Physic... ... s on regeneration from bu... scale leaves of Lilium spec...osum Thun.

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INTRODUCTION

During the past twenty years a vast amount of research work has been carried out on the culture of plant tissues or organs excised from parent bodies. Haberlandt's (53) theories in 1902 on the possibility of procuring growth in such isolated tissues were not put into practice until some twenty years later, when the work of Kotte (63,64) and Robbins (97,98) was partially successful and encouraged further research along these lines. Later work by White (123-125) on excised roots, and by Gautheret (43-46), Nobecourt (84-87), and White (126) on cambial tissue proved that excised plant material could be cultured on a suitable synthetic medium. From that time, an ever-increasing volume of research work has been done on the culture of plant tissue, and the technique has found application in the investigation of a variety of problems in plant physiology.

In 1939, White (126) obtained from the stem of a tobacco hybrid cultures of callus tissue which could be maintained indefinitely on a sterile agar medium of known composition. Under these cultural conditions the callus grew but did not differentiate into buds or roots. On other cultures, however, differentiation, and the direction of differentiation, could be regulated. Callus cultures in a liquid medium, where they were

submerged, produced numerous buds. This behaviour was attributed to a decrease in oxygen tension (127). Indolacetic acid or naphthaleneacetic acid added in small amounts to culture media have been found to prevent the formation of buds and to favour root differentiation (103). Larger amounts inhibited also callus growth, but this inhibitory effect could be overcome by increasing the relative concentration of sucrose and phosphate in the medium. Following this discovery, the effect of adenine and phosphorylated derivatives on differentiation were investigated, and it was found, using tobacco stem tissue, that adenine may be as specific for budding as is indoleacetic acid for rooting (104). The presence of adenine does not prevent root formation and development entirely, but it markedly decreases the number of adventitious roots and the amount of callus formed, while hastening the development and increasing the number of buds. The effects on tobacco tissue have been confirmed (78,108) and similar responses in pea epicotyls have been observed (42). These results indicate that the factors influencing growth and development may be numerous, and their interaction very complex. As yet, little is known about the mode of action of these substances, or about the cellular systems through which they exert their effect. It would appear that not only the con-

centration of specific compounds, but also the concentrations of other interacting factors, will determine the direction of differentiation. The reactive levels would seem to vary to some extent even between species (7).

In recent years, other factors have been discovered which promote the growth of excised plant tissues, notably that found in coconut milk. Coconut milk was originally incorporated in a culture medium for bacteria (12) where it was observed almost to double the production of certain species. It was later found to be beneficial for the culture of a variety of tissue (5, 94,95), and it has been used in the culture of several dicotyledonous embryos, and those of Ginkgo and Lilium (117). Van Overbeek and his co-workers found it to be essential for the growth of very young, excised Datura embryos (91,92), and Caplin and Steward (23,111) found that non-cambial tissue of the carrot tap root could be made to proliferate only if coconut milk was added to the medium. Its action was apparently only to stimulate growth, not to maintain it. Similar stimulation of growth by coconut milk was observed by Duhamet on Parthenocissus (32) and on crown-gall tissue (33), by Duhamet and Gautheret on Jerusalem artichoke (34), by Morel and Wetmore on monocotyledon tissue (81) and by Nickell (83) on plant virus tumour tissue. The active principle of coconut milk has been investi-

gated although not yet isolated (76,93), and is stated to be a factor other than indoleacetic acid (23). A similar factor has also been found in malt (11,70), in barley seed at the milk stage, and in other tissues (24, 70,76). Caplin and Steward (24) observe a common characteristic in the sources from which they obtained the "coconut milk factor" in "their ability to grow at the expense of, or to be nourished by, nucellar tissue, and to store nutrients for an eventual and very specific nutritive function."

Synergism of action of coconut milk and 2,4 - dichlorophenoxyacetic acid was observed in the stimulation of growth of potato tuber tissue (109). 2,4D is a well-known stimulator, especially of cambial tissue, but has little or no effect on the growth of potato parenchyma tissue. Coconut milk by itself has no power to stimulate the growth of such tissue either, but when these two substances are added together to the culture medium they stimulate growth to a remarkable extent. Recently Steward and Caplin (110) have found that the growth-promoting action of coconut milk on carrot tissue can be inhibited by extracts of potato periderm tissue, of onion bulb tissue, and others. The inhibitor would seem to be present in physiologically inactive or storage tissue, which suggests that the inhibitor, and/or the growth factor which it inhibits, may be part of a more

general mechanism than has hitherto been envisaged. Caplin et al. suggest that the prevalence of the inhibitory effects of such extracts may explain why parenchyma, which usually contains the main organic nutrients necessary for growth, often fails to grow, either because it lacks specific growth factors, or is prevented from responding to them by the presence of inhibitors. They suggest further that this may explain the action of 2,4D and coconut milk on potato tuber parenchyma, the 2,4D component releasing the potato tissue from the control of the growth inhibitor and so allowing it to respond to the growth factor contained in coconut milk.

In 1937, Bonner and English (36) isolated from bean pods a wound hormone which was heat-stable and water-soluble. Later, this substance was purified, found to be 1-decene-1,10- dicarboxylic acid, and named traumatic acid (16,17,37). Traumatic acid was capable of bringing about renewed growth activity in mature parenchymatous cells of the bean pod (17), inducing wound periderm formation in washed discs of the potato tuber, partially inhibiting the germination of tomato seeds (39), and acting as a growth factor for many algae (90). Davis (30) in 1949 reported that traumatic acid had no effect on the wound-healing of sugar maple, and considered that it may be specific for the bean pods

from which it was isolated.

Almost without exception, tissue culture experiments have dealt with herbaceous or woody dicotyledons representing several families. Work on monocotyledonous tissue has been for the most part neglected. Loo (71,72) and later Galston (41) successfully cultured tissue from the stem apex of Asparagus officinalis, and more recently Morel and Wetmore (81) cultured explants from the underground tuber of a tropical member of the Araceae. Kandler (58) and Almestrand (1-3) were unsuccessful in culturing the roots of several monocotyledons, some of which, although kept alive in vitro, did not proliferate, or did so feebly and for short periods.

The object of the present investigation was to culture excised monocotyledon tissue in vitro. To this end, tissue explants from the bulb scale leaves of Lilium speciosum were used. It is well-known that lily bulb scales, when isolated from the parent bulb, readily regenerate bulbils basally (25), and this behaviour is made use of by horticulturalists in the propagation of this plant. It seemed likely, therefore, that tissue excised from the bulb scale leaves of Lilium speciosum would prove amenable to culture on synthetic media. When this had been successfully accomplished, observations were made on the qualitative

effects of the several growth-regulating substances mentioned above on the differentiation of regenerated tissue. This was undertaken with a view to ascertaining whether the effects of these substances on the regeneration of lily tissue were similar to those previously observed by other workers on dicotyledon tissue.

Since substances affecting plant growth frequently alter plant respiration also, it was deemed advisable to carry out respiratory studies on lily tissue under the influence of compounds used in growth experiments. Simultaneously, comparable studies were carried out with carrot tissue, thereby providing a comparative study of their effects on monocotyledon and on dicotyledon tissue.

The wide-spread occurrence of natural plant growth-substances, in the light of their physiological activity, argues for their having some sort of universal role in plant metabolism. Since in many instances increased respiration and growth rates have been correlated with hormone concentrations increased to a maximum (beyond) which there are toxic effects), investigation has naturally followed into the question of whether hormones stimulate respiration. To date, despite numerous papers on this problem, there is surprisingly little evidence linking hormone concentration with stimulation of respiration. Prelininary investigations into the effect of indoleacetic acid on respiration led to some difference

of opinion (13,14,31,113). There seems now to be fairly general agreement that at levels of 1-10 mg. /l. indoleacetic acid stimulates respiration by 10-35% in Avena coleoptile segments and other tissues (9,15,28, 115,116). In some cases, however, growth was stimulated without a corresponding increase in respiration (14).

Kelly and Avery (60) found that 2,4D stimulated respiration in oat coleoptiles by 20% or more in concentrations of from 1-100 mg. /l., and in pea stems by 40% at 0.1-10 mg. /l. Other reports indicate similar increases in oxygen uptake in other plants (20,105,106). Conversely Taylor (114) and Hsueh and Lou (55) observed inhibitions of respiration through the action of 2,4D on certain seedlings.

From such diverse results it is apparent that the effects of growth-regulating substances on respiration are as yet not well-defined. Most of the work on respiration in recent years has been done using Warburg micro-respirometers to measure gas-exchanges. The technique lacks standardisation, and Audus and Garrard (4) have recently criticised the non-statistical approach to the whole problem. With physiological concentrations of auxin, plant respiration, which is normally low, is increased or depressed to a very slight extent. Audus et al. find that the minimal error of gas estimation using Warburg manometers is 10%, a figure which is

Part I

THE TISSUE CULTURE OF THE MONOCOTYLEDON

Lilium speciosum Thunberg

Materials and Methods.

Tissue used for culture experiments was obtained from the bulb scale leaves. The bulbs were taken from the same clone, and prior to use were grown out of doors in soil beds. Bulb scales were separated from the parent bulb by cutting through the point of junction of basal disc and scale with a sharp scalpel. Experimental explants were then obtained from these by boring out pieces 6 mm. in diameter from the extreme base, except where otherwise stated.

A sterilising fluid for these tissue pieces was prepared by stirring 10 g. of calcium hypochlorite in 100 ml. distilled water for five minutes and then filtering. The excised tissue was soaked in the filtrate for fifteen minutes before transferring to culture media.

The media employed in the following experiments were based on that of White (128), with additions of accessory growth factors.

Medium I incorporated:-	Nicotinic acid	0.5 mg./l.
	Aneurine HCl	0.1 mg./l.
	Pyridoxine	0.1 mg./l
	Sucrose	20 g. /l.
	Iron	Trace

Medium II was tested subsequent to the publication in 1951 of Morel and Wetmore's paper (81) on the culture of tissue from the underground stem of a monocotyledon, and was prepared by adding to White's basic medium the following compounds:-

Thiamine HCl	0.001 mg./l.
Nicotinic Acid	0.001 mg./l.
Ca-d-pantothenate	0.001 mg./l.
Biotin	0.0000125 mg./l.
i-inositol	0.1 mg./l.
Pyridoxine	0.001 mg./l.
Folic Acid	0.005 mg./l.
Sucrose	20 g./l.
Iron	Trace.

On the whole, cultures grew rather better on a solid medium than on glass-wool dipping into a liquid one, as well as being more easily handled in the former case, and all experiments mentioned hereafter deal with media gelled by the addition of 0.6% agar.

Tissue explants were cultured either in flasks or test-tubes to which the medium was introduced by burette. Vessels and contents were then plugged with non-absorbent cotton-wool, and autoclaved at 15 lbs. pressure for 15 to 20 minutes. After tissue and vessels had been sterilised, all subsequent manipulations were carried out in a sterile cabinet which was treated in the following manner.

Prior to use, all walls and bench surfaces were washed down with methylated spirits. The cabinet was

then steamed thoroughly and the steam allowed to settle. In this way the atmosphere was cleared of floating contaminants. When the atmosphere had cleared, methylated spirits were sprayed round the chamber, and after another steaming and spraying the cabinet was ready for use. Contamination in the many cultures set up in this cabinet was not more than 10% and usually less.

Explants were introduced into the culture vessels by means of forceps sterilised at 15 lbs. pressure for 15 - 20 minutes. Inoculated test-tubes or flasks were kept in ordinary daylight at laboratory temperature unless otherwise stated.

Results:

I. Controls.

Either medium proved to be a satisfactory synthetic base for cultures, which grew equally well regardless of which medium was used. Explants were cultured at intervals throughout a two-year period, and it was observed that the tissue showed a seasonal capacity for regeneration.

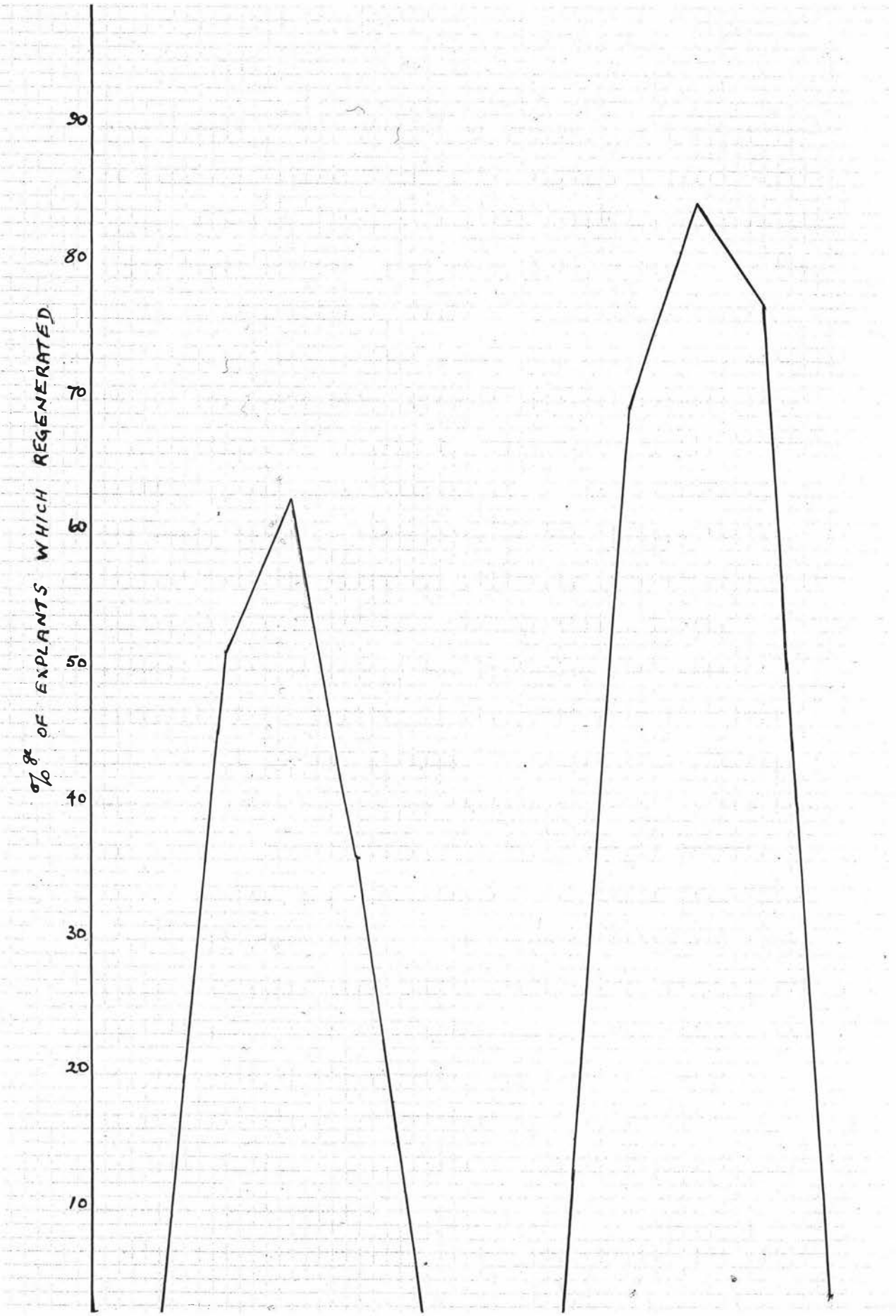
TABLE 1.

SEASONAL RESPONSE OF EXPLANTS OF *Lilium speciosum*.

	Spring	Summer	Autumn	Winter
Total No. of cultures	140	664	40	43
No. which regenerated	108	14	21	0

Graph I.

Seasonal regeneration of explants cultured in vitro.


% of EXPLANTS WHICH REGENERATED
90
80
70
60
50
40
30
20
10

From Table 1 and Graph I it may be seen that cultures grew best when excised during the spring or autumn months. At other times the tissue remained relatively unresponsive.

The first external sign of cellular activity is the appearance of a slightly swollen ring on the adaxial surface of the tissue disc about nine weeks after excision. This was obviously occasioned by internal changes, and was observed on sectioning to arise through cell-divisions occurring in sub-epidermal tissue. During the next three weeks small protuberances became clearly differentiated from the otherwise uniformly swollen ring, and proved to be bud initials. Two or three of these gradually enlarged during the following two weeks, apparently at the expense of the other potential buds, which did not develop further. A short time afterwards roots made their appearance at a point basal to the buds. Those roots, like the buds, had their origin internally, but from regions more deep-seated than those from which the buds differentiated. The vascular system of roots and buds was apparently quite distinct from that of the parent tissue. The elongating roots curved downwards along the side of the explant, and finally pushed into the agar substrate. Subsequent to the appearance of the roots, one or two leaflets elongated from the buds, bearing a green lamina.

That these regenerated plants were normal was shown

by separating them from the explant when they had developed more fully and planting them in sand in pots in a glasshouse. There they grew rather slowly, but in a normal fashion.

This cycle of regeneration is typical of that which takes place when whole bulb scales are removed from the parent bulb and allowed to propagate. In the case of the explants, however, the various stages of swollen ring formation, bud, root and leaf development take much longer to complete than in whole scales. Whole scales will complete the cycle in 5 - 6 weeks after their removal from the bulb, whereas explants take 15 - 16 weeks to reach the same stage of development in culture.

The effect of light on regeneration.

In some experiments, explants were placed in dark cupboards in the laboratory, while controls were left in light on laboratory benches. Temperature was not controlled in these experiments. Results, presented in Table 2, show that regeneration is not dependent on light.

TABLE 2.

REGENERATION IN LIGHT (L) AND DARKNESS (D) OF EXPLANTS EXCISED AT ALL SEASONS.

	L	D
Total No. of cultures	64	86
No. which regenerated	17	28

The effect of the presence of pigment on regeneration.

The lily bulb scale possesses an anthocyanin pigment in solution in the vacuole of the epidermal cells of both upper and lower surfaces. It was thought that this might in some way influence regeneration (40). To investigate this problem, the epidermis was stripped from excised tissue pieces. It was found impossible to do this without also damaging several adjacent layers of mesophyll tissue. None of the explants so treated regenerated, (Table 3), but this may have been due to excessive disruption of the tissue complex and not entirely, if at all, to lack of the pigmented epidermal layer.

TABLE 3.

REGENERATION OF EXPLANTS IN THE PRESENCE AND ABSENCE OF THE PIGMENTED EPIDERMIS.

	Epidermis intact	Epidermis removed
Total No. of cultures	94	64
No. which regenerated	67	0

Position on bulb scale as affecting regeneration.

Several experiments were carried out on tissue discs isolated from different parts of the bulb scale, namely, from apical, median and basal areas. It was observed in such experiments that there is a definite polarity in the regenerative capacity of these scales, since sections from the base almost always regenerated, those from the

middle very rarely did so, and apical tissue did not at any time proliferate (Table 4).

TABLE 4.

REGENERATION OF EXPLANTS, EXCISED IN SPRING, FROM DIFFERENT REGIONS OF THE BULB SCALE.

	Apex	Middle	Base
Total No. of cultures	26	26	94
No. which regenerated	0	1	67

Position on bulb as affecting regeneration.

Following these experiments, investigation was made on the problem of whether a lateral polarity exists within the whole bulb. Accordingly basal discs were excised from bulb scales of all physiological ages, from younger inner scales to outer mature scales. Results showed that the basal tissue of young scales regenerates as easily as does that of mature scales. Of 60 basal explants of all physiological ages grown in culture, 57 regenerated in 15 - 16 weeks.

Evidence of Transverse polarity within the bulb scale.

It was also observed that no matter in what position an explant was placed on the agar medium, regenerated bulbils always grew out from the adaxial surface. In one experiment several discs were placed on media with the abaxial surface downwards, and others with the adaxial surface on the substrate. In the latter case, regenerated buds formed on the side next to the medium

pushed into the agar base, as did the roots, and subsequent elongation of the leaflets tended to push the explant upwards, away from the agar surface.

II. Treatments.

All treatment experiments mentioned below were carried out on explants excised in spring months (September/November), since this had been already shown as being the time when excised tissue grew best in culture.

(a) Traumatic acid (T) treatment.

Traumatic acid, commonly known as the "wound hormone", is presumed to be produced in any wounded tissue prior to callus-formation over the injured surface. It was thought that this substance might be effective in promoting the formation of callus tissue in excised bulb scale discs. It was incorporated in the basal medium at a concentration of 20 mg./l. Experiments showed that, in the reactive period, excised tissue placed on such a medium produced a swollen ring, after twelve weeks of culture, on the adaxial surface of the tissue disc. At the end of a further 3-4 weeks two or three buds developed, and shortly afterwards roots, one or two per bud, appeared. After 21 weeks of culture, fully developed buds, with one or two laminate leaves, and one or two roots per bud, had differentiated.

The cycle of reganeration under the influence of T, although basically the same as that of control tissue,

takes five months to complete, in contrast to that of controls, which takes 3½ - 4 months to complete. The presence of T did not promote callus growth, and had a slightly delaying effect on the development of the swollen ring and subsequent differentiation. Of 66 explants cultured on media containing T, 48 regenerated.

(b) Indoleacetic acid (1AA) treatment.

Indoleacetic acid was added to media at a concentration of 0.2 mg./l., and it was observed that the course of differentiation was modified by its presence. The swollen ring made its appearance at the same time as in control tissue (i.e. after 9 weeks), but later growth was qualitatively different. No more than one bud developed from each piece of tissue, but as many as five long roots, with well-developed root-hairs, were associated with it. These results indicate that the addition of 1AA, although not affecting the initiation of growth, does influence the direction of subsequent differentiation in partially suppressing bud development and favouring root differentiation. The bulbil which formed on each disc produced one or two leaves, and growth was vigorous.

136 tissue explants were cultured in these 1AA experiments, and 90 regenerated in this fashion.

(c) Adenosine triphosphate (ATP) treatment.

The addition of ATP at a concentration of 40 mg./l.

to culture media did not alter the regenerative pattern, although growth and differentiation were rather slower than in control tissue. There was no change in the number of buds and roots formed, and the plants, which were completely regenerated in 21 weeks, were vigorous and healthy. 47 out of 60 explants regenerated completely.

(d) Coconut milk (CM) treatment.

CM at 15% (v/v) concentration had no effect on development, either in stimulating growth or in altering the pattern of regeneration. Fully developed bulbils were formed in 16 weeks in vitro, as was the case in control tissue.

(e) Effect of the combination T + IAA.

66 explants were cultured on media incorporating these compounds. Of these 19 failed to respond in any way, and the others produced the normal swollen ring after nine weeks of culture. Thereafter only 5 explants fully regenerated, while all further growth and differentiation were suppressed in the remaining tissue discs. This behaviour is in direct contrast to that which occurs in explants grown on media containing these two substances separately, where complete differentiation is observed. It would appear that, although neither substance prevents the initiation of growth, together they block the differentiation which normally follows swollen-ring production.

(f) Effect of the combination ATP + 1AA.

These compounds added in combination to excised tissue led to the production of fully regenerated plants in 44 out of 66 cultures. The remaining 22 failed to grow. The cycle in this case took only 12 weeks to complete. Since in explants grown on media containing 1AA alone such plants appeared after 15-16 weeks, and in tissues grown on media containing ATP alone in 21 weeks, there would appear to be some interaction between the two in combination which results in accelerated differentiation.

(g) Effect of the combination T + ATP.

The presence of T with ATP had an effect on regeneration similar to that observed with T + 1AA, in that although the swollen ring appeared in 9 weeks, no further growth or differentiation takes place. This was shown on 66 explants, 47 of which grew in culture, and all responded in this fashion, except one, which fully regenerated.

(h) Effect of the combination T + 1AA + ATP.

A similar result was obtained when T was combined with both 1AA and ATP in culture media. The number of explants which grew in vitro was rather poorer than in most experiments, being 56% of the total number, but in the responsive tissues there was no development formation of the swollen ring.

(i) Effect of CM combined with test substances.

CM, when added to media along with the five test materials either singly or in the combinations dealt with above did not in any way alter either growth rate, or the effect on development of these substances alone.

(j) Callus tissue cultures.

At no time was a callus culture of lily bulb scale tissue obtained. A very small amount of undifferentiated callus tissue is formed in the development of the swollen ring, but differentiation occurs at a very early stage. Many attempts were made to induce such callus tissue to grow when isolated from the parent tissue. It was excised from whole bulb scales which had been allowed to callus normally, and from explants which were forming the swollen ring in culture, and transferred to the culture media mentioned above. In no case, however, was its continued growth maintained.

Part II

SEASONAL SUGAR AND NITROGEN CONTENT IN THE BULB SCALE LEAVES OF *Lilium speciosum* Thun.

Materials and Methods:

During the period when tissue cultures of lily bulb scale tissue were being grown, estimations of the level of sugar and nitrogen present in the whole bulb were made at intervals. Extraction of sugar and soluble nitrogen were carried out after the method of Loomis and Shull (73). About 100 gm. fresh weight of lily bulb scales picked at random were broken into small pieces, dropped into boiling 80% ethyl alcohol and refluxed for one hour. At the end of that period the contents of the reflux flask were filtered. The solid residue was dried at 110°C. for at least 24 hours and subsequently used in the assay of insoluble nitrogen. The filtrate was concentrated to 3-4 ml. in a boiling water-bath, made up to 500 ml. with distilled water and used in the determination of reducing sugar, non-reducing sugar and soluble nitrogen content.

A. Estimation of sugar content.

The micro-method of Somogyi (107) was employed in the quantitative assay of both reducing and non-reducing sugars. This method depends on principles similar to those of the Fehling's test, but the amount of cuprous oxide formed by the reducing action of the sugar is more

accurately measured.

1. Reducing sugar estimation.

The filtrate was treated directly with the various reagents of the Somogyi method.

2. Non-reducing sugar estimation.

10 ml. of the filtrate were hydrolysed by boiling for 15 minutes with 1 ml. of 2N sulphuric acid. In this way non-reducing sugar was converted to reducing sugar. After the solution had cooled, been neutralised with 1N sodium hydroxide and made up to constant volume with distilled water, a normal estimation for reducing sugar was carried out on it. The value obtained expressed the total amount of reducing sugar in the solution, and by subtraction the amount of non-reducing sugar in the sample was obtained.

All values are expressed as mg./100 g. fresh weight.

B. Nitrogen Estimation.

A micro-Kjeldahl apparatus was used for the estimation of nitrogen. To each sample in the micro-Kjeldahl flask were added 5 ml. concentrated sulphuric acid and 0.5-1 gm. of a catalyst mixture, prepared by combining 20 gm. cupric sulphate with 80 gm. potassium sulphate. Digestion was allowed to take place, and the contents then transferred to a measuring flask and made up to constant volume with distilled water. 2 ml. aliquots of this solution were pipetted into a steam-distillation

apparatus, 2 ml. 40% sodium hydroxide added (75), and nitrogen in the aliquot steam-distilled into boric acid solution (96) as ammonia. This solution was then titrated against 0.1N hydrochloric acid, and values expressed as mg. N/100 gm. fresh weight.

1. Soluble nitrogen estimation.

The determination of soluble nitrogen content was carried out on 5 ml. samples of the extract obtained from refluxed bulb scales.

2. Insoluble nitrogen estimation.

0.5 gm. samples of the dried residue from the bulb scale extraction were used in the assay of insoluble nitrogen.

RESULTS.

The results of this investigation are plotted on Graph II. It may be noted that both reducing and non-reducing sugar levels rise gradually from low values during October-April (when plants are flowering and subsequently dying-back) to a peak lasting over July-September (when young roots develop and shoot-growth is active).

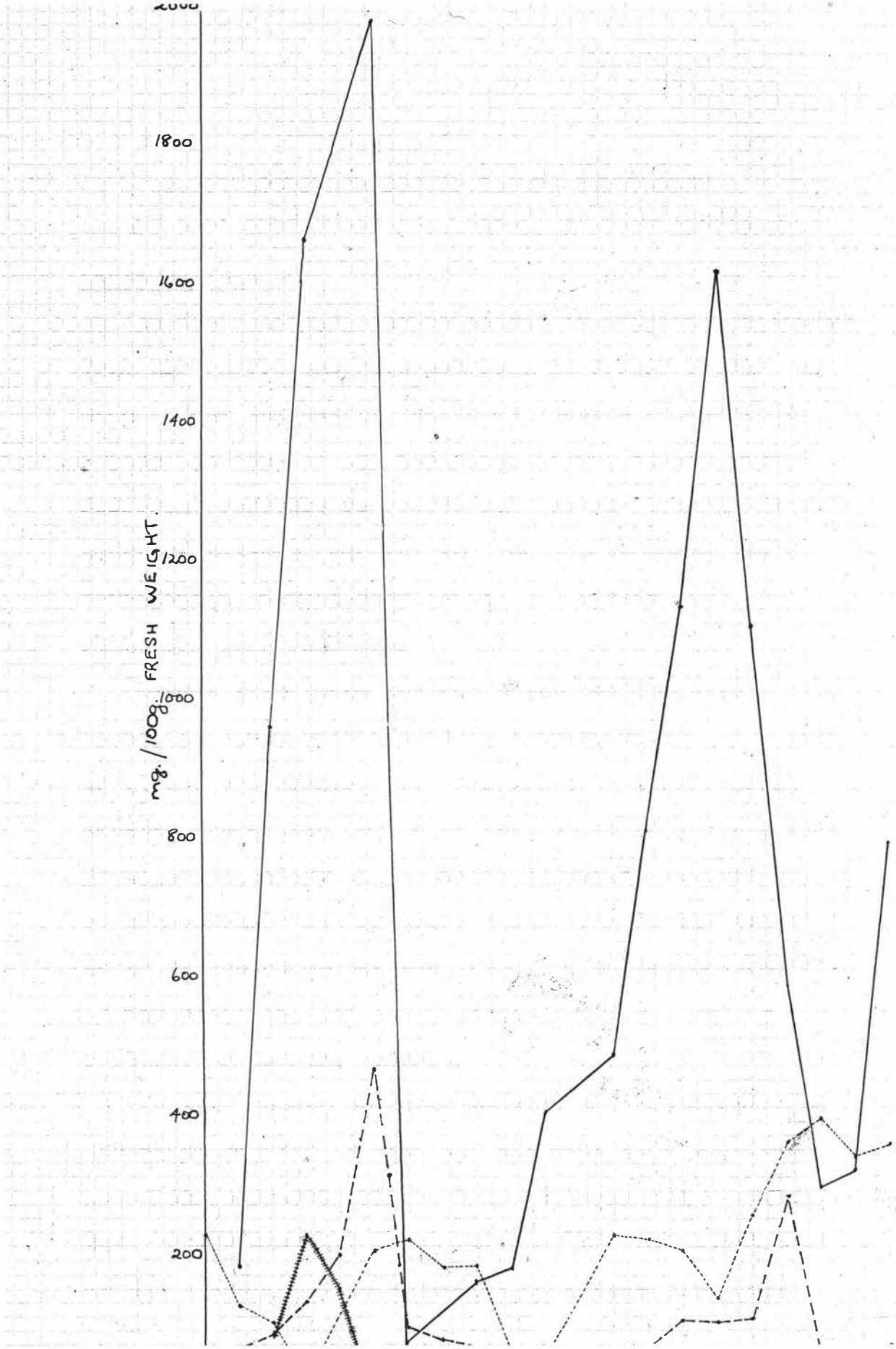
Insoluble nitrogen concentration exhibits two peaks during the year, the first over September-November (when there is much shoot-growth prior to flowering), the second during March-May (after flowering).

From tissue culture experiments, explants were found to regenerate over the periods September-November and

Graph II.

Sugar and nitrogen levels in the bulb scales of Lilium speciosum Thun. from April, 1951, to December, 1952.

——— non-reducing sugar.
– – – – – reducing sugar.
..... insoluble nitrogen.
~~~~~ soluble nitrogen.
~~~~~


1800
1600
1400
1200
1000
800
600
400
200
mg./100g. FRESH WEIGHT.

Part III

RESPIRATORY EFFECTS OF GROWTH SUBSTANCES ON LILY BULB SCALE AND CARROT TAP ROOT TISSUE.

Introduction.

In the following investigation on respiration, tissue from the carrot tap root and from lily bulb scales was used. The purpose of the investigation was twofold: (a) to find out if the monocotyledonous tissue gave a different response to growth-substances from the dicotyledonous tissue; and (b) to find out if there was a correlation between respiration and growth response using the same chemicals.

Oxygen absorption was the criterion observed in all cases, measured with a Warburg respirometer. A survey of literature dealing with respiratory studies using Warburg manometers indicates that there is a great lack of standardisation of technique affecting several points of procedure. Two of these would seem to the writer to be of paramount importance, namely the time of washing of cut sections, and the fluid used for suspending sections in Warburg vessels.

In many cases, workers studying the respiration of tissue slices do not wash cut sections at all before inserting them into the vessels (6,27,59,77). Others have washed them for intervals varying from a quick rinse to 650 hours. It is obvious from many experimental

results where tissue has been washed for hours, and even days, prior to the estimation of its respiratory rate that such treatment has a profound effect on respiratory activity. It is indeed recognised that such periods of washing serve to increase the subsequent respiratory rate to an appreciable extent. Schade et al. (6,101), working with potato tissue, and Stiles and Dent (112) with various storage tissues, observe that respiration increases with time of washing. It has also been stated that malonate stimulates the respiration of carrot tissue only after 200 hours' washing (118) and that potato discs had to be washed for 15-24 hours before ascorbic acid exerted an inhibitory action on anaerobic carbon dioxide production (122). Barrow et al. (6) observe that long periods of washing probably damage the cell membrane and cause the loss of water-soluble co-enzymes. It would seem to be abundantly clear, therefore, that this habit of long-washing is most undesirable where normal endogenous respiration is the direct object of study.

The technique followed in this laboratory involves washing cut sections for 30 minutes under a running tap before the estimation of their respiratory rate. The purpose of this short washing period is solely to remove the contents of damaged cells and any wound substances produced by the cutting process.

The type of suspending fluid for tissue slices in Warburg vessels appears also to be a controversial point. In cases where exogenous respiration is being measured tissue slices are suspended in sugar solution (9,59,60, 77) or nutrient solution (27). Where endogenous respiration is the object of study, most workers have used buffer solutions to supply a medium of constant pH. Several, however, have used distilled water (8,54,99, 112,119), and recently Hanly et al. (54), working with carrot tissue, and Audus et al. (4) with pea, have stated that they used distilled water in preference to buffer, which they consider affects respiration. The question would appear to be whether it is better to have a medium with a constant pH, but which might enter into the respiratory mechanism, or a medium in which the pH is not regulated but which will not react within metabolic systems, and so alter the respiration rate.

In the experiments described below, (where endogenous respiration is studied) tissue was suspended in distilled water for two reasons:

(a) because it is believed that, the cell being naturally buffered, the pH of the external solution will not affect internal cellular activity; and

(b) because the writer agrees with Hanly et al (54) that the constituents of buffer solutions react with cell contents, or become incorporated into metabolites,

in such a way as to radically affect the rate of respiration.

Materials and Methods.

The full technique employed in all the experiments described below, which involve the estimation of oxygen uptake only, was as follows:-

A total liquid volume of 2.5 ml. was added to each Warburg vessel. In control vessels, this was distributed in the following manner:

Main compartment	:	2.2 ml. distilled water.
Centre well	:	0.3 ml. 20% KOH on filter paper rolls.

and in experimental vessels:

Main compartment	:	1.87-1.70 ml. distilled water.
Centre well	:	0.3 ml. 20% KOH as above.
Side-arm	:	0.33-0.5 ml. test solution.

Lily slices were obtained from fresh bulb scales, which were cut transversely into sections 0.5 mm. thick with a hand-microtome. Carrot tissue slices were obtained from plugs, 6 mm. in diameter, extracted from the secondary phloem of freshly-dug carrots. These plugs were cut transversely with a hand-microtome into slices of 0.5 mm. thickness.

In both cases the tissue slices were transferred immediately to tap-water in a beaker, which was then covered with muslin and placed under a running tap for 30 minutes. At the end of this washing period the slices were blotted on filter paper and inserted into

The manometers were placed in a water bath maintained at 30°C. ± 0.05°C., and shaken at 108-112 oscillations per minute. After an equilibration period of 30 minutes the stopcocks were closed, and normal oxygen uptake estimated in all vessels for 90-120 minutes prior to tipping. Manometers were read at 30 minute intervals, and experiments were continued for 5-6 hours after tipping. In all cases the vessels were left overnight in the bath with the stopcocks open, and further estimation of oxygen uptake carried out for 5-6 hours on the second day. Similar observations were made on the third day also. Contamination, mostly bacterial, occurred in very few cases. Evidence of infection lay in cloudiness of the suspending fluid, and in disintegration of the cells of the tissue slices. Results from infected vessels were discarded.

Presentation of results.

As is often the case in experiments involving biological material, a considerable amount of variability in rate of tissue respiration on successive days was encountered. The effects of this were minimised by computing the data on a relative basis. The oxygen uptake of treated tissue is expressed as a percentage of that of comparable control tissue.

RESULTS.

Two experiments on lily and carrot tissue slices

were carried out to determine the normal course of oxygen uptake. Graph III illustrates these results. Both rates of respiration increase to a maximum, which is reached in carrot after about 20 hours, and in lily after about 30 hours. Thereafter the rates fall, gradually in carrot slices, and more abruptly in lily.

Since both tissues are derived from storage organs, they have a relatively abundant food supply available, and continue respiring for long periods in distilled water. The food materials get depleted in time, accounting for the fall in respiration towards the end of the experimental period.

CM. Coconut milk was added to vessels to give a final concentration of 15% ($^{v}/_{v}$) after tipping. There was an immediate response in both lily and carrot tissue, shown in increased oxygen uptake, an increase which reached a maximum on the second day and was still very pronounced on the third (Table 5, Graph IV).

Table 6 illustrates the statistical method for obtaining these, and all following values. (An appendix incorporating all analyses of variance may be found at the end of the text.)

Graph III.

Normal endogenous respiration of carrot tap root and lily bulb scale tissue slices, with standard deviation.

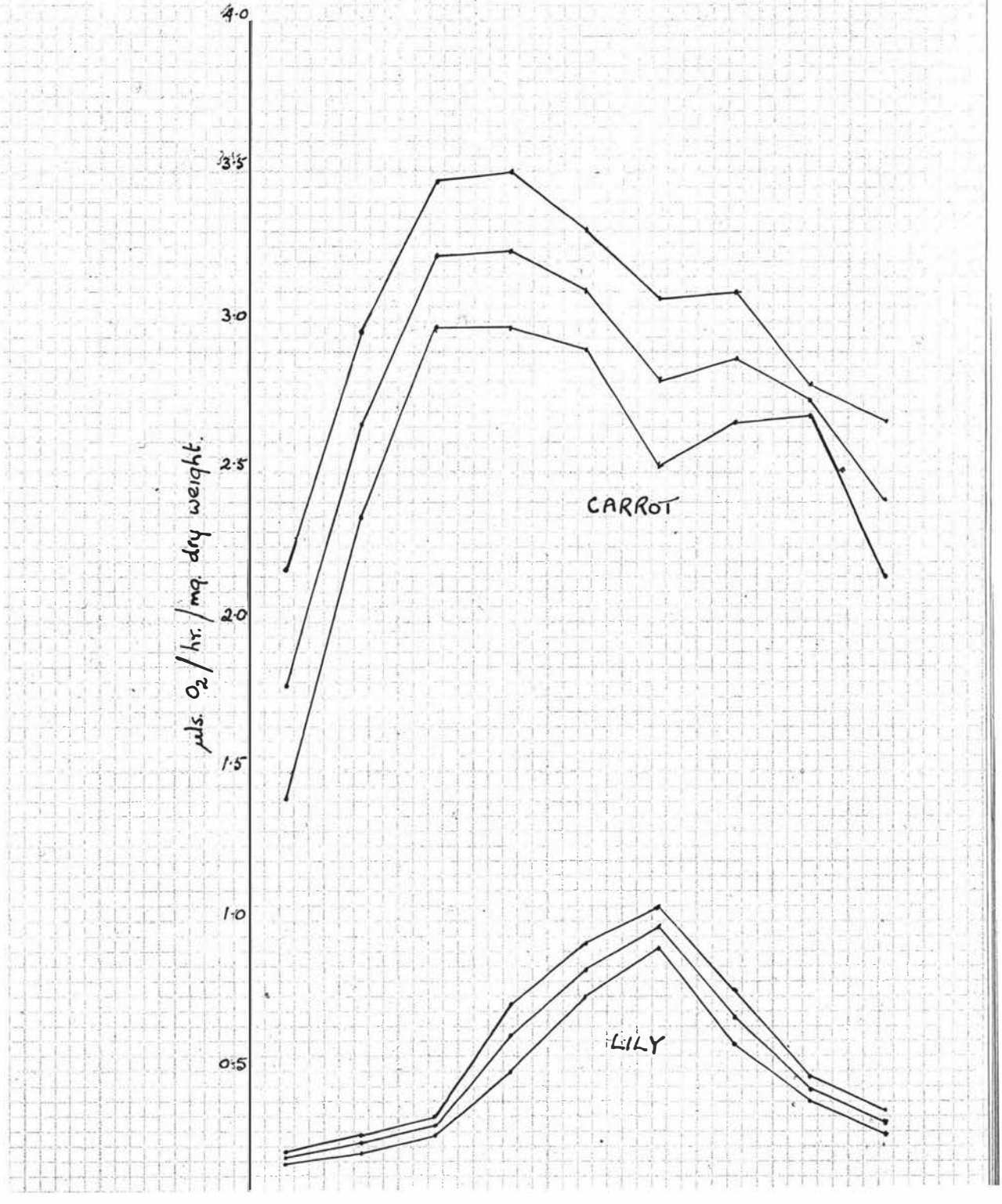

4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·5
µls. O2 / hr. / mg. dry weight.
CARROT
LILY

TABLE 5

RESPIRATION RATE IN uls O_2/mg. DRY WEIGHT /HR.
DIFFERENCES BETWEEN CONTROL AND CM VALUES.

Time	uls. O_2.	% increase	P.
Carrot			
1st day	1.22±0.30	96±24	0.005
2nd day	12.90±2.27	725±128	0.001
3rd day	9.29±0.22	683±16	0.001
Lily			
1st day	0.27±0.19	59±41	0.02
2nd day	3.21±1.07	406±135	0.005
3rd day	2.34±0.37	236±37	0.001

TABLE 6

STATISTICAL CALCULATION OF EFFECT OF CM ON
OXYGEN ABSORPTION IN CARROT TISSUE.

Control uls. O_2/mg. dry wt./hr. 1st day	2nd day	3rd day	CM uls. O_2/mg. dry wt./hr. 1st day	2nd day	3rd day
0.92±0.04	2.04±0.09	1.29±0.04	2.64±0.66	18.97±5.50	10.34±0.52
1.84±0.20	1.83±0.11	1.42±0.10	2.51±0.42	13.81±1.68	10.96±1.90
1.06±0.08	1.46±0.17		2.32±0.44	11.27±0.12	

Analysis of variance for 1st day:

Source	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	2.2204	1	2.2204	16.35	0.005
Error	0.5433	4	0.1358		
Total	2.7637	5			

Control mean = 1.27 Treatment mean = 2.49

Treatment difference = $1.22 \pm \sqrt{\frac{2(EMS)}{3}} = 1.22 \pm 0.30$

% increase = $\frac{1.22 \times 100}{1.27} = 96$

with standard error = $\frac{0.30 \times 100}{1.27} = 24$

Although the general type of response is the same in both cases, the oxygen uptake of carrot tissue is increased to a value relatively greater than that of lily tissue. Over the first six hours of treatment, the increase in oxygen absorption in carrot tissue reaches significance at the 5% level, and thereafter is significant to 1%. In lily tissue, P values over the three-day period are 0.002, 0.005, 0.001. It would appear from these figures that CM has a more profound effect on carrot tissue respiration than on that of lily. Furthermore, the high rate observed in carrot tissue on the second day of treatment is maintained on the third, while that of lily slices falls on the third day to nearly half of the rate previously exhibited on the second.

From Table 5 it is seen that the response in both tissues is variable within rather wide limits. Although this may in part be due to physiological difference between the tissues used in the various experiments, there is also the possibility that variation in the active principle of coconut milk itself may be responsible. The milk used was autoclaved to keep it sterile, and was stored in a glass flask. From time to time the storage vessel was opened to obtain aliquots of the contents for experiments, and in each case the remaining fluid was re-sterilised. It is thought that the repeated autoclaving may have had some effect on the constitution of

the milk which would account for the variability of the magnitude of its effect on the oxygen uptake of lily and carrot tissue.

CM + 2,4D:

The final concentration of CM was 15%, and of 2,4D 6 mg./l. The increases in oxygen uptake over three-day periods caused by the addition of these substances are presented in Table 7 and Graph IV.

TABLE 7.

CM + 2,4D. Resp. rate in uls. O_2/mg. dry wt./hr.
DIFFERENCES BETWEEN CONTROLS AND CM+24D VALUES.

Time	Uls O_2 Diff.	% increase over controls	P.
Carrot			
1st day	1.70±0.61	125±45	0.005
2nd day	24.18±3.61	1336±199	0.0001
3rd day	16.65±5.75	1224±423	0.005
Lily			
1st day	0.28±0.13	82±38	0.01
2nd day	1.98±0.50	319±81	0.001
3rd day	1.34±0.16	137±16	0.005

The individual response of the two tissues is in this case somewhat different after the first day. During the first six hours of treatment, the rate of oxygen uptake in both tissues is increased, but not significantly so over the CM response. Carrot slices increase oxygen absorption on the second and third days to levels which are almost double those observed when CM alone is added. These changes are highly significant

within treatments, especially on the second day (P=0.0001). The high rates reached on the second day are maintained on the third day also.

Lily tissue, subsequent to the first day of treatment, exhibits no enhanced rate of absorption over the CM rate. There is even a decrease in the level on the third day, when the absorption of oxygen falls to half of that consumed on the previous day.

CM + 1AA:

In Table 8 and Graph IV are given the respiration rates of carrot and lily tissue slices when 15% CM and 0.2 mg./l. 1AA are added to the substrate.

TABLE 8.

CM + 1AA Resp^{n} rate in uls. O_2/mg. dry wt./hr.
DIFFERENCES BETWEEN CONTROL AND CM+1AA VALUES.

Carrot

Time	uls O_2 Diff.	% increase over control	P.
1st day	1.56±0.74	153±73	0.02
2nd day	43.13±20.28	2679±1260	0.02
3rd day	16.01±4.61	738±212	0.01
Lily			
1st day	0.37±0.30	60±48	0.02
2nd day	5.04±2.13	504±213	0.02
3rd day	5.08±1.84	395±140	0.02

Increases in the consumption of oxygen in the presence of CM + 1AA were observed. In carrot tissue respiration was increased over that of the CM response on the second day (P = 0.02) but fell sharply on the

third to a level comparable with that of CM at the same time.

Lily respiration, after maintaining increased rates of oxygen uptake similar to those of CM during the first two days, decreased slightly on the third day.

CM + ATP:

15% CM and 40 mg./l. ATP increased the amount of oxygen absorbed by lily and carrot tissue slices (Table 9, Graph IV).

TABLE 9

CM + ATP Resp^{n} rate in uls. $^{O}2$/mg. dry wt./hr.
DIFFERENCES BETWEEN CONTROL AND CM + ATP VALUES.

Time	uls $^{O}2$ Diff.	% increase over control	P.
	Carrot		
1st day	1.09±0.42	68±26	0.02
2nd day	12.42±1.21	618±60	0.001
3rd day	5.99±1.56	281±73	0.01
	Lily		
1st day	0.12±0.08	23±15	--
2nd day	5.66±2.66	380±179	0.02
3rd day	3.93±1.70	266±115	0.02

While increasing oxygen uptake to an appreciable extent in both tissues (most significantly in carrot), the rates of absorption compared with those of the CM treatments were on a lower level. Carrot tissue respiration on the third day especially was markedly reduced. That of lily was depressed on the first day, subsequent rates being comparable with those due to CM addition.

CM + $AdSo_4$:

Ad So_4 at 20 mg./l. and CM at 15% concentration increased oxygen uptake in both tissues by the percentages given in Table 10 and Graph IV.

TABLE 10.

CM $AdSo_4$ Resp^n rate in uls. O_2/mg. dry wt./hr.

DIFFERENCES BETWEEN CONTROL AND TREATMENT VALUES.

Carrot

Time	Difference	% increase over control	P.
1st day	1.29±0.42	80±26	0.01
2nd day	14.02±2.30	698±114	0.005
3rd day	6.67±0.60	313±28	0.001
Lily			
1st day	0.34±0.14	64±26	0.02
2nd day	6.39±1.16	429±78	0.005
3rd day	1.23±0.31	83±21	0.01

Over the first two days, these increased rates of absorption were comparable with those obtained from the CM treatment alone. On the third day, oxygen uptake had fallen to approximately half that of tissue treated with CM.

Graph IV.

% increases in oxygen uptake, calculated on a dry weight basis, in lily bulb scale and carrot tap root tissue over three-day periods. Treatments as described in text. In each case the standard error is indicated by a vertical line.

Lily histograms unshaded.
Carrot histograms shaded.

% INCREASE IN O_2 UPTAKE OVER CONTROL VALUES.
3600
3200
2800
2400
2000
600
1200
800
400

Part IV

THE MORPHOLOGY OF REGENERATION OF BULBILS ON THE SCALE LEAF OF *Lilium speciosum Thun*.

Materials and Methods.

The morphology of regeneration of bulbils was investigated on scale leaves detached from bulbs. They were removed by cutting through the junction of scale and basal disc with a sharp scalpel, and were then placed in Petri dishes on filter paper moistened with distilled water. The morphology of one sample was studied immediately, and weekly samples of the remainder fixed and similarly investigated.

Basal portions of the scales were fixed in formalin-acetic alcohol, and dehydrated by the ethanol-tertiary butanol method of Johansen (57). Transverse and longitudinal sections were made, 14 u. thick, and stained with safranin and crystal violet according to the tannic acid-ferric chloride method of Northern (89).

RESULTS:

External Observations.

After one week the cut surface of the scale leaf turned brown, but there was no external sign of cellular proliferation. After two weeks, a ridge developed adaxially behind the cut surface, which at the end of the third week had developed protuberances at intervals along its length. By the end of the fourth week, these protuberances had developed into obvious buds. Roots

appeared, basal to the buds, during the fifth week, and young leaflets had elongated from the bud by the end of the fifth week.

Internal observations.

The scale leaf, at the time of excision from the bulb, is bounded by an upper and lower epidermis which are exactly similar in nature. They possess an anthocyanin pigment dissolved in the cell-sap of the vacuole. There is no palisade layer, the tissue between the upper and lower epidermis consisting of thin-walled parenchymatous cells containing numerous starch grains and oil globules. Leaf traces, varying in number from 4-5 in inner scales to 10-12 in outer scales, are embedded in this parenchymatous mesophyll. The xylem is composed of elongated vessels with spiral or reticulate thickening, and the phloem of thin-walled iso-diametric cells.

Sections from one-week-old regenerating scales indicate that the damaged cells of the cut surface have collapsed. The damaged area is cut off from intact cells by the formation of suberin along the walls of proximal parenchymatous cells, while meristematic activity has commenced in adjacent internal cells (Fig. 1). Cell-divisions take place parallel to the cut surface, forming a periderm, and also parallel to the adaxial epidermis at its basal end in sub-epidermal layers up to four cells internal to the epidermis. Starch disappears from those areas exhibiting meristematic

activity, presumably because it is used up in growth and development. The activity of the meristems gives rise to the ridge observed externally on the adaxial surface at the end of the second week. Subsequently, cell-divisions in any plane occur at localised points adaxially, the daughter cells at first increasing little in size before they again divide, so that the original cell-boundary remains plainly visible for some time, enclosing a small group of daughter cells (Figs.2,3). The enlargement of these cells gives rise to the protuberances observed on the adaxial ridge (Fig.4). From these the primordia of the first leaves develop, increasing more rapidly in size and number on the outer side, so that the young leaves curve over the stem tip, which is still elongating.(Fig.5). At about the same time as the leaf primordia are developing, roots form from deeper layers of tissue (Fig.6). They arise independently from groups of meristematic cells below the bud primordium, and usually near a vascular bundle (Fig.7). These meristematic areas become organised into a root-tip which pushes through the parenchymatous cells and emerges at a point basal to the bud (Figs.8,9). Tissue between these independent bud and root initials becomes meristematic and forms vascular elements which link the primordia (Fig.8). Although the fully-developed bulbil is entirely independent of the parent scale, it usually remains attached to it for some time, using up the food reserves stored in mesophyll tissue.

Walker(120) observed a similar pattern in the regeneration of bulbils on the scale leaves of two other species of Lilium

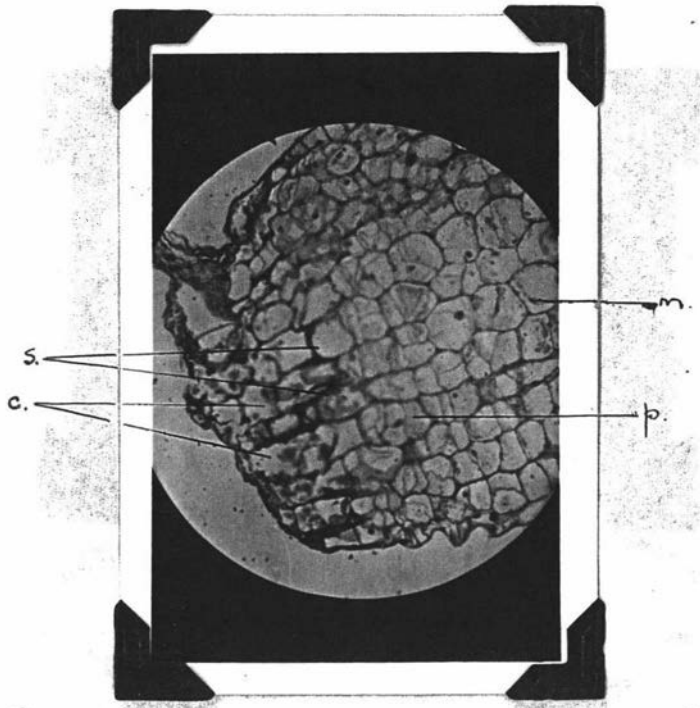


Fig. 1. L.S. of a scale leaf one week after excision showing periderm formation. Note the prominent nuclei and absence of starch grains in meristematic tissue. x66.
c:collapsed cells, s:suberised cells, p:periderm, m:mesophyll tissue.

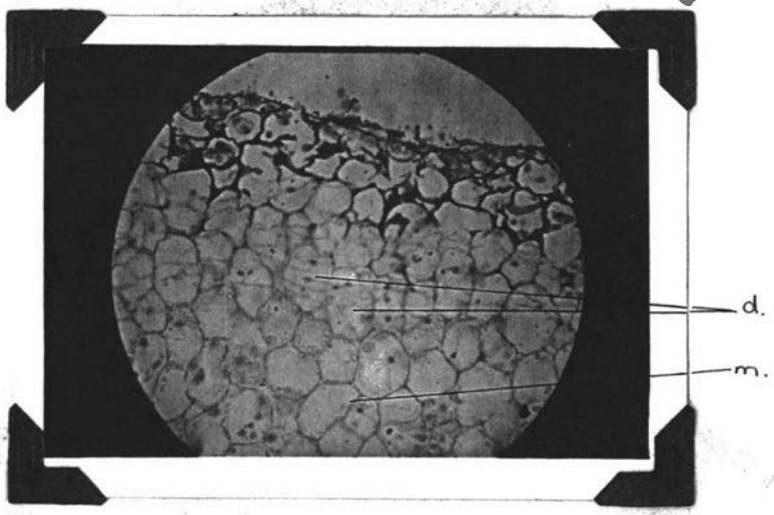


Fig. 2. L.S. at a later stage, showing early bud meristem development. x66
d:groups of daughter cells within the parent cell, m:mesophyll tissue.

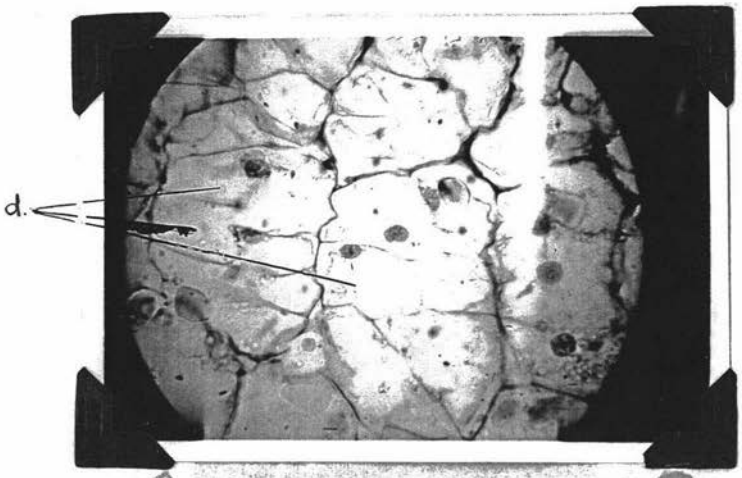


Fig. 3. A more magnified portion of Fig. 2 illustrating the mode of cell-division. x220
d:daughter cells.

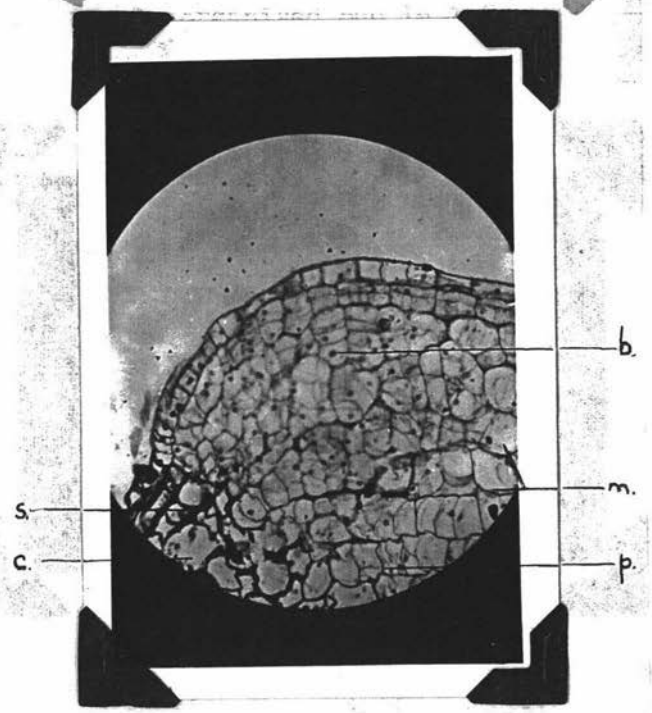


Fig. 4. A scale leaf in L.S. at a more advanced stage of bud development. x66
c:collapsed cells, s:suberised cells, p:periderm, m:mesophyll tissue, b:bud meristem.

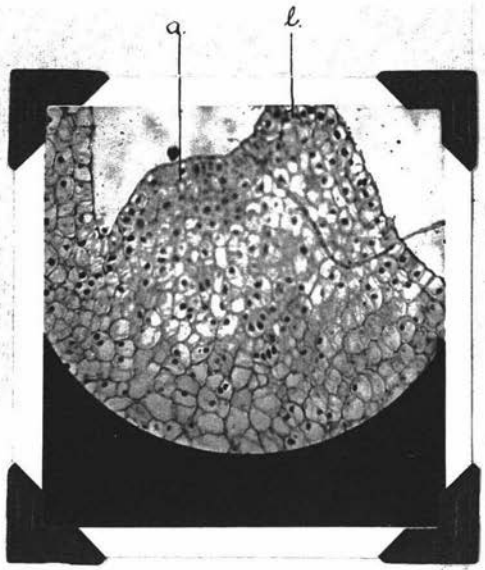


Fig. 5. An organised bud in T.S. x66
a:stem apex, l:leaf primordium.

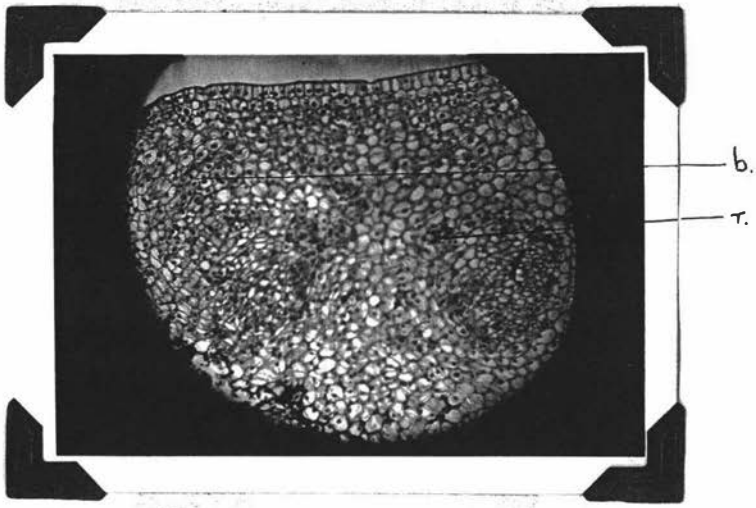


Fig. 6. T.S. of a regenerating scale leaf showing the redative positions of development of bud and root meristems. x40
b:bud meristem, r:root meristem.

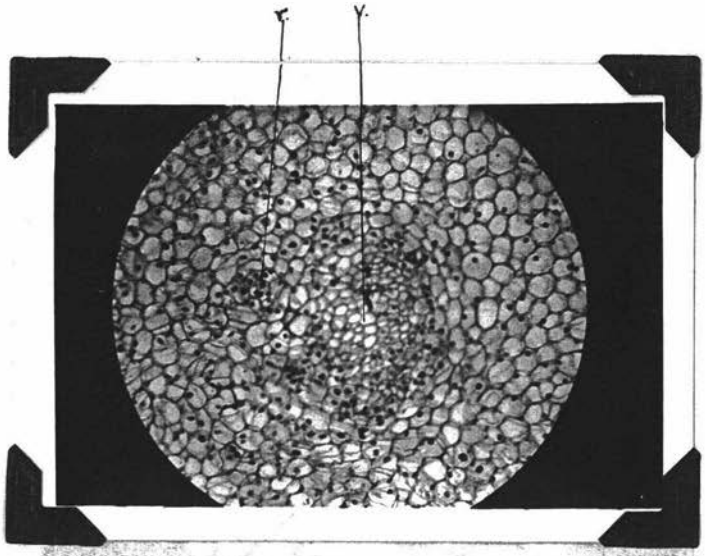


Fig. 7. The root meristem illustrated in Fig. 6 under higher magnification. r:root meristem, v:a vascular bundle of the scale leaf. x66

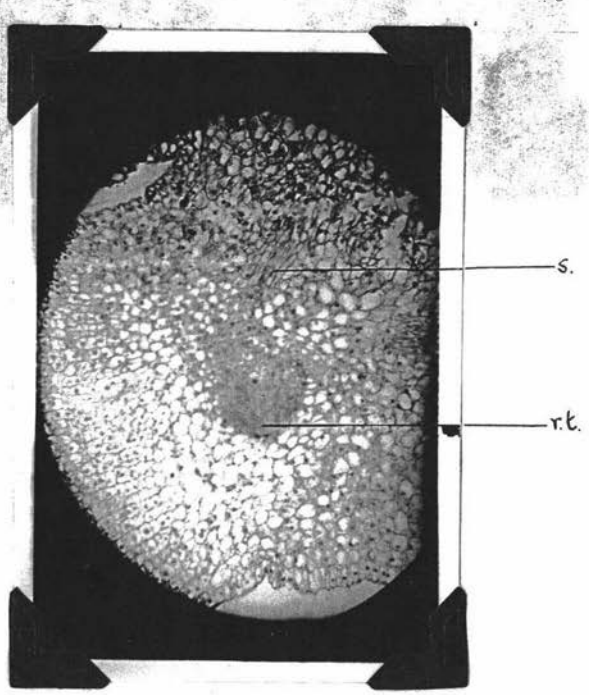


Fig. 8. T.S. of a regenerating scale leaf showing an organised root tip. x42
r.t.: root tip, s: differentiating vascular strands between root tip and bud.

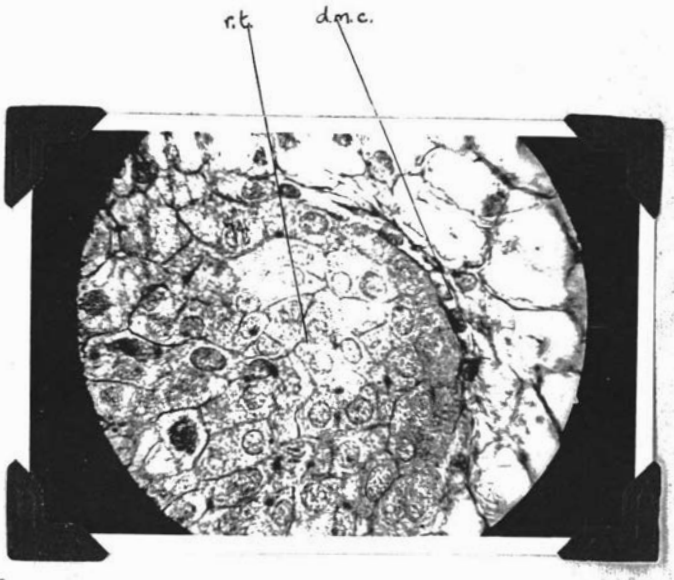


Fig. 9. The root tip illustrated in Fig. 8 more highly magnified.x210
r.t.:root tip, d.m.c.:disintegrating mesophyll cells.

DISCUSSION

I. Normal Response of Explants to Culture.

In many instances where tissue from dicotyledonous plants is cultured, cell-proliferation occurs readily, sometimes spontaneously, as in artichoke (65, endive (51), asparagus (41), and carrot (51,52,68,85), but quite often only in the presence of indolylacetic acid (46,47, 51,65,67,68). Explants from bulb scales of the monocotyledon Lilium speciosum Thun. have been shown here to proliferate and differentiate spontaneously in vitro. Due to the fact that the amount of callus formed on cultures was very small and would not grow when isolated, and also because differentiation within the callus appeared at a very early stage, observations have of necessity been of a qualitative rather than a quantitative nature. Lily bulb scales have no cambial region, and cell-proliferation occurs from parenchymatous mesophyll cells. The capacity of these cells to assume a meristematic state is seasonal, in that tissue which has been excised only during spring and autumn months will regenerate. A similar seasonal response has been observed in artichoke tissue (65), which spontaneously proliferated in vitro only during periods when the plant was in a vegetative condition. In the present work, lily tissue has been observed to grow and regenerate during vegetative phases of growth of the lily plant, i.e. when root and flower-bud differentiation was absent. It is of interest that in all cases regeneration

occurred solely from basal adaxial areas, regardless of the orientation of the tissue explant with regard to the surface of the culture medium. Such polarity of differentiation has been noted in several tissues - Carrot (48), endive(49), vine (80), and others (49) - and has been attributed (48,49) to the leaf-root polarisation of circulation of indolylacetic acid, in the plant.

II. Response of Explants to Growth-regulating Substances.

It has been previously noted (65) that the physiological state of the tissue at the time of excision determines its reaction to culture conditions and to indolylacetic acid. In the culture of carrot tissue there appears to be some confusion as to the necessity of indolylacetic acid for growth (51,85; 46,47,67,88), and it may well be that contradictory reports are the result of seasonal physiological variation. The addition of indolylacetic acid at 0.2 mg./l. to lily culture media had no effect on the growth per se of lily explants. Its presence did however influence subsequent differentiation in promoting root-development. This effect has been observed also in asparagus (41), carrot (22,47,68), and other tissues (26), and has been demonstrated by Skoog and his co-workers (78, 104) to interact in tobacco tissue in a complex which involves at least sugar and adenine.

The addition of adenosine triphosphate to lily explants did not in any way alter the regeneration of the tissue; it had only a slight delaying action on the process. When adenosine triphosphate was added along with indolylacetic acid, however, development was hastened over that of control tissue. It would appear from this that a system influencing differentiation similar to that present in tobacco tissue is active also in Lilium speciosum explants. Recently it has been suggest that such a system acts through nucleic acid metabolism (78).

Traumatic acid has been reported to have an inhibitory effect on the germination of tomato seeds (39), and in this work it has been observed to exhibit certain inhibitory qualities regarding the differentiation of lily tissue. The inhibition is not apparently exerted by traumatic acid itself (since explants grown in its presence completely differentiate) but by its effect on a growth-regulating mechanism involving adenosine triphosphate and indolylacetic acid. Addition of traumatic acid to media containing ATP and/or IAA prevents complete differentiation. That it does not inhibit cell-division is apparent from the fact that in all cases callus tissue formed on explants, but differentiation did not follow in the majority of cases.

Coconut milk has been shown to contain a growth

factor which promotes cell-division in a wide variety of excised tissue, including that of carrot (23), Parthenocissus sp. (32), crown-gall (33), artichoke (34), virus tumour (83), and potato parenchyma (109). No stimulation was observed when this substance was added to lily tissue in culture. Steward and Caplin (110) have found that inhibitors of the coconut milk factor are present in certain mature tissues, such as the storage tissue of potato and onion, and there are other indications that the coconut milk factor (11,24,70,76, 93) and its inhibitors (76) may be of fairly general occurrence in plants. Certain growth substances appear to counteract the effect of the inhibitors. 2,4D and CM act synergistically in the proliferation of potato parenchyma (109), and IAA partially overcomes the low activity which is found in some coconut meat extracts (76).

Morel and Wetmore (81) cultured tissue from a monocotyledonous tuber which grew vigorously on a medium which incorporated naphthaleneacetic acid and CM. In the present work, experiments in which 0.2 mg./l. IAA were added to culture media along with CM did not result in any increase in cell-proliferation. There are two possible explanations for this failure to stimulate growth: (a) the coconut milk factor is not active on lily tissue; and (b) the factor is prevented from

exerting its effect by the presence of inhibitors in the tissue. Should the latter alternative be correct, then a concentration of IAA of 0.2 mg./l. is not sufficiently strong to counteract the inhibitory action.

III. Sugar and Nitrogen Content of the Lily Bulb related to Cultural Response of Explants.

Explants have been already shown to exhibit a seasonal response in their behaviour in culture. Graph I clearly indicates that regenerative capacity is limited to spring and autumn months, more especially to the former. Explants obtained from bulbs during winter and summer for the most part fail to proliferate in culture.

Graph II, illustrating sugar and nitrogen levels over the two-year period which also covers the tissue culture experiments, correlates in some respects with the cultural response of explants. Both reducing and non-reducing sugar content show one peak yearly. Low levels are recorded for non-reducing sugar content over October-November, rising gradually during December-April, and abruptly thereafter to reach a high level over August. There is then an equally abrupt decline to the low October-November level.

Reducing sugar peaks show a similar, but slightly delayed, curve to those of non-reducing sugar, although on a much lower level.

Insoluble nitrogen content of the bulb has two peaks

yearly, those peaks corresponding with the periods when explants regenerate, namely March-May and September-November. Between these periods, insoluble nitrogen content is low.

It would appear, therefore, that the ability of explants to grow in culture is directly correlated with insoluble nitrogen content of the bulb at the time of excision of tissue. It is of note, however, that the high recorded levels of insoluble nitrogen correspond, within limits, with low levels of non-reducing sugar, and it is possible that not only high insoluble nitrogen concentration, but also a relationship between high insoluble nitrogen and low non-reducing sugar concentration plays some part in influencing the behaviour of explants in culture.

IV. Comparative respiratory Response of Lily and Carrot Tissue Slices.

Stiles and Dent (112) have observed that the respiration of beetroot, carrot and potato discs, after short preliminary washing periods, starts at a low level, gradually increasing to reach a maximum after about twenty hours. They give reasons for concluding that tissues inside a bulky storage organ are under a very low oxygen and high carbon dioxide tension. Both condition a low respiration rate. When exposed, tissue discs are then in a high oxygen-low carbon dioxide environment, and it is to be expected that

there is a rise in respiration rate. That this rise is gradual is probably due to the fact that metabolic changes are accomplished gradually. Subsequent fall in the rate of respiration is most likely due to depletion of substrate.

Graph III illustrates the time-increase obtained here in the oxygen uptake of carrot and lily tissue slices. Both are derived from storage organs, and it is likely that the reasons given by Stiles and Dent account for this curve. Subsidiary causes might be temperature change, mechanical handling, and leaching out of inhibitors (129, 130).

That coconut milk has a marked effect on the endogenous oxygen uptake of both lily and carrot tissue is obvious from the figures presented in Table 5. It has been suggested to the writer that these increases in oxygen uptake may be due to sugars present in the milk. Preliminary experiments on lily tissue, however, indicate that over the first six hours of application there is no effect on oxygen uptake by 2% and 5% sucrose solutions. It would appear from this that the enhanced rate of oxygen absorption observed over a similar period with coconut milk is not caused by sugars. Respiration rates on the second and third days in lily tissue supplied with sucrose show increases up to, but not exceeding, 40%. It is possible that this proport-

of increase in uptake caused by coconut milk on the second and third days of experiment is due to sugars, but this does not by any means account for the major part of the oxygen absorbed under these conditions Table 5). It would seem that another factor, or factors present in coconut milk produce the accelerated rates of oxygen uptake in the tissues under investigation.

Criticism may be levelled at the length of the individual respiratory experiments described here, due to the possibility of bacterial infection occurring in the tissue slices during this long period. As has been mentioned elsewhere, detection of bacterial infection lay in cloudiness of the suspending fluid, and in loss of firmness of the tissue discs. In every case where either or both of these criteria were observed, results were discarded. Nevertheless, the objection may still be raised that bacterial infection could have been intra-cellular and not detectable by the criteria mentioned above. In view of this, the results of the first six hours only of any experiment are absolutely valid. The later results are presented and discussed here also, bearing in mind the reservation that they may have been infected.

In the experiments in which growth-regulating substances were supplied along with coconut milk, there

is one notable difference in the effects on carrot and lily tissue, that of CM + 2, 4D. Initially, in both tissues, there is an increase in oxygen uptake of 20-30% over that obtained with coconut milk alone. This stimulation is that which might be expected (60) with 2,4D alone at the concentration used, and could possibly be additive to that caused by coconut milk. On the second and third days after application carrot tissue absorbs oxygen to an even greater degree (500-600%), while lily tissue respiration falls by about 10%. It is known that the effect of 2,4D on tissue metabolism is to accelerate starch hydrolysis and the utilisation of sugars. It may be, therefore, that carrot tissue slices have a greater carbohydrate reserve than those of lily, which get depleted early and result in a decreased level of oxygen uptake through starvation. The more abundant reserves of the carrot would enable respiratory metabolism to continue in this tissue at a higher level through the increased availability of respiratory substrate.

In general, both lily and carrot tissue react in a similar manner to the remaining experimental solutions. CM + 1AA increases oxygen uptake over the CM effect, while both CM + ATP and CM + $AdSo_4$ decrease oxygen absorption. The fact that 1AA increases oxygen uptake is not surprising, but the inhibitory effect of

both CM + ATP and CM + $AdSo_4$ relative to the CM response is rather unexpected. CM + $AdSo_4$ is strongly inhibitory on the third day of experiment, while CM + ATP is inhibitory in lily tissue over the entire experimental period. The more strongly inhibitive properties of the combination CM + ATP may be attributed to the presence of ribose. The ATP molecule is composed of adenine, ribose and three phosphate radicles, and it is likely that the ribose element would be readily split from the molecule. Ribose at relatively low concentration is strongly inhibitory to growth, and it is within the bounds of possibility, therefore, that the presence of ribose causes ATP to exert the depressing effect on oxygen uptake. It is not likely that adenine is the inhibitory factor, since $AdSo_4$ has no such marked effect.

It may be concluded from all these respiratory results, then, that the monocotyledon and dicotyledon tissues react in a similar manner to the test materials supplied, although to a different degree, and have similar respiratory mechanisms.

SUMMARY

1. Explants from the bulb scales of Lilium speciosum Thun. have been cultured in vitro, where they proliferated and differentiated to regenerate new plants in 15-16 weeks.

2. The regenerative capacity of such explants has been shown to be seasonal and of a localised nature. Apparently it is correlated also with the vegetative state of the plant and with insoluble nitrogen content of the bulb scale tissue at the time of excision.

3. Indolylacetic acid at 0.2 mg./l. concentration promoted root differentiation and reduced bud differentiation in cultured explants.

4. Adenosine triphosphate at 40 mg./l. concentration did not alter the normal pattern of regeneration, but delayed its completion from 15-16 weeks to 21 weeks.

5. Adenosine triphosphate and indolylacetic acid added simultaneously to culture media resulted in the complete regeneration of new plants in 12 weeks, thus hastening the process over that occurring in control tissue.

6. Traumatic acid at 20 mg./l. concentration did not influence regeneration in explants.

7. Traumatic acid in the presence of indolylacetic

acid and/or adenosine triphosphate did not prevent cell-proliferation, but blocked differentiation.

8. Coconut milk was ineffective in stimulating cell-proliferation.

9. Oxygen uptake in carrot and lily tissue slices increases with time to a maximum, reached in carrot after 20 hours and in lily after 30 hours, thereafter falling off. The gradual rise is possibly caused either by changing oxygen and carbon dioxide tensions or leaching out of inhibitors, and the decline to gradual depletion of substrate.

10. The effect on oxygen uptake in lily and carrot tissue of coconut milk, and of coconut milk combined with certain growth-regulating substances, is essentially similar, from which it is concluded that both the monocotyledon and dicotyledon tissues have similar respiratory mechanisms.

11. Morphologically, regeneration of new plants from bulb scale tissue takes place from mesophyll tissue, buds arising from more superficial layers, and roots from deeper layers, usually near a vascular bundle.

APPENDIX

ANALYSES OF VARIANCE:

CM Treatment : CM 15% (v/v)

CARROT

1st day:

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	2.2204	1	2.2204	16.35	0.005
Error	0.5433	4	0.1358		
Total	2.7637	5			

2nd day:

Treatment	249.8731	1	249.8731	32.28	0.001
Error	30.9615	4	7.7404		
Total	280.8346	5			

3rd day:

Treatment	86.3971	1	86.3971	860.39	0.001
Error	0.2006	2	0.1003		
Total	86.5977	3			

CM Treatment : CM 15% (v/v)

LILY

1st day:

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	0.2160	1	0.2160	2.05	0.02
Error	1.0519	10	0.1052		
Total	1.2679	11			

2nd day:

Treatment	25.7924	1	25.7924	9.01	0.005
Error	22.9060	8	2.8633		
Total	48.6984	9			

3rd day:

Treatment	8.2134	1	8.2134	40.92	0.001
Error	0.8029	4	0.2007		
Total	9.0163	5			

CM+24D Treatment : CM 15% (v/v); 2,4D 6mg./l.

CARROT

1st day:

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	5.7630	1	5.7630	7.78	0.005
Error	4.4433	6	0.7406		
Total	10.2063	7			

2nd day:

Treatment	1169.8284	1	1169.8284	44.80	0.0001
Error	156.6699	6	26.1117		
Total	1326.4983	7			

3rd day:

Treatment	277.2225	1	277.2225	83.98	0.005
Error	66.0185	2	33.0093		
Total	343.2410	3			

CM+24D Treatment : CM 15% (v/v); 2,4D 6mg./l.

LILY

1st day:

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	0.1568	1	0.1568	4.87	0.01
Error	0.1932	6	0.0322		
Total	0.3500	7			

2nd day:

Treatment	7.8012	1	7.8012	15.49	0.001
Error	3.0207	6	0.5035		
Total	10.8219	7			

3rd day:

Treatment	1.7956	1	1.7956	72.70	0.005
Error	0.0493	2	0.0247		
Total	1.8449	3			

CM+1AA Treatment: CM 15% (v/v), 1AA 0.2 mg./l.

CARROT

1st day

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	2.4336	1	2.4336	4.48	0.02
Error	1.0874	2	0.5437		
Total	3.5210	3			

2nd day:

Treatment	1859.7657	1	1859.7657	4.52	0.02
Error	823.0272	2	911.5136		
Total	2682.7929	3			

3rd day:

Treatment	256.4803	1	256.4803	12.08	0.01
Error	42.4628	2	21.2314		
Total	298.9431	3			

CM+1AA Treatment : CM 15% (v/v), 1AA 0.2 mg./1.

DILY ?

1st day:

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	0.1407	1	0.1407	1.54	0.02
Error	0.1832	2	0.0916		
Total	0.3239	3			

2nd day:

Treatment	30.4521	1	30.4521	6.71	0.02
Error	9.0796	2	4.5398		
Total	39.5317	3			

3rd day:

Treatment	24.2557	1	24.2557	7.17	0.02
Error	6.7652	2	3.3826		
Total	31.0209	3			

CM+ATP Treatment: CM 15%, ATP 20mg./l.

CARROT

1st day

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	1.1881	1	1.1881	6.81	0.02
Error	0.3490	2	0.1745		
Total	1.5371	3			

2nd day

Treatment	154.2564	1	154.2564	104.97	0.001
Error	2.9389	2	1.4695		
Total	157.1953	3			

3rd day

Treatment	35.8203	1	35.8203	14.75	0.01
Error	4.8572	2	2.4286		
Total	40.6775	3			

CM+ATP Treatment : CM 15%, ATP 20mg./l.

LILY

1st day

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	0.0133	1	0.0133	2.22	--
Error	0.0120	2	0.0060		
Total	0.0243	3			

2nd day

Treatment	32.0923	1	32.0923	4.53	0.02
Error	14.1636	2	7.0818		
Total	46.2559	3			

3rd day

Treatment	15.4057	1	15.4057	5.34	0.02
Error	5.7752	2	2.8876		
Total	21.1809	3			

CM+$AdSo_4$ Treatment : CM 15% (v/v); $AdSo_4$ 20 mg./l.

CARROT

1st day

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	1.6513	1	1.6513	9.02	0.01
Error	0.3662	2	0.1831		
Total	2.0175	3			

2nd day

Treatment	196.5604	1	196.5604	37.17	0.005
Error	10.5757	2	5.2879		
Total	207.1361	3			

3rd day

Treatment	44.4223	1	44.4223	124.22	0.001
Error	0.7152	2	0.3576		
Total	45.1375	3			

CM+$AdSo_4$ Treatment : CM 15% (v/v); $AdSo_4$ 20 mg./l.

LILY

1st day

Source of Variance	Sums of Squares	Degrees of Freedom	Mean Square	F.	P.
Treatment	0.1123	1	0.1123	5.43	0.02
Error	0.0414	2	0.0207		
Total	0.1537	3			

2nd day

Treatment	40.8321	1	40.8321	30.12	0.005
Error	2.7109	2	1.3555		
Total	43.5430	3			

3rd day

Treatment	1.5129	1	1.5129	15.63	0.01
Error	0.1936	2	0.0968		
Total	1.7065	3			

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