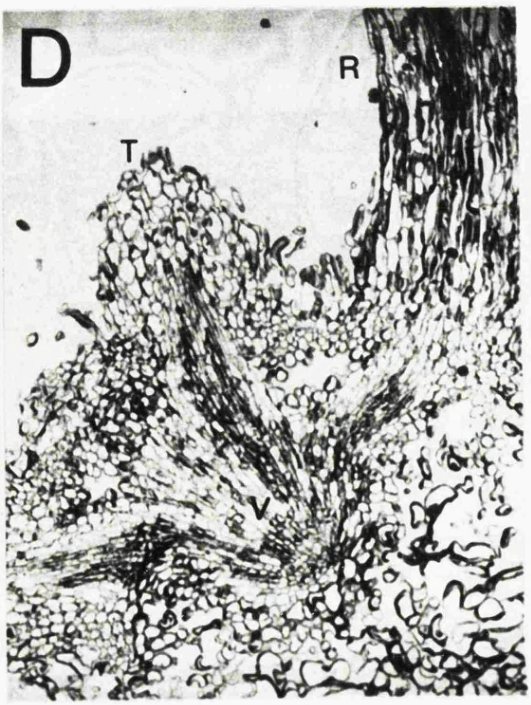
(x40)

Plate XVI









vigorous clump from within the callus mass, or as numerous small leaves and buds on the surface of the callus. It is this second type of shoot formation (or, more properly, phyllogenesis, since the first structures to arise were leaf-like) which is described further below.

The sections shown in Plate XVII were cut from a culture similar to that in Plate IX, E, F, where there is a mixture of callus and tiny green leaves with perhaps one vigorous shoot. Leaf-like or phylloid structures appeared to have developed directly from the outermost layers of callus. Vascular tissue was not necessarily associated with these structures but could generally be found in the callus immediately below. Such phylloid structures did not always develop into shoots, although those shown in Plate XVII, A, B and E do resemble buds. Roots were produced independently of the phylloid structures and a plantlet was formed if a vascular connection arose between a clump of vigorous leaves and nearby roots. Leafy structures were only observed on the upper surfaces of the callus, and often they could not be recognised as leaves or shoots during the early stages of formation (see Plate IX, A - D).

V. Anatomy of leaves of regenerated plants

Transverse sections were cut from the leaf blades of plantlets regenerated from callus tissue, to determine whether they had any abnormalities in their internal anatomy. Plate XVIII shows the internal structure of leaf blades from two such plants, A - C (which had just been transferred from culture vessel to plant pot) and D - F (which had been potted for 23 days). Differences are apparent in the thickness of the cuticle: the plant which had been in the plant pot

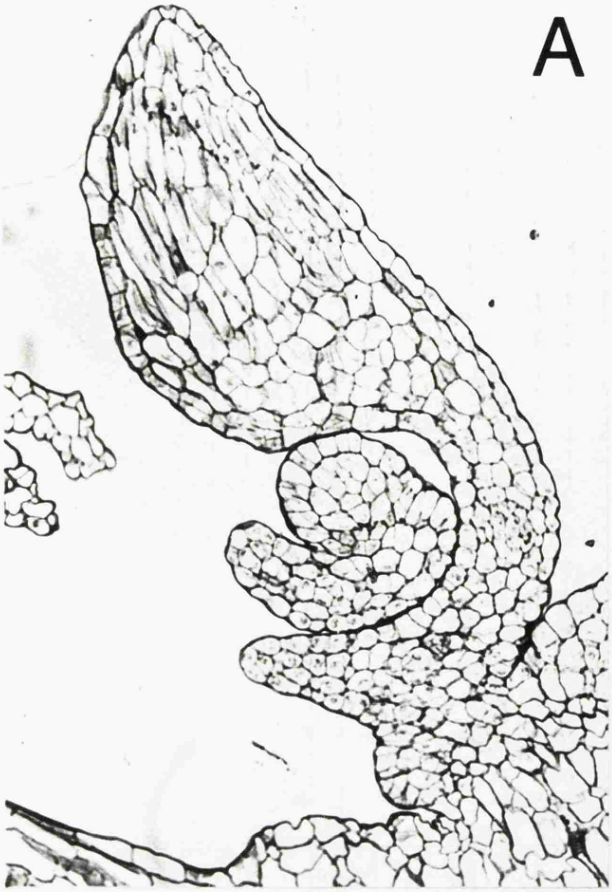
Plate XVII

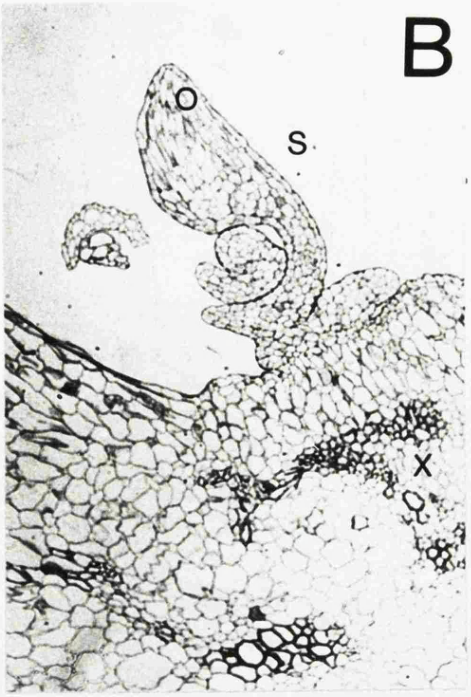
The internal structure of portions of phyllogenic callus.

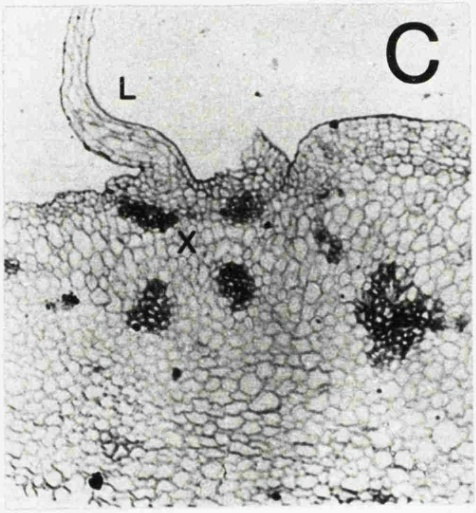
Sections were cut from plastic-embedded pieces of callus taken from a culture of Maris Ranger, similar in appearance to that shown on Plate IX,E, having many tiny green leaves and buds over its surface. The photographs show several leaf-like (L) and bud-like (S) structures, which appear to form from the outermost layers of cells. Groups of xylem cells are visible nearby in the callus (X) mass, but do not appear to be arranged into vascular strands.

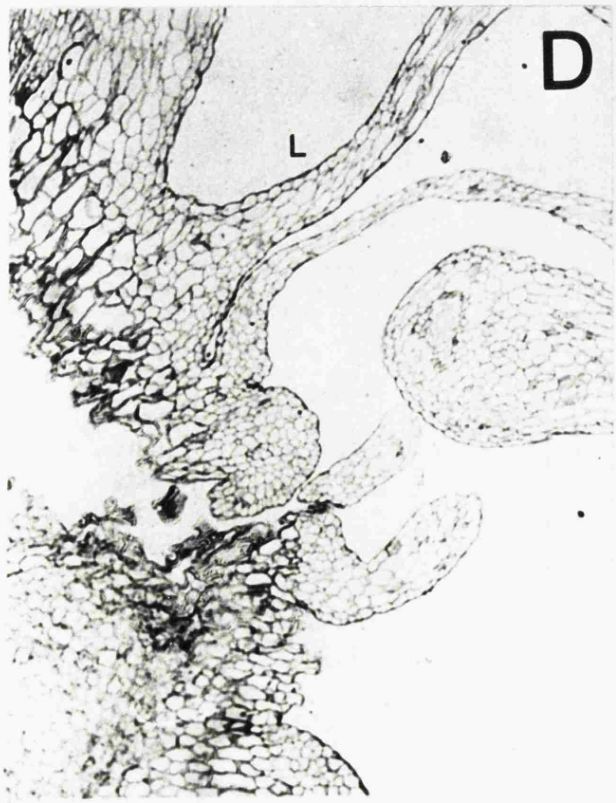
(A magnified x150; B, C, D and E are x60.)

Plate XVII









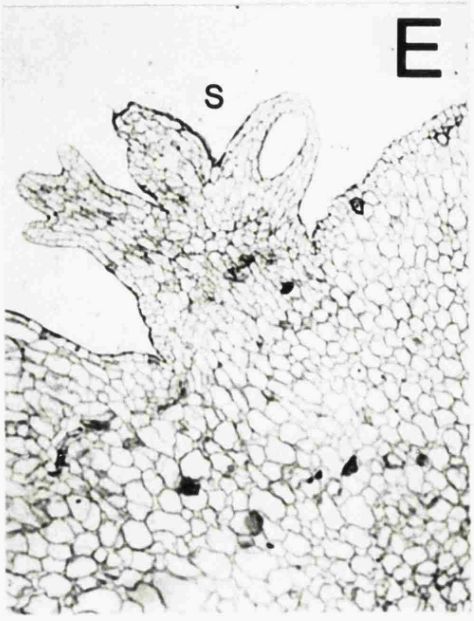


Plate XVIII

The internal anatomy of leaf blades from 2 plants generated from wheat cultures *in vitro*.

A, B and C show details of the structure of a leaf of *6 WUK 3* wheat; the plant had just been transferred from the culture vessel to a plant pot.
D, E and F show the structure of a leaf from *Maris Ranger* wheat; the plant had been transferred to the plant pot from the culture vessel 23 days previously.

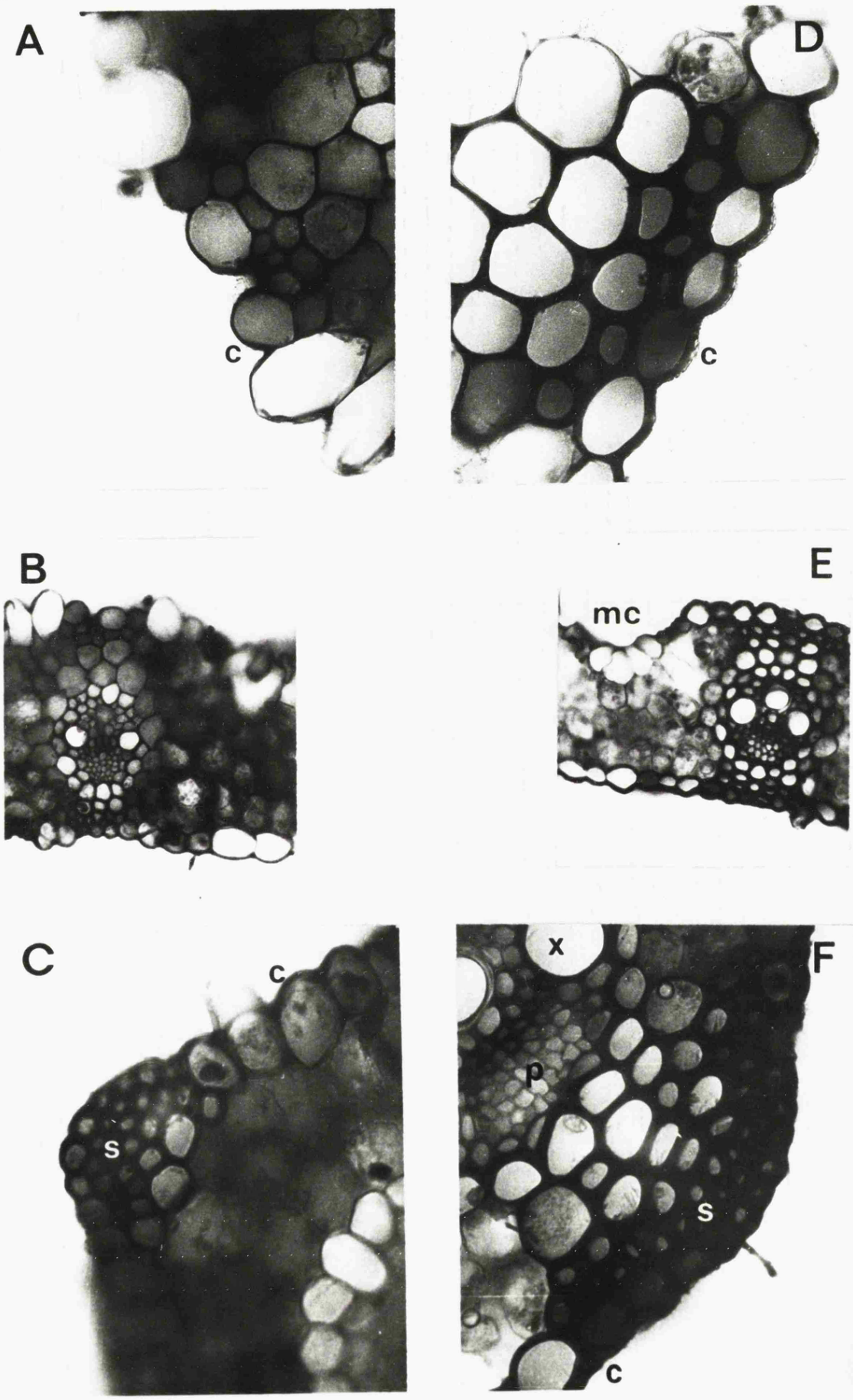
In both cases, the internal structure of the leaf is very similar to that of a plant grown from grain, and all the same cell types are present. However, the leaf of the plant just removed from the humid atmosphere of the culture vessel, A - C, has a less regular epidermis and a thinner cuticle.

A and D are sectioned cells of the upper part of the leaf;(x160)
B and E are transverse sections through the leaf, including a vascular bundle. (x64)
C and F are sections through part of the leaf including the lower epidermis. (x160)

c = cuticle
mc = motor cells
s = sclerenchyma
p = phlcem
x = xylem vessel

Sections were hand cut and stained with Paragon.

Plate XVIII



for 23 days had much more thickening of both upper and lower epidermal cuticles. The cells of the epidermal layer of A - C were not as regular in shape as those of D - F, perhaps due to the absence of cuticle. In both regenerated plants, the leaf structure is basically identical to that of plants grown from seed, and all the same cell types are present. The only apparent difference is in the thickness of the cuticle and, as Plate XVIII shows, this increases after the plant has been grown in 'open' conditions. The thin nature of the cuticle makes maintenance of a humid atmosphere essential for regenerated plants when they are first removed from the culture vessel. It was found necessary to gradually introduce regenerated plantlets to less humid conditions.

Grout, 1975, reported that regenerated plantlets of Brassica oleracea had less surface wax on their leaves than plants grown from seed, and he concluded that this was due to the environmental conditions of the culture vessel, since seedlings grown in culture vessels also had less wax.

Accordingly, material was prepared from both regenerated and seed-grown plants, for the Scanning Electron Microscope (as described in Materials and Methods). The results are shown in Plate XIX. A and B show the wax needles on the upper surface of a leaf from a side-tiller of a mature, pot-grown plant (from grain); it can be seen that the distribution of wax needles is uneven. C shows the wax on the surface of a regenerated plant which has been in a plant pot for 19 days, and D shows the leaf surface from a similar plant which had been transferred from the culture vessel for 1 day. Both C and D are of the same magnification, so it appears that the newly-transferred plantlet (D) had smaller

Plate XIX

Scanning electron micrographs of the upper surface of leaves taken from both grain-grown plants and plants regenerated *in vitro*.

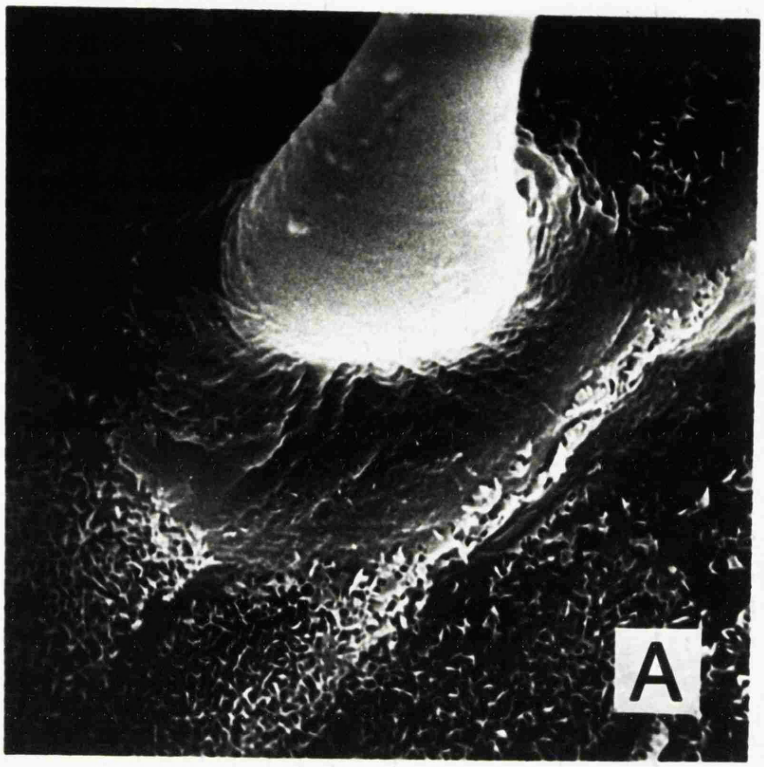
A, B. Wax needles on the upper cuticle of a leaf taken from the side-tiller of a mature *Maris Ranger* plant grown from grain in a plant pot.
(A: x3200; B: x8000)

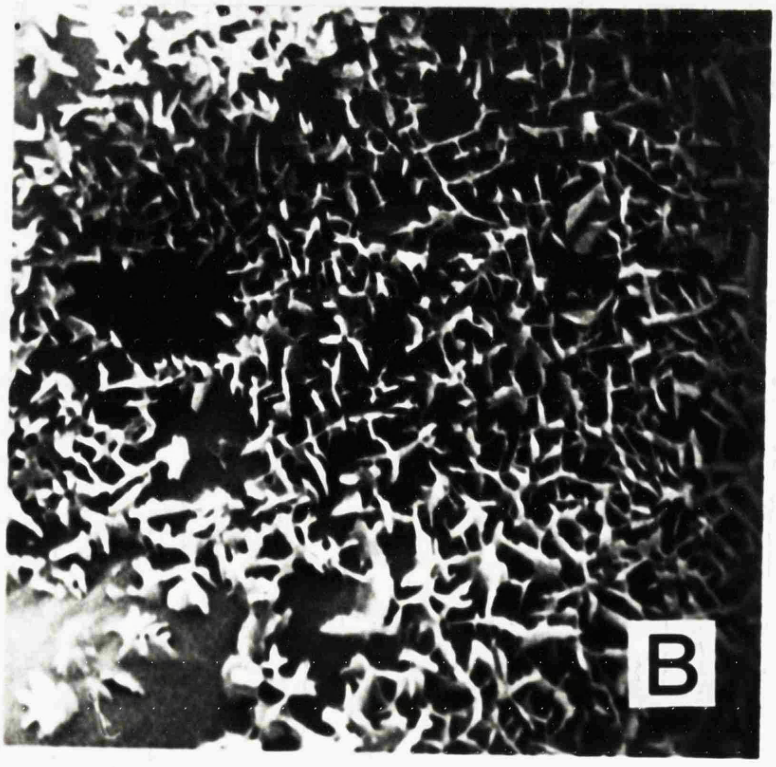
C, D. Wax needles on the surface of leaves taken from plants regenerated from tissue cultures of *Maris Ranger*, and transferred from the culture vessel to a plant pot 19 days (C) or 1 day (D) previously.
(C: x8000; D: x8000)

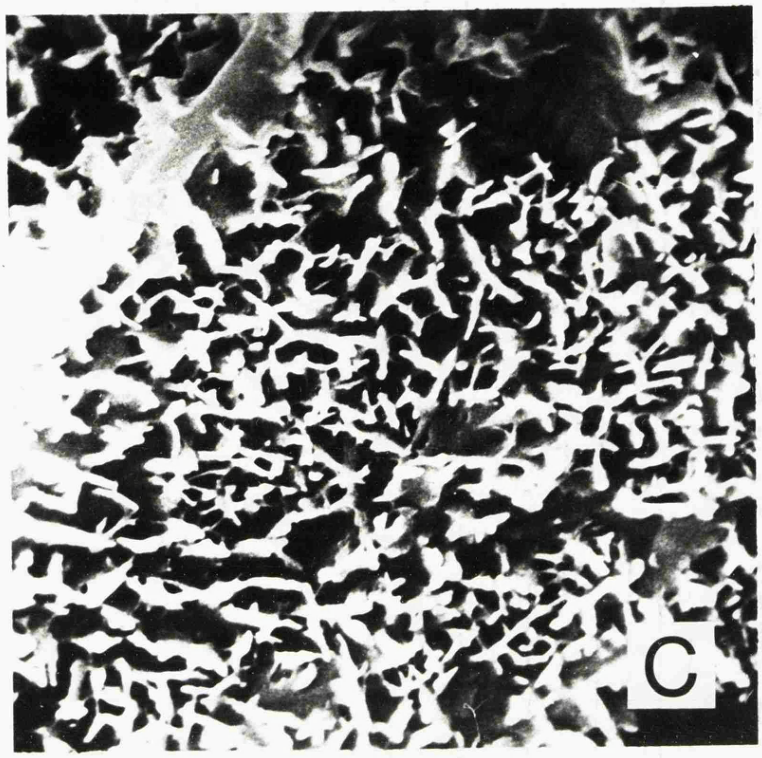
E, G. Wax needles on the upper surface of leaves from *Maris Ranger* grain which was germinated on agar (lacking hormones) in a culture vessel. (x8000)

F, H. Wax needles on the upper surface of leaves taken from plants regenerated from cultures of *Maris Ranger*, which had been growing in the culture vessel for 46 (F) or 45 (H) days.
(F: x2000; H: x8000)

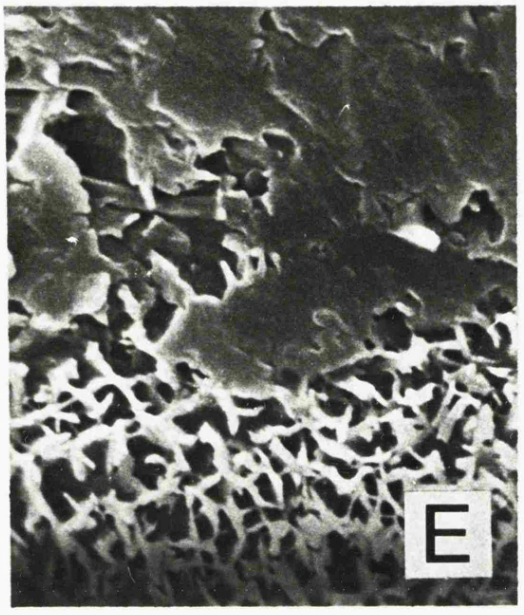
Plate XIX

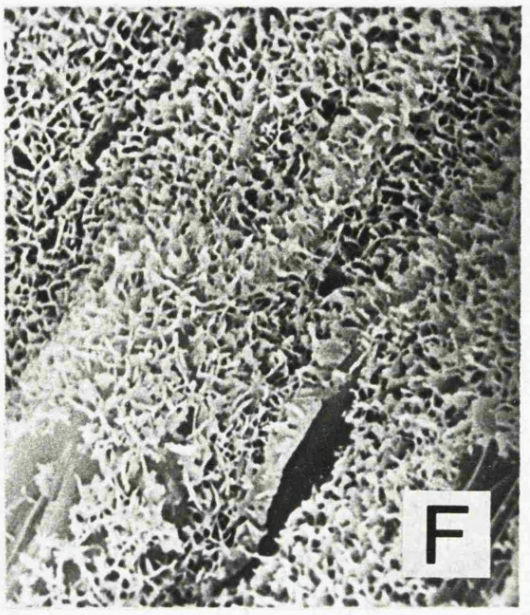


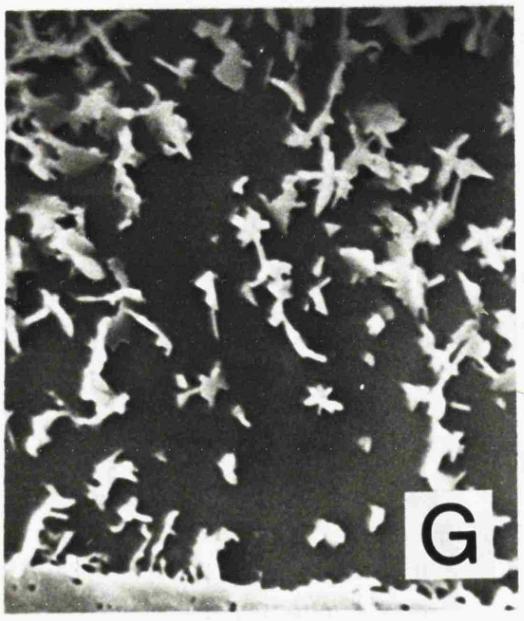


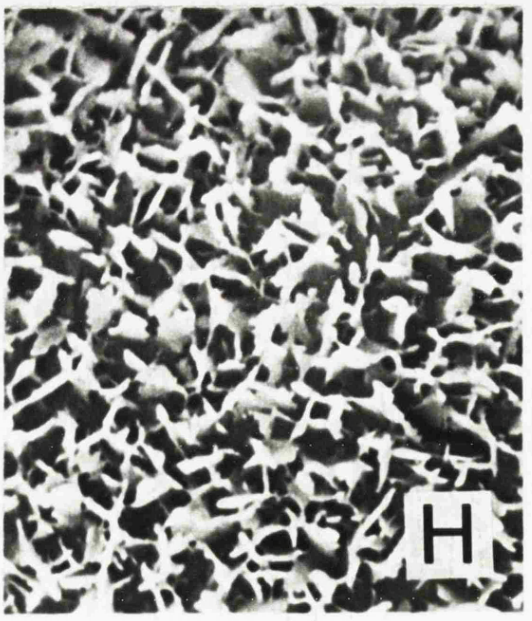

C


D


E


F


G


H

and more thinly-distributed wax needles. Again, the presence of wax was uneven. E and G show the leaf surface of a seedling grown under sterile conditions in a culture vessel, and again the needles are small and unevenly distributed. However, in F and H, the leaf surfaces from regenerated plants, which have been kept in the culture vessel for 46 and 45 days respectively, are thickly covered with wax needles.

To summarise, transverse sections of leaves from regenerated plants showed a normal anatomical structure, but had a thin cuticle which thickened when the plant was removed from the culture vessel and potted. Although Scanning Electron Microscopy revealed the presence of wax needles on the leaf surfaces of plants grown entirely in culture vessels (whereas Grout, 1975, reported their absence in regenerated plants), there is some evidence to suggest that plants in culture vessels may have smaller needles more thinly distributed, than potted plants.

SECTION THREE: Conclusions

A. Summary of Results

Below are listed the main findings to emerge during the present study of wheat tissues cultured *in vitro*. These are not always presented in the same order as the experiments from which they originated, since often the findings from one experiment were also relevant to those from another. Some of the points will be elaborated upon in the General Discussion which follows.

A. Callus tissues on semi-solid media

I. Initiation of callus from the embryo

(i) Genotypic differences in the yield of callus were observed between 10 varieties of wheat. These included hexaploid, tetraploid and diploid species, and although the total fresh weight obtained was not strictly related to ploidy level, the two *Aegilops* varieties (*A. squarrosa* and *A. speltoides*) (2n = 14) showed by far the least proliferation in culture.

(ii) Progressive depletion of components of the basic medium showed that the only essential factor for callus initiation from the embryo was the presence of 2,4-D. However, mineral salts, vitamins, sucrose and other components of a complete medium, were necessary for sustained proliferation.

(iii) The standard level of FeEDTA contained in Murashige & Skoog (1962) medium (5.6 mg/l iron) proved to be the optimum for callus initiation.

(iv) When immature embryos, complete with scutellum, were excised (9 - 25 days after anthesis) callus formed from the embryo axis rather than the scutellar tissue. 'Older' embryos tended to form callus more readily, but even embryos excised only 9 days after anthesis were capable of both germination and callus production *in vitro*.

II. Initiation of callus from other parts of the plant

(i) Each of the nodes along the plant stem was capable of callus formation. However, only those internode segments immediately above the most recently-formed nodes, were able to produce callus. Longitudinal splitting of the excised node increased callus formation from the cut surfaces. The optimum level of 2,4-D for callus formation varied depending on the original position of the node on the plant: a high level of 2,4-D (10.0 - 20.0 mg/l) produced the best callus on the first node below the ear and on young nodes from side-tillers, while 5.0 - 10.0 mg/l 2,4-D was the optimum for the other 'older' nodes. Callus production was improved if the segments of stem in culture were orientated in the same direction as they had been on the plant. If a cytokinin such as 2iP or Kinetin at 5 mg/l, was added to the medium, the explants produced less callus and became necrotic. 2,4-D at 1.0 mg/l gave the optimum production of callus.

(ii) All parts of the young seedling proved capable of callus initiation. The roots formed callus most readily but it grew slowly.

III. Growth of the callus

(i) Passage length was found to affect the amount of undifferentiated tissue present: the longer the cultures were left undisturbed, the more organogenesis was observed. Regular sub-culture at 3, 5 or 8 week intervals showed a decline in the growth rate of the cultures over several passages. There were signs that the growth rate was stabilising after several transfers, but at a low level. This decline in growth rate indicated the inhibiting influence of an unidentified factor which was either built up or depleted with time in culture.

(ii) The replicates of any particular treatment were found to vary in their response to culture. In some experiments, the level of variation was such as to make quantitative studies on callus growth difficult. The use of 20 replicates gave results which could be better statistically analysed, but limited the scope of the experiments.

(iii) No significant differences in callus growth were found when 4 media were used which differed in their mineral salt composition, indicating that wheat callus has no exacting mineral requirements.

(iv) The effect of increasing the organic content of the medium was different in the first passage from later passages. In Passage I, when compared with medium containing the standard level of vitamins, and lacking casein hydrolysate (CH), the addition of 100 mg/l CH had no significant effect on callus production. Medium containing both 100 mg/l CH and a 10-fold increase in vitamins also showed no enhancement of callus growth. However, in medium lacking CH but having an increased (x 10) vitamin content, the yield of callus was enhanced. In subsequent passages, callus growth was increased by the addition of either 100 mg/l CH or 10-fold vitamins, showing that the requirements for optimum growth may be different in the first passage from later passages.

(v) Addition of 10% coconut milk or 25 mg/l Yeast Extract to the medium, during the second passage, had no effect on callus growth.

(vi) When compared with medium containing 1.0 mg/l 2,4-D, the addition of 4.0 mg/l Tryptophan increased the fresh weight during P. I. However, if the medium contained 1.0 mg/l 2,4-D plus 4.0 mg/l Tryptophan and also 5.0 mg/l CPA, the extra auxin had the effect of decreasing the fresh weight (but only in cultures grown in the dark). In a later passage, the addition of Tryptophan to medium containing a low level of auxin appeared

to enhance rhizogenesis.

(vii) The presence of auxin was essential for callus initiation and growth. Use of 1.0 mg/l 2,4-D gave the optimum yield of undifferentiated tissue from the original explant material, while suppressing most of the extension growth of the embryo axis (below this level the embryo axis developed at the expense of callus production, while at higher concentrations of 2,4-D, callus yield was affected). CPA was found to have a slightly weaker auxin action than 2,4-D, since a higher level (3.0 mg/l) was necessary for complete suppression of the embryo axis, during the first passage. During later passages, only 1.0 mg/l CPA was needed to inhibit organogenesis. Cultures left undisturbed on medium containing 5.0 mg/l CPA were observed to remain visibly undifferentiated for at least 14 weeks. In general, whileincreasing the concentration of auxin made the cultures respond in a uniform manner and inhibited organogenesis, it also caused some decrease in the callus growth rate.

(viii) During P. I, the addition of 2.5 mg/l 2iP or 5.0 mg/l Kinetin to medium containing 1.0 mg/l 2,4-D had no significant effect on callus yield, but did favour extension growth of the embryo axis. In later passages, the addition of up to 5.0 mg/l of either Kinetin, Benzyl adenine or Zeatin, again had no effect on fresh or dry weight of the cultures. However, despite the presence of 2.0 mg/l CPA in all these media, several roots were produced and this was interpreted as due to the action of the cytokinin. Although there was no response to the different levels of each cytokinin tested, there was a difference between the individual cytokinins, in that cultures on medium containing BA showed the greatest increase in biomass.

(ix) Factors affecting the "doubling time" (time taken for a culture to double its biomass) were found to be complex. Increasing the passage length tended to decrease the "doubling-time", while the more transfers a culture had undergone, the longer the "doubling-time" tended to be. Increasing the concentration of CPA was found to progressively lengthen the "doubling-time". The "doubling-times" obtained compared favourably with those calculated from earlier published data for wheat cultures.

IV. Organogenesis in callus on semi-solid medium

(i) When cultures were transferred to medium lacking auxin, the amount of organogenesis (root formation and greening) which occurred was related to the hormone content of the medium which the cultures had been on previously. Cultures previously on medium containing 5.0 mg/l 2,4-D showed more organisation in the absence of auxin, than those previously on medium containing 5.0 mg/l CPA. Thus, the inhibiting carry-over effect of CPA was greater. Whether the cultures were incubated in darkness, or low or high light intensity, did not influence the amount of organogenesis, but did affect the type of differentiation, since under high light intensity, more greening of both callus and roots was evident.

(ii) To obtain maximum organisation of the culture, auxin needed to be omitted altogether; suppression of differentiation increased with increase in the 2,4-D level. The presence of 2.0 mg/l 2-NOA had the same effect on differentiation as when no 2,4-D was present, showing it to have a much weaker auxin action than 2,4-D.

(iii) When cultures were placed on media containing Kinetin plus either IAA or NAA in different ratios, there was little difference in response. All the cultures formed roots and showed greening of both callus and roots, although differentiation had occurred to a lesser extent where

5.0 mg/l NAA + 1.0 mg/l Kinetin were present. Only one culture formed shoots (out of 120) on medium containing 2.0 mg/l NAA + 2.0 mg/l Kinetin.

(iv) Two experiments were carried out which involved the culture of immature embryos, excised 9 - 25 days after anthesis. Although the scutellum was left attached to the explant, this was not observed to proliferate in culture. Little difference was noted between the different 'ages' of embryos with regard to their ability to undergo organogenesis. Cultures of one of the two varieties of wheat tested, 6 wuk 3, tended to become organised more readily than those of the other variety, 6 wuk 4. Inclusion of 2.0 mg/l 2iP in the medium tended to cause browning of the tissue. Organogenesis occurred when the cultures were transferred to medium containing either 0.25 mg/l 2,4-D or no auxin at all. While root formation occurred readily, shoots were produced more rarely. Cultures already showing some root formation and greening when transferred to medium lacking auxin, proved more likely to produce shoots than cultures showing no organisation at the time of transfer. In several cultures, the original embryo explant remained completely 'dormant' for 2 passages, before finally showing elongation of the shoot and root axis. This embryo axis extension could take place in the presence of 2.0 mg/l 2,4-D, but occurred more readily in the absence of hormones.

(v) Information collated from many experiments showed that shoot formation from callus tissue could occur in the presence or absence of hormones, and even on medium containing 5.0 mg/l CPA. On the other hand, rhizogenesis would only occur when the auxin level was low or omitted. Since visibly undifferentiated cultures were capable of producing shoots,

it was suggested that these apparently homogeneous cultures could contain endogenous primordia. The fact that shoot formation was most often seen during the second passage of culture, suggested that part at least of this shoot production could be due to development of meristems originating from the embryo explant which had remained inside the callus (and therefore not visible) during the first transfer to fresh medium.

Shoots formed independently of root formation were observed in cultures derived from both mature and immature embryos, and also from plant stem node segments and rachis segments.

B. Tissues cultured in liquid medium

I. Inoculation into liquid medium

(i) When pieces of callus were placed into flasks of liquid medium, a high rate of agitation (120 - 135 rpm) of the horizontal rotary shaker proved to be the most important factor for rapid dispersion of the callus. Varying the light intensity, or the level of 2,4-D or sucrose did not appear to affect dispersion.

(ii) Excised embryos placed into liquid medium, formed callus more slowly than they did on semi-solid medium.

(iii) Cultures of wheat tissue in liquid medium grew very well (nearly tripled their mass in 3 weeks), but dispersion was not good and the cultures consisted of aggregates often greater than 1mm diameter.

(iv) Raising the level of 2,4-D from 1.0 mg/l to 5.0 or 10.0 mg/l caused a decrease in aggregate size, but when these cultures were returned to the original level of 2,4-D the aggregates again increased in size.

(v) Increasing the sucrose level to 40 g/l had no effect on aggregate size and little effect on growth.

(vi) Decreasing the 2,4-D level enhanced rhizogenesis and greening.

(vii) Tissues cultured in liquid media containing different ratios of Kinetin and either NAA or IAA, all produced long roots. Greening of aggregates was also observed, but again this was not related to one particular medium.

(viii) No signs of shoot production were observed from cultures in liquid medium.

C. Anatomy of cultured tissues

I. Formation of callus from the embryo

(i) Callus formed in the region at the base of the coleoptile. The amount of shoot extension depended upon the auxin level of the medium, but root extension was seen only at very low levels of 2,4-D (less than 0.5 mg/l).

(ii) The callus formed as a loose outer monolayer of cells continuous with the epidermis of the plumule and radical (if present), and surrounding an inner core of compact tissue containing many tracheids.

(iii) Even apparently undifferentiated primary cultures contained many cell types, originating from the original embryo tissues, within the callus mass.

(iv) During the first few weeks of proliferation at least, the very young shoot buds retained their intact structure while callus formed nearby.

II. Formation of callus on root explants

(i) The original stele of the root retained its structure.

(ii) Callus appeared to be initiated from among the outer cells of the stele (pericyclic or endodermal origin?)

(iii) The original cortical cells became highly expanded although some of the epidermal cells retained their shape.

(iv) The callus appeared homogenous in its early stages, but soon began to produce further roots.

III. Callus formation on shoot explants

(i) The meristemmatic regions of callus were associated with the vascular tissue of the original leaf material.

(ii) Much of the original leaf material remained unchanged.

(iii) The callus appeared homogenous, at least in its early stages of formation.

IV. Types of callus observed

(i) Rhizogenic callus.

Two types of roots were observed: thin white roots with laterals, and fatter green roots. Both types had root hairs and an internal structure similar to that of seminal roots. Green roots were found under high light intensity, and were associated with localised green areas of callus at their base.

(ii) Phyllogenic callus.

Shoots were formed either as a single clump from within the callus mass, or as numerous small leaves and buds arising superficially. These appeared to develop from the outermost layers of callus. Roots formed independently of the shoots (if at all) and only where roots became associated with a shoot was a plantlet formed.

V. Anatomy of leaves of regenerated plants

Leaves from plants regenerated in culture were found to have a normal internal anatomy. When first regenerated, the cuticle was thin and the epidermal layer irregular; there was evidence to suggest that the wax needles present on the upper epidermis were smaller and more thinly distributed than on pot-grown plants. These features are thought to be partly due to the humid environment of the culture vessel.

B. General Discussion

Within the last 10 years, successful initiation of callus and regeneration of plantlets have been reported for most of the cereals. However, although callus can be obtained with ease, the conditions necessary for the induction of organogenesis are as yet ill-defined. During the present study, we found callus initiation to be possible from all parts of the wheat plant, except that part of the internode immediately below the node (P.48) and the expanded leaf blade. Endosperm culture was not attempted, but Trione, Jones & Metzger, 1968, tested 13 varieties of Triticum vulgare on 20 different media and reported no proliferation of the endosperm on any of these; there have been no published accounts of wheat endosperm proliferation in vitro. However, in maize, the endosperm of immature grain has proved capable of sustained divisions on medium lacking auxin (Sun & Ullstrup, 1971) and was maintained in culture for nearly 2 years, during which time no differentiation was noted. Nakano, Tashiro & Maeda, 1975, reported plant regeneration from cultures of immature endosperm of rice, and although only a low proportion of explants proliferated to callus and just 2 plants were obtained, this is evidence that cereal endosperm (at least in its immature state) is capable of both proliferation and organogenesis, given the right conditions of culture. Again, the rice endosperm required no plant hormones for initial proliferation, presumably because of high endogenous levels of these, since auxin was necessary for sustained growth in culture.

The majority of workers have used parts of the germinating embryo or young seedling as a source of callus. In these reports, many different concentrations and types of auxin and cytokinin have been used,

but as we have shown in the present study (P.46) for wheat tissues, a fairly low level of 2,4-D (1.0 mg/l) used alone is adequate for dedifferentiation and callus proliferation. The fact that these workers have successfully employed many different media for the culture of wheat tissues, is in itself evidence that wheat has no special nutrient requirements for in vitro culture. However, both in the present study and in published data, growth of the tissue was rarely as rapid as that reported for some dicotyledonous culture systems, indicating that optimal conditions for growth have not as yet been determined. It may be that there is a fundamental physiological or biochemical difference between Monocotyledons (or, more particularly the Gramineae) and Dicotyledons, since in many respects the latter group have shown themselves to be more amenable to in vitro studies. While the excised roots of Dicotyledons such as tomato can be maintained in culture indefinitely, on a fully defined medium, Street, Carter, Scott & Sutton (1961) have demonstrated that despite organic supplements to the medium, excised wheat roots show a steady decline in growth rate over several passages. Similarly, it has been noted in the present work, that although callus proliferates extremely well from the explant, in the first passage, during subsequent passages the rate of growth declines rapidly to a low level (P.54). Caramiello & Bianco (1970) have reported how wheat callus transferred to fresh medium at regular intervals over 13 months showed a steady decline in increase in fresh and dry weight (although still having a gain in total size). This was thought to be partly due to a corresponding decrease in surface area (as the total size increased) of callus in direct contact with the medium, thus restricting the nutrient supply. If the reason for the decline in growth is due to genetical factors, or to the absence of a specific nutrient, then it may be

possible to select for cells within the colony which are able to divide more rapidly than the rest on the medium provided. This kind of selection operates each time a piece of callus is subcultured to fresh medium, when the experimenter discards unhealthy-looking areas of the culture and transfers only those parts apparently growing actively. All too often, published reports do not include a description of how the procedure of transfer to fresh medium is carried out; this kind of information is particularly valuable where morphogenetic studies are involved and changes are described in the appearance of the culture when incubated on a particular medium. The nature and appearance of the piece of tissue used as inoculum has been emphasized throughout the present study of wheat tissues *in vitro*, as this has direct bearing on the response involved. In experiments to determine the optimum conditions for callus growth, the data presented for increases in fresh weight would be misleading if there was no indication of the appearance of the cultures involved; it was usually those cultures which had produced a mass of roots which showed the greatest increase in biomass.

In the present study, variability in response to culture among replicates has often been commented upon. Also, genotypic differences were observed among 10 varieties of wheat tested for callus production (P.29); one particular variety (*6 wuk 3*) tended to produce shoots more readily than the others, while callus from another variety (*Maris Ensign*) showed a characteristic lack of greening. Thus far, no variety unable to form roots in culture has been noted.

Several authors have reported genotypic differences in response to culture. Yamaguchi & Nakajima (1966), investigated the

variation in totipotency of cultured tissues derived from carrot roots, and found that response to auxin concentration varied from root to root, but not among cultures derived from the same root. Further variation was introduced since the totipotency of all the cultures declined with the number of transfers. Cummings, Green & Stuthman (1976) investigated callus initiation and plant regeneration from 25 genotypes of oats, and noted that while all but 2 were capable of callus formation, only 16 genotypes gave callus capable of plant regeneration (in the second passage). As well as this variation between genotypes (although it should be noted that only a small number of cultures were used from each genotype) they reported a difference in capacity to regenerate plantlets which was related to the part of the plant used as explant. No detailed data was presented, but excised apical meristems were claimed to be most active in this respect, and mature embryos to have less ability to regenerate plantlets than immature embryos. In the present study, regeneration of plantlets was observed from wheat callus derived from both mature and immature embryos, and also from nodes of the mature plant stem and parts of the young inflorescence (see Table 2.25). The fact that the majority of plantlets were generated from callus from the embryo simply reflects the greater use made of this tissue as a source of callus. It is not possible from the present work, to assess the comparative regenerative capabilities of the different parts of the plant. Chin & Scott (1976) compared the behaviour of callus derived from the root, embryo and inflorescence of wheat, and observed that only recently-initiated embryo callus would produce both roots and shoots: the root callus only produced more roots, and that callus from the inflorescence remained undifferentiated. They suggested that the reason

why regenerative capacity decreased with the age of the culture was due to the primary callus containing meristems from the explant which were suppressed by auxin and expressed when this was removed. Caramiello & Montacchini (1970) after histological and autoradiographic examination of callus formation from the wheat embryo, claimed that callus was formed by dedifferentiation of already-existing proparenchyma cells, and that the meristematic cells present in the explant were sensitive to disturbance and therefore temporarily inhibited. In agreement with this, we have previously noted (P.86) that the roots and shoot of the embryo axis may remain 'dormant' for 2 passages before undergoing extension. We have further suggested that, since wheat is a tillering plant, there are most probably other tiny shoot primordia at the base of the plumule and a similar temporary inhibition and later activation of the growth of these could explain at least some of the so-called 'regenerated' shoots which appeared in early passages.

From the mature embryo of wheat, the callus is known to originate in the region of the cotyledonary node (Caramiello & Montacchini, 1970). We have observed that callus formation from the shoot and root of the young seedling appeared to originate from among the cells of the vascular tissue (P. 105). Apart from the works of Caramiello (wheat), and Maeda (rice), no other workers have published histological examinations of the processes of callus formation and subsequent differentiation. Unfortunately, much of the work of Maeda and co-workers is published in Japanese, and is therefore difficult to follow, but he has drawn up a scheme for the development of zones of differentiation and root formation in culture, based on observations of callus derived from rice grain (Nakano & Maeda, 1974a), and involving

the formation of a peripheral meristem around the callus. In a second paper (Nakano & Maeda, 1974b), the morphology of rice cultures giving rise to shoots is described; shoots are reported to arise either after addition of Kinetin to the medium, or after omission of auxin. In the present studies on wheat tissues in vitro, no shoot-forming response was elicited to cytokinins. However, Caramiello & Montacchini (1973) observed bud formation with medium containing 2 mg/l Kinetin, but they reported higher concentrations than this to be either ineffective or toxic; no quantitative data was presented. Adachi & Katayama (1969) reported regeneration of 2 plantlets from wheat cultures derived from cylinders of coleoptile; the shoots appeared during the second passage on medium containing 1 μM each of IAA, Kinetin and inositol. Also, Prokhorov, Chernova & Filin-Koldakov (1974) have reported shoot regeneration in 3 cultures of Triticale, derived from embryo tissue, cultured on medium containing IAA and Kinetin (0.5 - 3.0 mg of each). Other workers have obtained plantlet formation without the use of cytokinin. Green & Phillips, 1975, observed plant regeneration from cultures of immature maize scutellar tissue after simply lowering the auxin level. By a similar process, we have obtained plantlets from wheat callus derived from immature embryo tissue (P. 80) in which, although the scutellum was left attached, it appeared to play no part in proliferation or organisation of the callus. The only reported example of embryogenesis in the grasses was achieved after removal of auxin from cultures of Bromus inermis (Gamborg, Constabel & Miller, 1970). Thus, there is conflict as to whether cytokinin is necessary for the differentiation of shoots.

In contrast, there is complete agreement among published work concerning rhizogenesis; that root formation occurs readily and is

controlled by the auxin concentration of the medium. Reducing or removing the auxin, or allowing the level to become depleted during a long passage length, has the effect of stimulating rhizogenesis. Mascarenhas, Pathak, Hendre & Jagannathan (1974) have also reported that wheat tissue grew as a mass of roots in liquid medium but as a mixture of callus and roots on the same medium solidified with agar, and this effect was reversible (suggesting the influence of availability of some gas). They also observed that gibberellic acid and low auxin favoured rhizogenesis while abscisic acid suppressed root formation.

The work of Skoog & Miller (1957) showed that it was the balance between Kinetin and IAA which was the important factor controlling whether buds or roots developed from tobacco pith cells induced to form a callus. Both auxin and cytokinin were required for active cell division. For these tobacco cells, a relatively high auxin:cytokinin ratio was needed for rhizogenesis. With regard to cereal cultures, the situation could be similar (that relative levels of two phytohormones control the form of morphogenetic expression), since they have no essential requirement for an external supply of cytokinin (indicating, perhaps, the synthesis of endogenous cytokinin) and external auxin is the controlling factor for rhizogenesis. There is other evidence that differences in endogenous levels of plant hormones can be determinative in the type of response of callus to externally supplied hormones. For instance, Yamaguchi & Nakajima (1973) demonstrated how the endogenous level of cytokinin can affect behaviour in culture. They observed that sweet potato tubers put into culture immediately after harvesting formed roots on the primary callus more slowly than when tubers which had been stored for several months were used. These differences were found to

be due to the endogenous cytokinin level, which had either become activated or had accumulated during storage. They showed that if the callus was supplied with a high level of cytokinin, then roots formed, but if placed on medium containing a low level of auxin, then buds developed. Further, they demonstrated that the addition of abscisic acid antagonised the cytokinin effect and allowed bud formation. The endogenous Kinetin level was found to vary among the cultivars tested (a possible explanation for the genotypic differences referred to earlier). Thus since root and shoot formation has clearly been demonstrated to be controlled by the balance between endogenous and exogenous hormone levels in both tobacco and sweet potato, we may postulate that the same 'rule' also applies to cereal cultures. The readiness of wheat cultures to form roots, together with their lack of response to cytokinin in the medium, suggests that the tissues already contain an adequate level of cytokinin. Thus, future studies with wheat tissues should include a study of their endogenous phytohormones, how they are affected by conditions of culture, and how such changes are correlated with capacity for morphogenesis.

Cheng, in 1975, reported that applying a high level of cytokinin directly onto a piece of Douglas Fir callus, enhanced bud development. In this case, the culture was known to be already rich in endogenous auxin, and the initially high level of cytokinin gradually decreased until a balancing-point between the two hormones was reached where bud development was favoured. Using a similar technique, it would be interesting to know whether application of abscisic acid (or some other antagonist of cytokinin) to wheat callus would enhance bud formation through an antagonistic action on the endogenous cytokinin.

If endogenous levels of phytohormones are important for future response of the callus in culture, then the initial physiological state of the explant must also be important since this will affect the initial presence of these substances and possibly the capacity for their synthesis by the cultured tissue. It is well known in work involving anther culture of wheat that superior results are obtained with field-grown material which is in "better" physiological condition than material grown in the greenhouse. Similarly, better response to culture might be shown by wheat cultures derived from plants in the best possible condition. The medium used for the callus initiation might also be critical, for example, too low a level of hormones would force the explant to deplete its own reserves and so alter the physiological state for future passages. In the present work, callus was routinely initiated using the basic medium containing 1.0 mg/l 2,4-D; future experiments should investigate whether use of a higher or lower auxin level would alter the regenerative ability of the cultures (we have noted, P.89, that shoot formation did occur after treatment with 5.0 mg/l CPA). Furthermore, while every auxin (or cytokinin) has a general effect by which it is classified as an auxin (or cytokinin), each individual substance also has its own specific mode of action; it may be that use of one particular auxin may elicit a far better response in bud-formation than the others. Alternatively, shoot-formation in the Monocotyledons may prove to be mediated by some hormonal factor, other than an auxin or a cytokinin, as yet unrecognised.

An interesting observation requiring further research was that sometimes, after one vigorous clump of shoots had developed from a piece of callus, and the culture was then left undisturbed for several

weeks, areas of the neighbouring callus would produce numerous small leaves and buds. It would be informative to determine whether grafting a small shoot into a piece of visibly unorganised callus would have the same effect. A similar phenomenon may be involved in studies described by Walkey & Woolfitt in 1970, involving the culture of meristem tips of cauliflower. Generally, meristem tip culture produces only one newly-developed plant per explant. However, Walkey & Woolfitt reported that a single meristem tip (comprising the apical dome and one or two leaf primordia) of cauliflower placed in liquid medium developed basal callus containing several meristematic areas capable of forming shoots. If kept in gently agitated liquid medium, these meristematic areas would continue to proliferate, but would readily develop to plantlets if suspended over liquid medium on filter paper bridges. Thus the meristematic regions continuously multiplied; the same technique was described earlier by the same authors (Walkey & Woolfitt, 1968) when they obtained a large number of clonal plants from a single meristem tip of Nicotiana rustica. The question to be resolved is whether the already-present leaf or bud primordia release a substance which promotes development of further meristemstic regions. The fact that these cultures of cauliflower and Nicotiana rustica were able to replicate their meristematic areas, while similar explants of other species have so far generally given rise only to single plantlets, may be due to the conditions of culture affecting the apical dominance of the original meristem. The fact that the initial explants were gently agitated in liquid medium may have been the important factor in causing a switch from normal extension growth to less organised proliferation in the initial meristem.

In the first detailed report of embryogenesis in culture, Halperin (1966) emphasised the importance of the chemical composition of the medium for the type of morphogenesis obtained. He reported that cultures derived from the petiole of carrot could be induced to form embryoids in the presence of reduced nitrogen in the medium, but if this was omitted, then only rhizogenesis occurred. The work of Skoog & Miller (1957) has also drawn attention to the importance of a reduced nitrogen supply in relation to morphogenesis of tobacco cultures. The media used in the present study with wheat tissue contained reduced nitrogen in the form of ammonium salts, and also in some cases organic nitrogen was supplied as casein hydrolysate or L-asparagine. However, in no case could the presence of regenerated plantlets be ascribed to the presence of such nitrogenous substances with any certainty.

Many other environmental and cultural conditions such as pH, light, temperature and hormone supply, operating during the callus induction period, may critically affect the type of primary callus produced. There are bound to be many differences of this kind between the experiments published from different laboratories, and conflicts among these published reports may be partially resolved by careful scrutiny of these inductive conditions in so far as full details are published. Differing auxins and different concentration ranges have been used to obtain callus from explants, and such auxins have both stimulating effects on cell division and inhibiting effects on differentiation. The primary callus may therefore fall into one of two categories. One in which the cells have been induced to proliferate by auxin but have lost (or failed to acquire) competence to respond to hormonal treatment later in culture and produce whole plants. A second

in which the primary callus contains many cells capable of expressing totipotency although transiently suppressed by auxin; such cultures are able to produce shoots and roots once the 'inhibiting' auxin has been removed. In practice, primary cultures are highly likely to contain both these types of callus.

As Halperin (1969) in a review of morphogenesis in cell culture, has pointed out, assumptions about the rôle of a particular growth substance should not be made unless a thorough histological study of the tissue involved has been made prior to hormone treatment. Such studies are seldom made, or at least this information has not been included in published reports. However, as Halperin points out, the issues are further complicated since the potential organ-forming cells are usually not identifiable as such at an early stage; certainly root and shoot primordia are indistinguishable in their initial state. The question of whether roots and shoots produced in culture originate from single cells stimulated to express their totipotency by the right hormonal balance, can be regarded as a question of strictly academic interest. It can be argued that such information is not essential; it is only necessary to be able to produce reliably the desired morphogenetic changes in sufficient abundance. Unfortunately, we are still in the position of having to report that it is not yet possible to accurately define the conditions of culture required to induce plantlet regeneration from wheat callus. We are inevitably made aware of the fundamental questions regarding the induction of morphogenetic potential, to which at present we do not have satisfactory answers. This poses the research worker with a difficult question of strategy. Should

investigations be centred on cultures which readily lend themselves to plant regeneration, in the hope that a more complete understanding of the morphogenetic processes which these cultures undergo may enable the more 'difficult' cases to be solved? Alternatively, should an entirely empirical approach be continued, testing many combinations of conditions of culture and growth substances, in the hope that a lucky break will occur which will solve the particular regeneration problem? Perhaps both kinds of study will have to be pursued simultaneously, so that the first will certainly inform the second. The work reported in this thesis, together with current work in other laboratories, would seem to bring us closer to a solution to the problem of induction of plant regeneration in cereal culture. The growing pool of information at least eliminates unproductive avenues of approach, and suggests new lines of investigation to make this aim possible.

APPENDIX

I. Composition of Media used for the Culture of Wheat Tissue *in vitro*:

Components	"M" Final conc^{n}. (mg/l)	"G" Final conc^{n}. (mg/l)
KNO_3	1900.0	as "M"
NH_4NO_3	1650.0	"
$CaCl_2.2H_2O$	440.0	"
$MgSO_4.7H_2O$	370.0	"
KH_2PO_4	170.0	"
$MnSO_4.4H_2O$	22.3	"
$ZnSO_4.7H_2O$	8.6	"
H_3BO_3	6.2	"
KI	0.8	"
$Na_2MoO_4.2H_2O$	0.25	"
$CoCl_2.6H_2O$	0.025	"
$CuSO_4.5H_2O$	0.025	"
Nicotinic acid	0.5	1.3
Aneurine HCl	0.1	0.25
Pyridoxine HCl	0.1	0.25
Glycine	3.0	7.7
Calcium pantothenate	-	0.25
L-asparagine	-	1980.0
FeEDTA (mg of iron)	5.6	5.6
Meso-inositol	100.0	-
Sucrose	30.0g	20.0g
pH	5.5	6.0

"M" medium after Murashige and Skoog (1962);

"G" medium after Green and Phillips (1975).

Components	"B5" Final conc^{n}. (mg/l)	"S" Final conc^{n}. (mg/l)
KNO_3	2500.0	405.0
$NaH_2PO_4.2H_2O$	150.0	-
$(NH_4)_2SO_4$	134.0	-
$MgSO_4.7H_2O$	250.0	245.0
$CaCl_2.2H_2O$	150.0	-
$Ca(NO_3)_2.4H_2O$	-	710.0
KH_2PO_4	-	70.0
NaCl	-	60.0
$MnSO_4.4H_2O$	13.19	-
H_3BO_3	3.0	0.57
$ZnSO_4.7H_2O$	2.0	0.3*
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.04	-
$CuCl_2$	-	0.27
$CoCl_2.6H_2O$	0.025	-
KI	0.75	0.75
$MnCl_2$	-	0.49
Nicotinic acid	1.0	5.0
Aneurine HCl	10.0	1.0
Pyridoxine HCl	1.0	1.0
Glycine	-	30.0
FeEDTA (mg of iron)	28.0	5.6
Meso-inositol	100.0	100.0
Sucrose	20.0g	30.0g
pH	5.5	5.5

*in place of 0.62mg/l $ZnCl_2$.

"B5" medium after Gamborg, Miller and Ojima (1968);

"S" medium as used by Mascarenhas (1971), after Smith (1967)

Components	"C" Final conc^{n}. (mg/l)
$MgSO_4.7H_2O$	360.0
$Ca(NO_3)_2.4H_2O$	200.0
Na_2SO_4	200.0
KNO_3	80.0
NH_4NO_3	80.0
KCl	65.0
$FeSO_4.7H_2O$	34.75*
$NaH_2PO_4.2H_2O$	16.50**
$MnSO_4.4H_2O$	4.5
$ZnSO_4.7H_2O$	1.5
H_3BO_3	1.5
KI	0.75
Nicotinic acid	0.5
Aneurine HCl	0.1
Pyridoxine HCl	0.1
Glycine	3.0
Yeast extract	1500.0
Casein hydrolysate	200.0
L-Tryptophan	2.0
2,4-D	0.5
IAA	1.75
1-NAA	0.20
Glucose	30.0g
pH	5.8

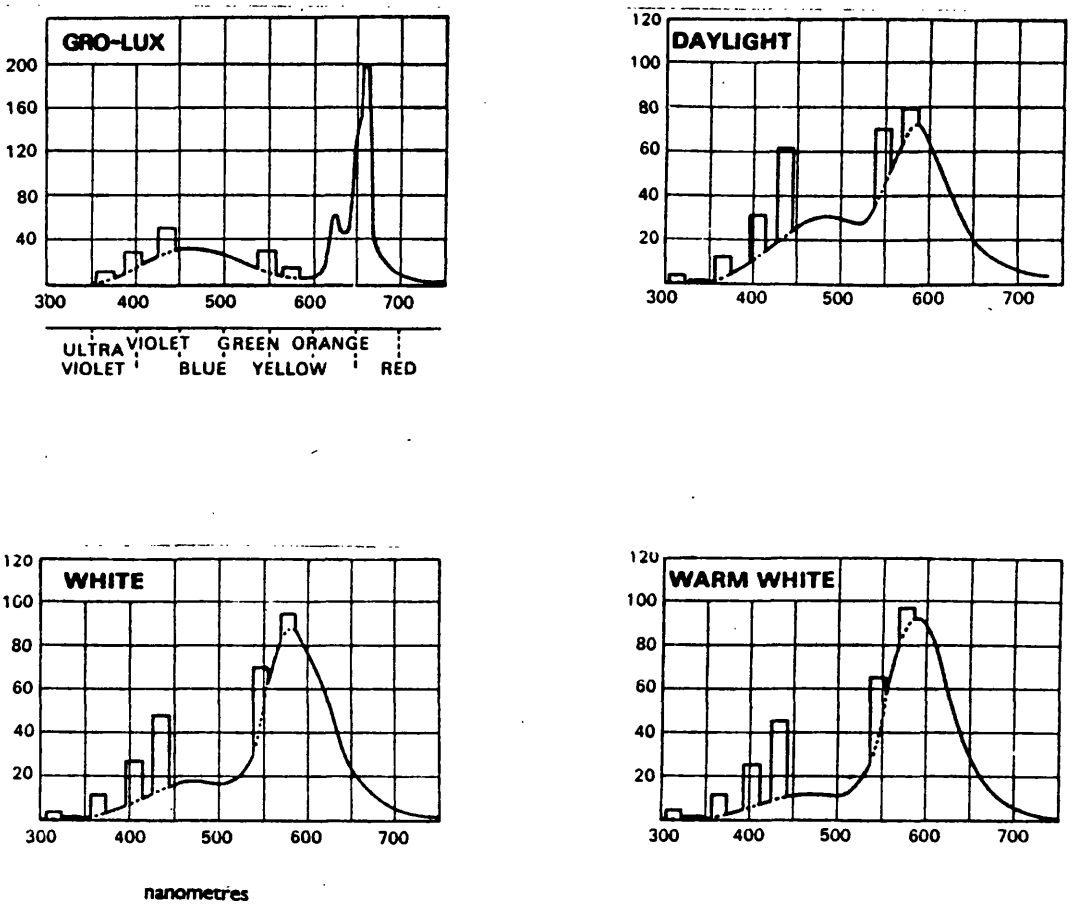
*equivalent iron to $Fe(SO_4)_3$; **in place of $NaH_2PO_4.H_2O$.

"C" medium after Caramiello (1970).

II. Growth Substances used in the Media, and their Solvents.

Abbreviation	Name	Solvent
BA	6-Benzyl amino purine 6-Benzyl adenine	1N NaOH
CH	Casein Hydrolysate Acid-hydrolysed Casamino acid	Water
CPA	p-Chlorophenoxyacetic acid	Absolute Alcohol
CM	Coconut milk (boiled, filtered and stored frozen)	-
2,4-D	2,4-Dichlorophenoxyacetic acid	Absolute Alcohol
IAA	Indole-3-acetic acid	"
Kinetin	6-(furfuryl amino)purine	1N NaOH
2iP	6-(3-methyl-2-butenyl-amino)purine 6-(γ,γ-dimethylallyl-amino)purine $N^6(\Delta^2$-isopentenyl)adenine	acidified water
1-NAA	α-Naphthalene acetic acid	90% alcohol
2-NOA	β-Naphthoxy acetic acid	Absolute Alcohol
Tri	Triacanthine 3-(3-methyl-2-butenyl)adenine	1N HCl
Tryp	L-Tryptophan	30% alcohol
Zea	Zeatin	1N NaOH

III. Spectral Composition of Fluorescent Tubes used in Controlled Environment Rooms and Growth Cabinet.



Controlled Environment Room 1 : Daylight, White.

Controlled Environment Room 2 : Warm White, Grolux.

The Growth Cabinet had tubes of a similar type to Warm White.

IV. Varieties of Wheat used for *in vitro* Culture.

		Ploidy (2n=7)	
Triticum aestivum :			
	Ardent	6n	R
	Champein	"	R
	Holdfast	"	R
	Koga II	"	R
	Kolibri	"	R
	Maris Ensign	"	R
	Maris Ranger	"	C
	MSxR (Male Sterile cross with M.Ranger)	"	R
	Troll	"	R
	6 WUK 3	"	R
	6 WUK 4	"	R
Triticum dicoccum		4n	C
Triticum durum :			
	Castelmonte	4n	R
	Indian Runner	"	C
	Stewart	"	C
	Rosetta	"	R
Triticum monococcum		2n	C
Triticum spelta		6n	C
Triticum timopheevi		6n	C
Triticum turgidum		4n	C
Aegilops squarrosa		2n	C
Aegilops speltoides		2n	C

C = grain obtained from The Plant Breeding Institute, Cambridge.

R = grain obtained from The Lord Rank Research Centre, High Wycombe.

All of these varieties of Wheat proved capable of callus proliferation from the tissues of embryos excised from the mature grain.

ADDENDUM

A Project concerning Wheat Anther Culture

The investigations described below were carried out at the Lord Rank Research Centre, High Wycombe, in May 1974.

Although successful regeneration of haploid plants has been achieved through anther culture of all the major cereals, to date yields have been low and success sporadic. The following project intended to investigate the viability of wheat pollen during the early stages of anther culture. Because of the large numbers of anthers involved, a reliable technique which was also quick and simple to perform was needed to screen the pollen, and for these reasons differential staining was chosen. Preliminary tests were carried out using several stains on fresh wheat anthers, to determine the most suitable method.

Preliminary tests using different stains on fresh anthers

Acetocarmine

While this stain does not distinguish between living and dead cells, it was an excellent stain for the nucleus and cytoplasmic contents. Acetocarmine (0.4% in 45% acetic acid) was chosen for routine staging of the anthers prior to culture.

Phenosafranin

To minimise plasmolysis of the pollen grains, the stain was made up as a 0.1% solution in 0.5M sucrose. The phenosafranin should stain dead cells a very dark red and living cells a lighter red.

Methylene Blue

This differential stain was made up in the same way as phenosafranin; dead cells should stain very dark blue.

Although both phenosafranin and methylene blue did show differential staining, there were several problems involved in their use as vital stains. They depend upon the assumption that only the most darkly-stained cells are dead, whereas empty pollen grains were generally lightly-stained because of their lack of contents. Also, there were degrees of intensity of staining, so that in many cases it was difficult to decide if a cell should be placed in the 'dead' category. Further, some cells with apparently normal healthy-looking cytoplasmic contents stained deeply. Different anthers have slightly different osmotic requirements, even if they are all at the same developmental stage, so there was always a proportion of plasmolysed cells present. Plasmolysis tends to make the damaged cells stain darkly, yet these were probably healthy living cells before staining. Another factor was that the pollen grains stained more deeply the longer they were in the stain, so that a standard time of staining (5 min) was required. Table A1 shows the percentage of dead pollen grains scored using these differential stains on freshly-excised anthers of two varieties of wheat. The 3 anthers of one floret would be expected to vary little in their content of dead pollen, yet for all 5 florets tested, there was a fairly wide difference between the anthers. Also, the proportion of dead pollen grains was rather higher than expected, and this cast doubt on the reliability of the two substances as vital stains.

Table A1: Dead pollen (= deeply stained) as a percentage of the total pollen grains from fresh anthers of wheat

Stain	Ear	Floret	Anther	% Dead	Variety of wheat
Pheno-safranin	A	1	1	26.4	6 wuk 4, bottom of spike, binucleate pollen.
			2	40.0	
			3	31.2	
		2	1	42.4	
			2	40.2	
			3	28.4	
Methylene Blue	A	1	1	37.6	Kolibri, ears, just emerging from flag leaf; uninucleate pollen; florets from middle of spike.
			2	44.8	
			3	73.2	
		2	1	29.2	
			2	42.3	
			3	31.8	
	B	1	1	44.2	
			2	45.0	

Iodine

This stain was only used with mature pollen, to detect the presence of starch. Cells with an accumulation of starch must be viable to this stage, and using iodine the viability of mature anthers was assessed as 95 - 99% living pollen grains (Dr. L. Ayre, personal communication).

3,2,5-Tetrazolium Chloride

A 1% solution in 0.5M sucrose was used to stain cultured anthers. However, the stain (which works by an enzymic reaction) needed at least an hour to develop a definite pink colouration of the nucleus, and so proved too lengthy. Also, all the pollen grains tended to stain to the same intensity, giving little differentiation.

Feulgen

Feulgen staining (as described in Materials & Methods) was used for anthers taken out of culture at regular intervals and fixed in Formalin-acetic acid (FAA) (see Exp. 2). The contents of the nucleus stained very clearly.

Fluorescein Diacetate (see Widholm, 1972)

This stain was chosen for use in Exp. 1, and problems which arose out of its use are discussed below. A stock solution of 5 mg/ml acetone was stored frozen and excluded from light; a few drops of this added to 0.5M sucrose solution was used to stain the anthers. The stain tended to be unstable and had to be kept cold while in use. The response of a cell to the stain depends on an enzyme reaction: the non-polar fluorescein diacetate (FDA) ester passes into the cell rapidly and is hydrolysed by intracellular esterase, giving a polar product, fluorescein. This does not pass in or out of the cell, and so accumulates; stimulation with UV light causes fluorescence. Hence, by this method, a cell is defined as living if it possesses active esterase.

The presence of the dye in the cell depends upon its membrane properties: if the membrane is damaged, there will be leakage of fluorescein back to the medium. Also there could be some degree of binding of the fluorescein to intracellular macromolecules. Both of these factors would lower the intensity of fluorescence. Differences in fluorescence from cell to cell could therefore be due to differences in membrane properties or cell contents. In practice, a fairly wide range of response to the stain was shown by each anther. It was found that

the walls of pollen grains which had been boiled for 10 minutes still glowed green under UV, although the walls of empty pollen grains (which had been dead far longer) showed a yellow non-fluorescent colour. Some pollen grains had fluorescing walls and the entire cell contents were glowing softly, as if the dye had penetrated the vacuole (a typical living cell should only show fluorescence of the nucleus and cytoplasm). In other cases, only either the nucleus or the cytoplasm was glowing. To obtain some degree of standardisation despite these varied responses to the stain, only cells containing a fluorescing nucleus were scored as 'living'. Table A2 overleaf shows the percentage of 'dead' pollen grains scored in fresh anthers of Champlein variety of wheat. The anthers were examined at the uninucleate stage of development of the pollen. It can be seen that the viability is much lower than expected. While there is variation among the 3 anthers of each floret, in each case, two of the anthers had a very similar viability. Fluorescein diacetate was chosen as the most suitable of the available differential stains, as it gave comparable viabilities for each of the anthers examined.

The Experiments

Two experiments were carried out, one to follow the viability of the pollen in culture, and the other to detect nuclear changes during the period of culture, using fixed samples. For both experiments, the ears from which the anthers were taken were at an early stage of development and fully enclosed by the flag leaf. Hence it was not necessary to use a sterilising agent: the anthers were simply excised from the florets under the aseptic conditions of a bench laminar air-flow unit, using sterilised instruments.

Table A2: Dead pollen (= non-fluorescing) as a percentage of the total pollen grains of freshly-excised wheat anthers, stained with Fluorescein diacetate.

Ear	Floret	Anther	% Dead
A	1	1	40.0
		2	54.1
		3	40.0
	2	1	25.5
		2	45.0
		3	25.5
B	1	1	55.0
		2	32.1
		3	50.0
	2	1	48.0
		2	35.0
		3	46.0

Medium was prepared as described in Materials & Methods, and dispensed in 10 ml aliquots to Sterilin tubes. Two media were used for purposes of comparison, and these were taken from papers where haploid plants had been obtained by wheat anther culture: "Chinese" (Ouyang, Hu Han, Chuang, Tseng, 1973) and "French" (Picard & de Buyser, 1973).

The following table shows the differences between the two media:

	"Chinese"	"French"
Mineral salts	after Murashige & Skoog, 1962	after Miller, 1963
FeEDTA	as in Murashige & Skoog, 1962	as in Murashige & Skoog, 1962
Vitamins		(after Fujii, 1971*)
Glycine	3.0 mg/l	20.0 mg/l
Thiamine HCl	0.4 mg/l	1.0 mg/l
Pyridoxine HCl	0.1 mg/l	5.0 mg/l
Nicotinic acid	0.5 mg/l	5.0 mg/l
Lactalbumen hydrolysate	250.0 mg/l	-
Mesoinositol	100.0 mg/l	100.0 mg/l
Sucrose	6%	12%
2,4-D	2.0 mg/l	1.0 mg/l
Agar	14 g/l	20 g/l
pH	5.5	5.9

* as used by Clapham 1971

Two varieties of Triticum aestivum were used Ardent and Champlein, and the plants were grown in the greenhouse under a 15°C night and 21°C day temperature regime. Only the 5 spikelets from the middle (most advanced) part of the ear were used, and of these, anthers were taken from only the two basal florets (again, more advanced than the other three). Each floret contained three anthers, each at the same stage of development. One of these anthers was stained with acetocarmine to check the stage of development, while the remaining two were excised aseptically and put into culture. All the anthers used were at the uninucleate stage, with a clearly visible vacuole. For both experiments, two media were being used, so two anthers from one basal floret were placed on "Chinese" medium, and two anthers from the second basal floret were placed on "French" medium. Altogether, 5 pairs of anthers were placed in each Sterilin tube, each of these pairs having come from a different ear of wheat. In this way, each culture tube contained a mixture of the available material, and it was hoped this would reduce the effects of variation between ears and individual spikelets.

The culture tubes were incubated under conditions of 16 hrs light and 8 hrs dark (15 - 23°C). At regular intervals, a sample tube containing 10 anthers of each variety on each medium was either scored for viability (Exp. 1) or fixed in FAA and later stained and examined (Exp. 2).

Experiment 1: Viability of pollen in cultured anthers

A culture tube of each treatment was sampled regularly after 2 to 14 days incubation. Each of the 10 anthers in the tubes was stained with fluorescein diacetate and examined under UV light through the micro-

scope. 'Living' pollen and cells fluoresced bright green: the fluorescence took a couple of minutes to develop and the intensity tended to fade after exposure to the UV radiation. Percentage viability was scored for each anther by counting random fields at x 40 magnification until roughly 100 pollen grains had been scored. The results are summarised in the histograms of Figs. A1 and A2. Despite attempts to minimise variation, a wide range of response was found between the 10 anthers in each tube, and while some anthers contained many fluorescing pollen grains, others had very few. A difference can be seen between the two varieties of wheat: Champlein anthers gave a more uniform response after 2 days in culture, the majority of the 10 anthers having about 50% living grains on both media. Ardent anthers after 2 days incubation showed a more variable and slightly lower viability. The viability of the Champlein anthers fell off very rapidly after 2 days in culture and only one or two anthers had more than 20% living pollen. There were signs that the pollen of Ardent anthers on "French" medium was surviving better than that on "Chinese" medium up to the 6th day of culture. No similar effect of medium could be detected for Champlein. In both varieties of wheat, there was always at least one anther per treatment which had a higher viability than the others. Also, even after 14 days in culture, when most of the pollen grains had degenerated, a few anthers contained a very small proportion of brightly-fluorescing pollen grains. It may well be that these few surviving pollen cells represent the proportion which might eventually form callus or embryoids under the right conditions of culture, and this could explain the low success rate reported in the literature. However, as stained by fluorescein diacetate, at most the pollen contained two nuclei, showing that while the first pollen mitosis occurred readily in culture, further

Fig. A1: % of Viable Pollen Grains in each Anther of Ardent Wheat Cultured on two Media and Sampled at Different Intervals.

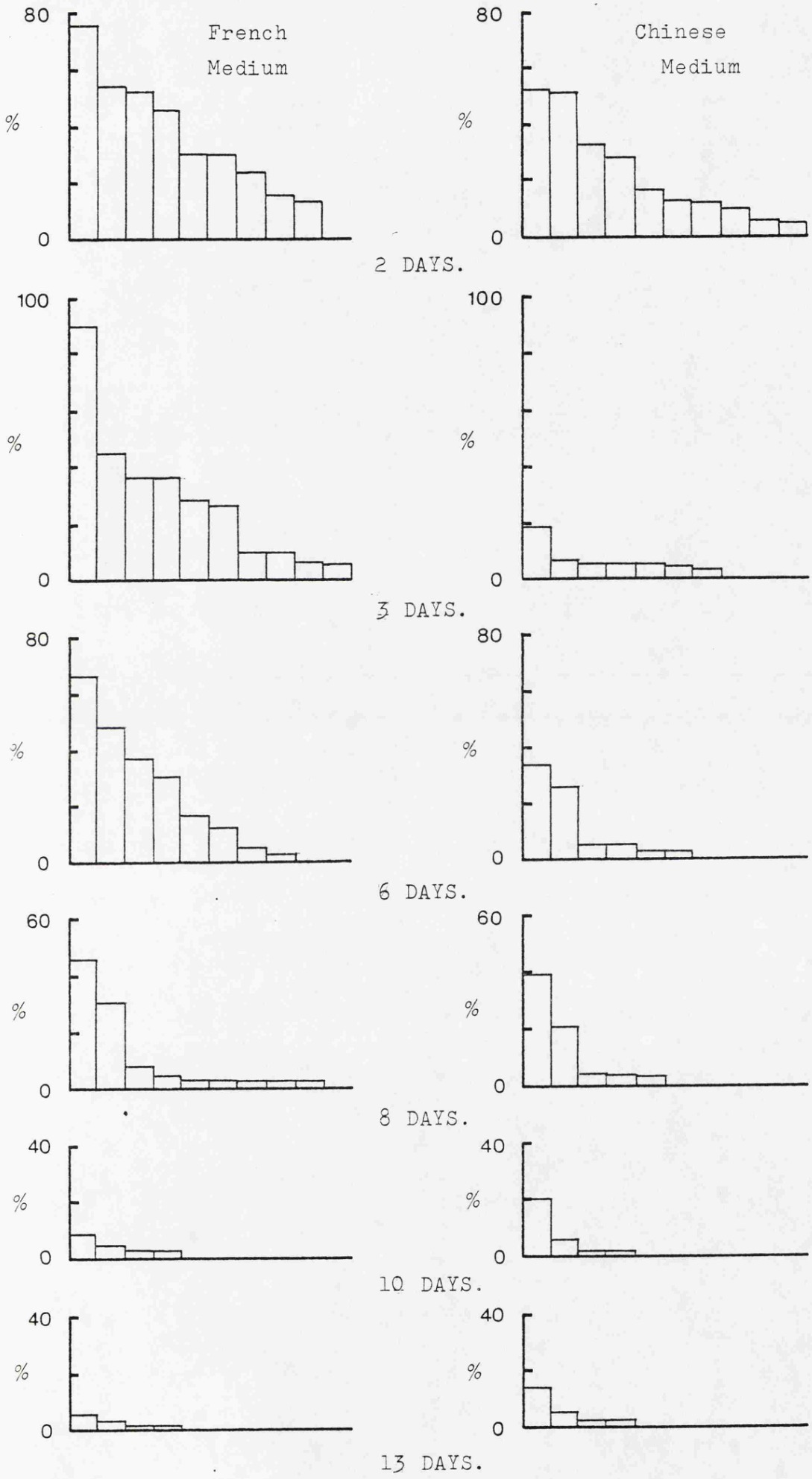
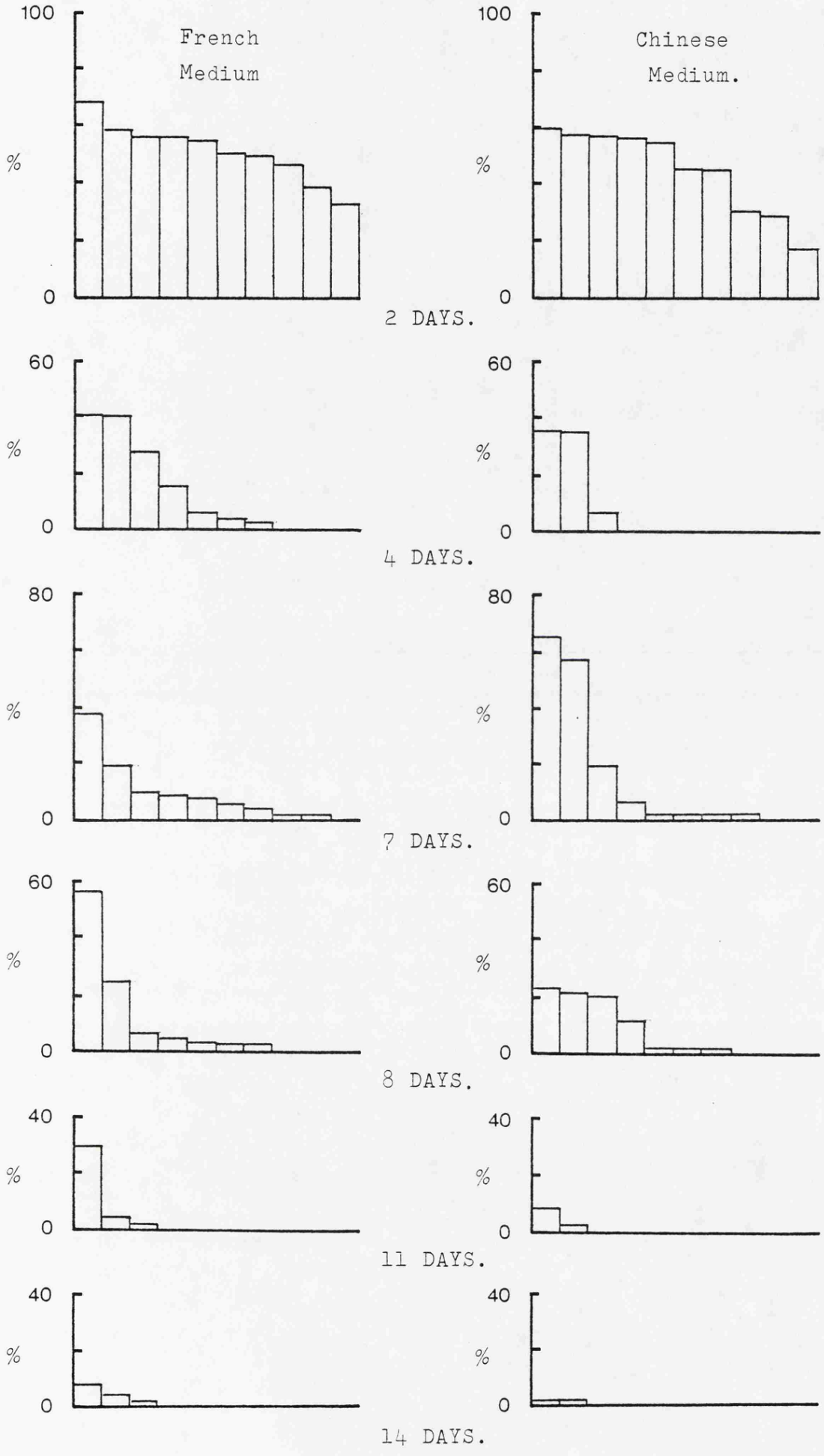


Fig. A2: % of Viable Pollen Grains in each Anther of Champlein Wheat Cultured on two Media and Sampled at Different Intervals.



divisions were extremely rare. The behaviour of the nucleus during culture will be further discussed in Exp. 2.

One of the factors to emerge from this experiment was the question of whether fluorescein diacetate could be used as a reliable vital stain for wheat pollen, since the viability as determined by this method rarely exceeded 60%, even in the earliest days of incubation. Some pollen grains had a brightly-glowing nucleus, but little cytoplasm, while others had fluorescing cytoplasm but no distinguishable nucleus (even though the nucleus could be seen when the same cell was viewed under bright-field illumination). These types could represent stages in the degeneration of the pollen cells, with, in some cases, the nucleus becoming disorganised ('dead') before the cytoplasm. There were always some plasmolysed pollen cells present, which may have been lysed before or after the stain was added. Most of these were empty, and therefore presumably had been 'dead' for some time. In those where cell contents were still present, if these contents were glowing then this suggests that enough stain had been taken up, before plasmolysis occurred, to give a positive response.

In most of the anthers examined, the cells of the anther wall retained their nuclei and cell contents for longer than the pollen cells. Thus, in many cases, a brightly fluorescing anther wall was enclosing empty pollen. However, in a small number of cases, it was the pollen which was glowing while the anther wall cells had degenerated.

Although the fluorescein diacetate proved unsatisfactory with respect to providing a clear distinction between living and dead cells, because of the wide range of response obtained, it was able to

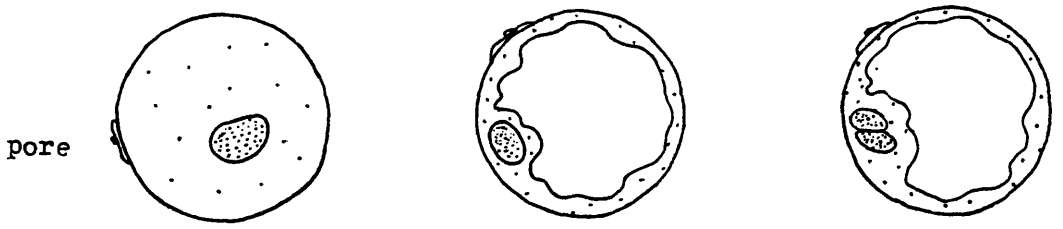
demonstrate how quickly the activity of the pollen cells declined when excised wheat anthers were placed on artificial medium. There were indications that the "French" medium may have been more beneficial than the "Chinese" medium with regard to survival of the pollen (of one of the varieties of wheat tested) in the early days of culture. Since the two media differed greatly from one another, this effect cannot be ascribed to one particular nutrient. No pollen cells with more than 2 nuclei were observed after 14 days in culture.

Experiment 2: The appearance of the pollen in cultured anthers

In this supplementary experiment, to obtain more information about what was happening to the pollen in culture, only one variety of wheat was used, Ardent. Otherwise, the procedure was the same as for Exp. 1, using the same two media. One culture tube of each medium was sampled at regular intervals, up to 18 days incubation. The 10 anthers of each sample were fixed in FAA (see Materials & Methods) and stored at 5°C until required. They were then hydrated through a series of alcohol, hydrolysed at 60°C for 12 min with 1N HCl, and then stained with Feulgen for at least 2 hours. Each anther was squashed in 45% acetic acid and the contents carefully examined under high power magnification on the microscope.

No quantitative measurements were taken; the main purpose was to observe changes in the appearance of the nucleus over several days in culture. The normal pattern of wheat pollen development in vivo, is briefly outlined overleaf (see also Plate A1):

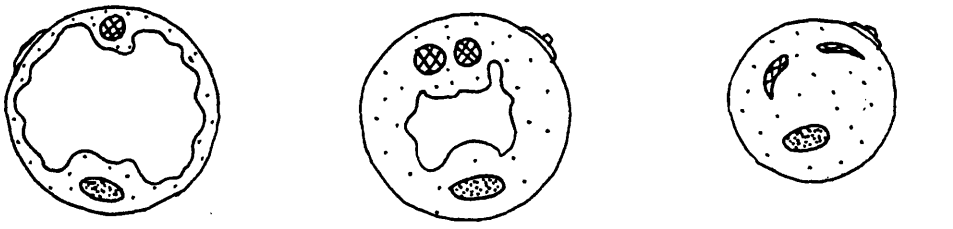
Development of wheat pollen *in vivo*:



a) Uninucleate, no vacuole

b) Vacuolate

c) Division of nucleus



d) Condensation of one nucleus to form the generative nucleus.

e) Division of generative nucleus

f) Mature state: one vegetative and 2 sperm nuclei

Several deviations from this pattern were noted, but there were also indications that many pollen grains underwent the normal process of development during the period of culture. The main observations are summarised below:

Day 0

All the anthers put into culture contained normal, healthy uninucleate pollen cells; very few of these were empty.

Day 1

"French" medium: already several binucleate grains were present and 3 anthers contained some trinucleate pollen (these anthers also contained the greatest proportion of binucleate pollen). When an

Plate AI

The photographs were taken using pollen which had been in culture for different lengths of time, but they illustrate the normal development of pollen as it would occur *in vivo* :

a. Uninucleate pollen cells having distinct vacuoles. Anthers were put into culture when the pollen was at this stage. (Day 1; 'French' medium)

b. Early anaphase of the first pollen mitosis. (Day 1; 'Chinese' medium)

c. The two large nuclei have moved to opposite sides of the cell. (Day 4; 'French' medium)

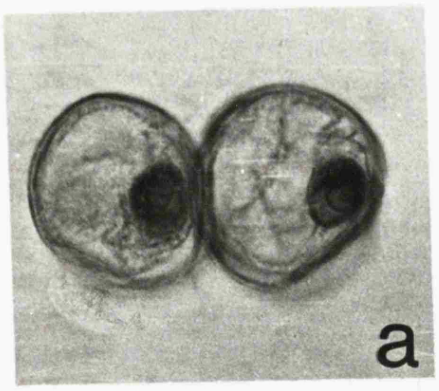
d. One nucleus (g) has started to condense, while the other (v) remains diffuse. (Day 1; 'Chinese' medium)

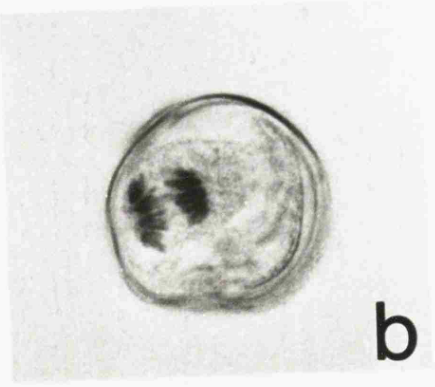
e. Clear distinction between the small generative (g) nucleus and the larger vegetative nucleus, in which two nucleoli are visible. (Day 6; 'Chinese' medium)

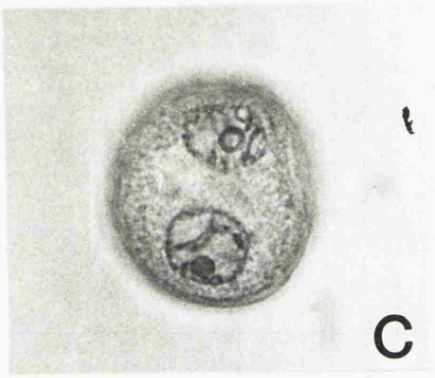
f. A group of binucleate pollen cells, one of which (arrowed)is undergoing division of the generative nucleus. (Day 4; 'Chinese' medium)

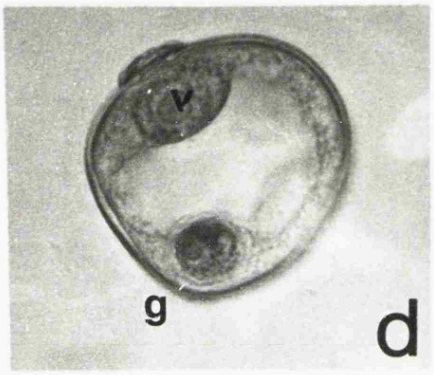
g. The final stage where the pollen grain contains one vegetative and two generative nuclei, and the vacuole is reduced. In the mature state, the two sperm nuclei are sickle-shaped. (Day 4; 'French' medium)

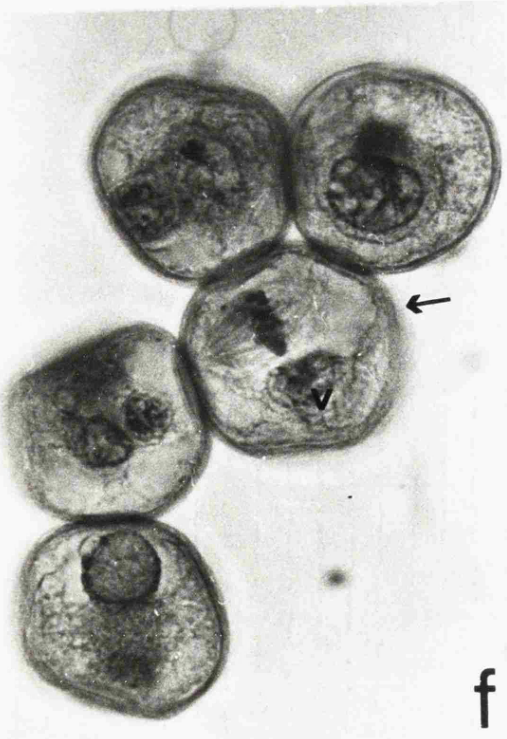
Plate A1

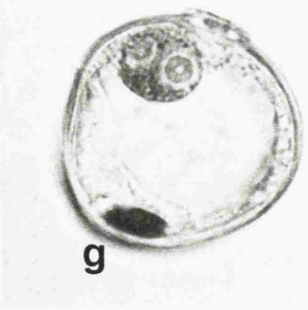


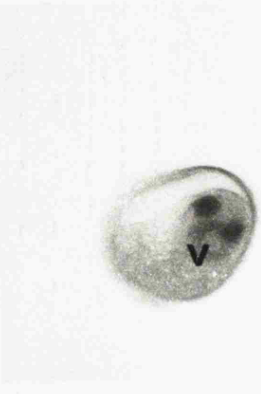












estimate was made for each anther of the number of dividing or binucleate pollen cells, there was a great difference from anther to anther, the range being from 2% to 50 or 60%. One anther had a very large proportion of empty pollen. Where two nuclei were present, both nuclei were the same (large) size.

"Chinese" medium: a similar picture to that described for "French" medium, but more anthers had a predominance of uninucleate pollen, and only one anther had a very low number of trinucleate pollen cells.

Day 4

"French": all 10 anthers had several binucleate and one anther had trinucleate pollen cells also. A tendency was noted for the 2 nuclei to be of unequal size as one of them condensed to become the generative nucleus. A greater proportion of degenerating or empty pollen was present in each anther.

"Chinese": similar to "French", but again more uninucleate pollen present.

Day 6

"French": one anther contained 7 pollen grains having 4 nuclei each. The same anther had many trinucleate pollen cells and some mitotic figures. Some of these trinucleate cells contained 3 condensed nuclei. Where 4 nuclei were present, there were 2 large and 2 small nuclei, showing that the vegetative nucleus had undergone division (except for one which had 4 small nuclei). This anther contained no uninucleate pollen. The other anthers contained a larger proportion of empty pollen than on Day 4.

"Chinese": no anthers contained pollen with more than 3 nuclei, and only one anther had trinucleate cells, the others having mostly binucleate or empty pollen.

Day 7

"French": a lot more anthers contained mostly empty pollen, although the anther wall cells were still well-studded with nuclei. Only 2 anthers had trinucleate pollen, and none had 4 nuclei.

"Chinese": most anthers had predominantly binucleate or empty pollen, although one anther also contained a few trinucleate pollen cells.

Day 8

Both media: 4 anthers had many binucleate and trinucleate pollen cells, and 2 (on "French" medium) had 4-nucleate cells and actively dividing nuclei. More pollen grains tended to have shrunken cytoplasm. Many of the anther wall cells were losing their nuclei.

Day 12

"French": all the anthers contained several empty grains, but some also had uninucleate, binucleate and a low proportion of trinucleate grains also.

"Chinese": most anthers contained only empty pollen; only one anther had binucleate and trinucleate pollen cells.

While much of the anther wall was degenerating, some areas still had many nuclei (as did the filament, also) and appeared to be regions of active division.

Day 18

"French": 6 anthers contained empty pollen only, the other 4 anthers had a majority of empty pollen cells, but also uninucleate or trinucleate cells.

"Chinese": 7 anthers had empty pollen only, the other 3 anthers had mostly binucleate pollen cells; only one trinucleate cell was noted.

Plate A2 shows some of the abnormal pollen cells observed in culture, while Plate A3 shows the degenerating pollen.

Several interesting features emerge from these observations of fixed pollen from cultured anthers. The first point is that while the pollen observed after staining with fluorescein diacetate had at most 2 nuclei, these pollen cells stained with Feulgen frequently had 3 or more nuclei. Since both experiments were conducted under similar conditions of culture, medium and using the same variety of wheat, there is the possibility that if more than 2 nuclei were present, not all of these had the capacity to respond actively to fluorescein diacetate. The originally uninucleate pollen cells had the ability to undergo at least one division (on both media tested) after only a day in culture. There was a general trend for the surviving pollen to continue development from the uninucleate to the trinucleate stage, although the binucleate stage was the one most frequently seen. During this development, the anthers on "French" medium seemed to be slightly in advance of those on "Chinese" medium (which generally had a greater proportion of uninucleate pollen). Also, it was only on "French" medium that 4-nucleate pollen was observed (on Days 6 and 8). Thus, there was evidence that this richer medium was able to support the first few divisions in culture, but neither of the 2 media was adequate for sustained division of the

Plate A2

Abnormal types of pollen development observed *in vitro*:

a. Binucleate and trinucleate pollen with normal-looking nuclei. (Day 6; 'French' medium)

b. Arrowed cell contains 3 generative nuclei. (Day 6; 'French' medium)

c. Pollen cell having 2 large and 2 small nuclei. (Day 6; 'French' medium)

d. Arrowed cell has 3 large nuclei sharing a tripolar spindle (Day 6; 'Chinese' medium)

e. Several trinucleate cells; the arrowed cell has 4 condensed nuclei. (Day 6; 'French' medium)

f. A trinucleate cell with 3 vegetative nuclei. (Day 1; 'Chinese medium)

Plate A3

Later in culture, the cytoplasm and nucleus show signs of degeneration:

a. Pollen grain in which the cytoplasm has nearly gone, although the nucleus is still distinct. (Day 4 'Chinese' medium)

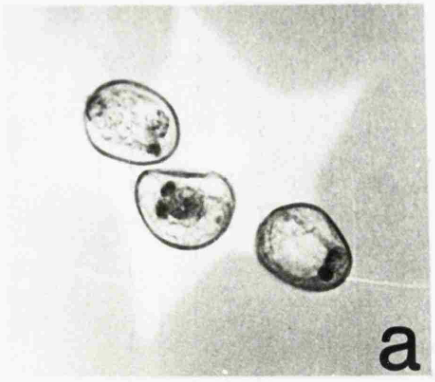
b. Many empty or nearly-empty pollen cells. (Day 4; 'Chinese' medium)

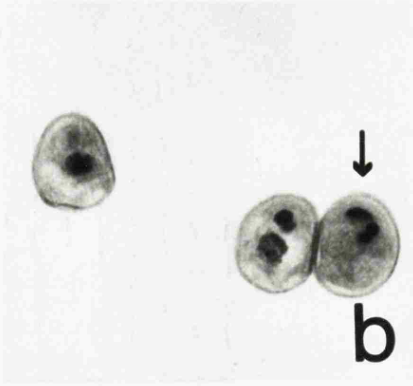
c. Nucleus still densely-stained although the other cell contents are degenerating. (Day 1; 'French' medium)

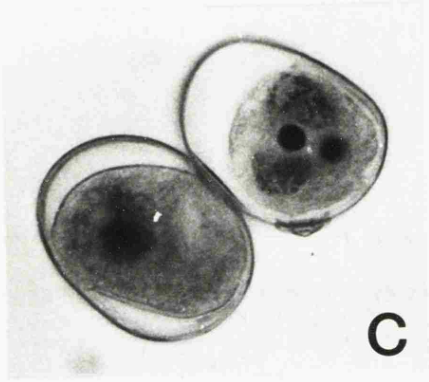
d. Part of the anther wall, showing an area of active division (many densely-stained nuclei). (Day 6; 'Chinese' medium)

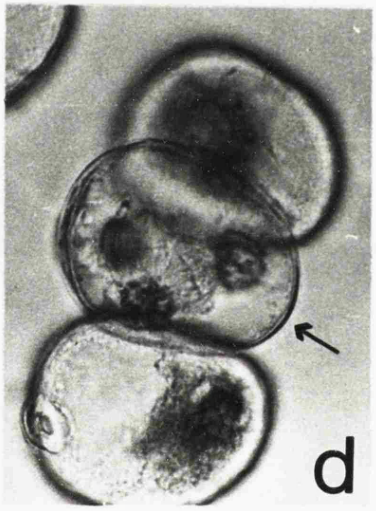
e. Many of the anther wall cells (a) are losing their contents; empty pollen inside the anther. (Day 4; 'Chinese' medium)

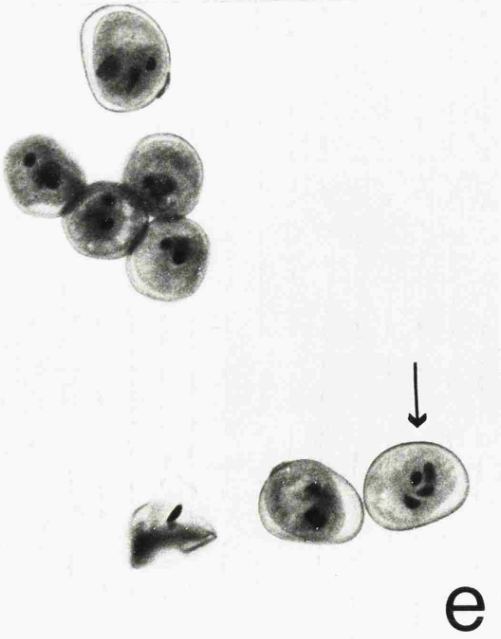
A 2

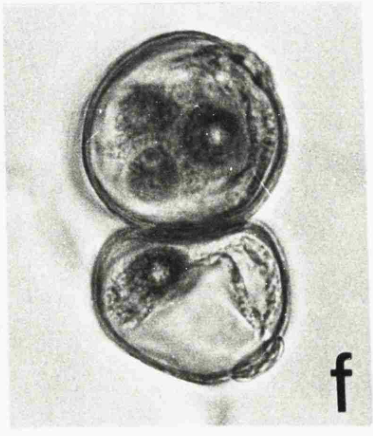




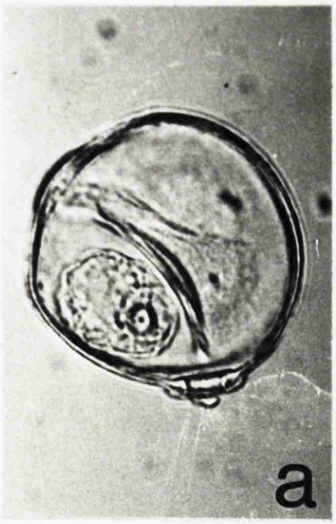


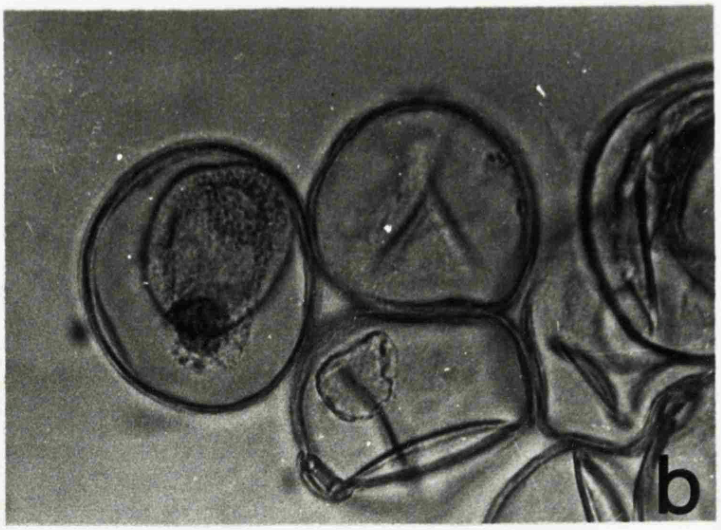


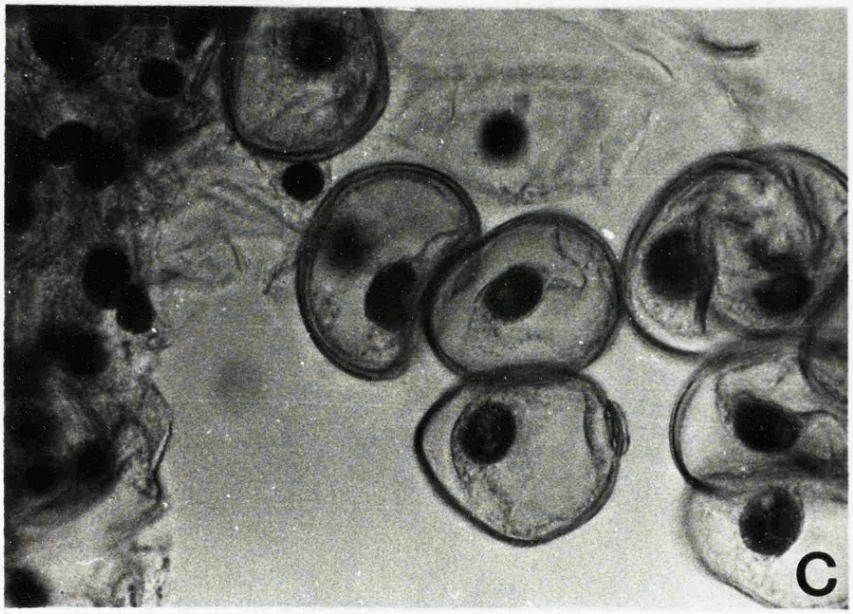


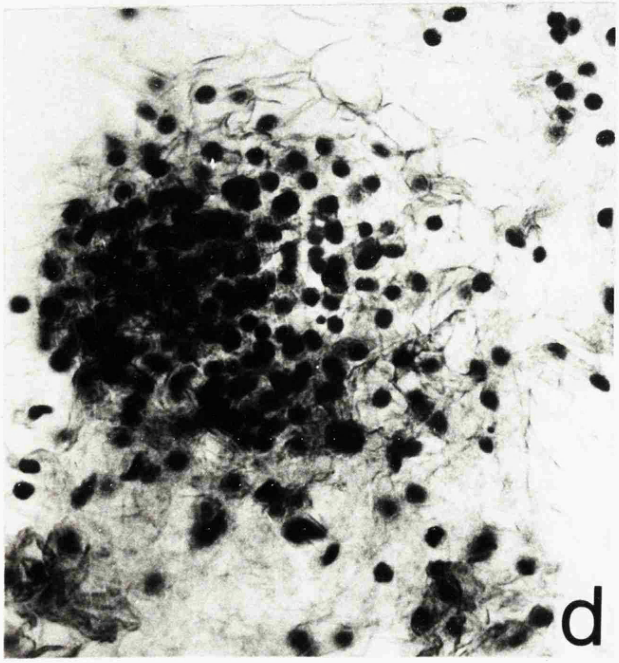


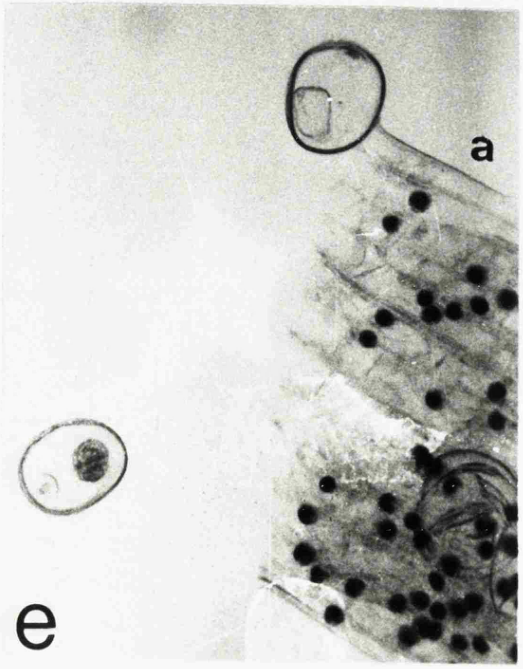
A 3











pollen and by Day 6 most of the anthers contained only dead pollen cells. The variation found from anther to anther on the same medium, could be influenced by their state of development when the cultures were set up, since although all the anthers were at the uninucleate stage, this is a relatively long phase in pollen development, and there is no way of determining whether or not the single nucleus is about to divide. Pollen cells having abnormal nuclear development were only found in very small numbers. It may be that such abnormalities do occur at a low frequency during pollen development in the normal *in vivo* situation, but this needs to be confirmed by further investigations.

The previous investigations illustrated the extremely low survival of pollen in cultured anthers. Yet, even under these manifestly unfavourable conditions, a few pollen cells were capable of multiple divisions. Similar observations have been noted in published reports of cereal anther culture. Wang, Chu, Sun, Wu, Yin & Hsü (1973) reported events similar to those which we have observed in the early stages of anther culture: they noted the presence of many normal-looking binucleate pollen cells by Day 5, and that most pollen was empty by Day 10. They found a low frequency of pollen cells having 4 or 5 nuclei, and obtained callus from 1% of the wheat anthers inoculated, after 20 - 45 days incubation. In this report, and also in later work with *Triticale* anthers (Sun, Wang & Chu, 1974), it was the vegetative nucleus which divided to give the callus or embryoid, while the generative nucleus only divided a few times at most. Clapham (1971) also indicated that it was the diffuse vegetative nucleus which divided to form pollen callus in *Lolium* and *Hordeum*. While Clapham and most other workers have noted that the best stage for anther excision is just before the first pollen

mitosis, Fujii (1969) successfully obtained pollen callus from wheat anthers at the earlier tetrad stage. While noting that the stage of development is important, Picard (1973) stressed that the time of year is also a vital factor, presumably because this affects the physiological state of the plant, especially if grown under natural conditions. Picard also recommended the use of a high level (10 - 12%) of sucrose to slow down the process of degeneration in culture.

In the present study, we observed only a slight difference in response to culture between anthers from two varieties of Triticum aestivum. However, Fujii (1969) observed greater genotypic differences. He reported that while only 3% of cultured anthers of Triticum aegilopoides gave rise to callus, 18% of the anthers of Triticum dicoccoides gave pollen callus, but he obtained no callus at all from the hexaploid wheats tested. Wang, Sun, Wang & Chien (1972) also reported different degrees of success with anthers from several genotypes of Triticale, the highest proportion of anthers giving pollen callus being 17%.

Thus, with cereal anther culture, as with cereal tissue culture, there is as yet no reliable method for inducing plantlet formation, and where callus or embryoid production does occur, the yield is always low. The present observations of wheat pollen from cultured anthers demonstrated that there is a very low proportion of pollen cells capable of survival and limited division, under the conditions tested. It may be that these viable pollen cells differ in some physiological way from the rest of the pollen (pollen dimorphism has recently been recorded in anthers of Barley: Dale, 1975), and this

may be a naturally-occurring phenomenon rather than induced by culture. However, one of the first objectives of future investigations must be to prolong the viability of the pollen in culture, and so increase the chances of successful induction of multiple divisions.

(Papers referred to in the Addendum will be found in the Bibliography).

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'The initiation, growth and morphogenesis of wheat tissue cultures' by Jane F. O'Hara

Abstract

Investigations were made into the behaviour of wheat tissues in vitro, with regard to the initiation of callus and its subsequent growth and morphogenesis, and with particular emphasis upon defining the factors leading to plant regeneration.

Despite quantitative genotypic differences, consistent callus initiation was possible from all parts of the embryo (mature and immature), the young seedling and mature plant, with the exception of the scutellum, the expanded leaf blade and certain segments of internode (endosperm cultures were not tested).

Auxin was essential for callus initiation and growth; the concentration of 2,4-D required for optimum yield of callus depended on the source of explant. Primary cultures had slightly higher auxin requirements than older callus. Typically the primary callus grew rapidly, but increase in fresh weight declined during subsequent passages. Use of several different media had little effect on growth. The time for the culture to double its biomass ("doubling-time") was affected by passage length, hormone concentration and age of the culture.

Addition of cytokinins was found to elicit little response other than a slight increase in rhizogenesis. Rhizogenesis was readily stimulated by decreasing the auxin level, but shoot formation was sporadic. A low proportion of shoot-bearing cultures was observed in many separate experiments, under different conditions of hormone treatment and using callus

derived from explants of many sources. Shoot formation was more common during the second passage, and occurred either as a single clump from within the callus, or (more rarely) as superficial leafy outgrowths. The former type of shoot formation is suggested to be due to expression of auxin-inhibited primordia, originating from the explant, continuing development once auxin is depleted.

Histological examinations revealed the source of callus initiation from different types of explant, and showed the internal structure of organogenetic cultures.

Wheat cultures grew well in liquid medium, but as aggregates greater than 1.0 mm diameter; aggregation was little affected by the level of auxin in the medium. In liquid medium, rhizogenesis and greening were the only types of differentiation observed.

A project of limited scope involving study of the behaviour of uninucleate pollen in cultured wheat anthers demonstrated the low incidence of further mitosis and the rapid loss of pollen viability.