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Title	Primordial development in Phaseolus
Author	Pereira, Maria de Fatima D. Aleixo
Qualification	PhD
Year	1978

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"PRIMORDIAL DEVELOPMENT IN *PHASEOLUS*"

by

Maria de Fatima D. Aleixo Pereira

Thesis presented for the Degree of

Doctor of Philosophy

University of Edinburgh

1978

The work described in this thesis is the original work of the author except where specific reference is made to other sources. It has not been submited, in whole or in part, for any degree at any other University.

Maria de Fatima D. Aleixo Pereira

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENT	i
ABSTRACT	ii
CHAPTER I INTRODUCTION	1
CHAPTER II MATERIAL AND METHODS	8
1. Material	8
1.1 Foliar organization in *Phaseolus vulgaris*	8
1.2 Choice of cultivar	8
1.3 Choice of seed size	10
2. Methods	13
2.1 Cultural details	13
2.2 Developmental analysis	13
2.2.1 Examination of the apices	13
2.2.2 Apical developmental stages	14
2.2.3 Histological preparations	16
2.2.4 Estimation of apical volume	16
2.2.5 Estimation of cell number	16
2.2.6 Estimation of cell volume	18
2.2.7 Comparative growth rate (COM)	18
2.3 Estimation of leaf area	19
2.4 Determination of leaf shape	19
2.4.1 Leaf shape index	19
2.4.2 Leaf types	20
2.5 Chemical treatments	20
2.5.1 Treatments applied to mature seeds	20

Page

2.5.2 Treatments applied to immature seeds 23

2.6 Replication 24

CHAPTER III PRELIMINARY RESULTS 25

1. Introduction 25

2. Growing conditions and plant development 26

3. Removal of one cotyledon and the primary leaves 29

4. Reproductive development 36

5. Discussion 48

CHAPTER IV EFFECTS OF INHIBITORS OF NUCLEIC ACID AND PROTEIN SYNTHESIS ON PRIMORDIAL INITIATION AND DEVELOPMENT 54

1. Introduction 54

2. Effects of 5-bromodeoxyuridine (BUDR) on primordial and further development 57

2.1 Effects on primordial development 57

2.2 Effects on further development 66

3. Effects of 2-thiouracil (2-TU) on leaf development 68

4. Effect of cycloheximide on leaf development 72

5. Discussion 75

CHAPTER V EFFECTS OF GIBBERELLIC ACID (GA_3) ON LEAF DEVELOPMENT 78

1. Introduction 78

2. Effects of treatment applied to mature seeds 81

Page

3. Effects of treatment applied to immature seeds 87

4. Discussion 90

CHAPTER VI EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D), OTHER AUXINS AND 2,3,5-TRIIODOBENZOIC ACID (TIBA) ON LEAF DEVELOPMENT 97

1. Introduction 97

2. Effects of 2,4-D on leaf development at different stages 101

2.1 Effects of 2,4-D on adult leaves 101

2.2 Effects of 2,4-D on primordial development 113

2.3 Effects of 2,4-D on cell division and expansion 126

2.3.1 Effects on the whole apex 126

2.3.2 Effects on different parts of the apex 131

3. Effects of other auxins on primordial development 143

3.1 Effects of indole-3-acetic acid (IAA) 143

3.2 Effects of naphtaleneacetic acid (NAA) 145

4. Effects of 2,3,5-triiodobenzoic acid (TIBA) on primordial development 148

5. Discussion 150

CHAPTER VII DISCUSSION 157

1. Uptake, penetration and movement of externally applied substances 157

Page

2. Primordial initiation and control of leaf form 159

3. The control of leaf shape 166

4. Conclusions and future work 168

REFERENCES 171

ACKNOWLEDGEMENT

The author would like to express her gratitude to her supervisor, Dr. J. E. Dale, not only for his wise guidance but also for his understanding during all phases of this work.

Thanks are also due to the following people:

- The technical staff of the Department of Botany, especially to Mr. A. Thompson for his help in this work and particularly for his general helpful attitude.

- Mr. J. Goodall from the Electrical Engineering Department for operating the SEM.

- Mrs. M. Donaldson for typing this thesis.

The author is also grateful to all those who helped directly or indirectly during the last three years making her stay in Edinburgh most pleasant and enjoyable.

Some institutions must also be aknowledged: Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) - Rio de Janeiro, Brazil for providing financial support and Universidade Estadual de Campinas - Campinas, Brazil for giving a leave of absence which made these studies possible.

ABSTRACT

Initiation and further development of primary and trifoliate leaves of P. vulgaris were studied. The major experimental approach involved the use of exogenously-applied substances. These were of two types: inhibitors of nucleic acid and protein synthesis and plant growth regulating substances. The effects of these substances on primordial initiation and leaf form (gross modifications affecting, for example number of leaflets) and shape (minor changes affecting, for example width, length, and lobing of leaves) were examined.

Inhibitors of nucleic acid and protein synthesis delayed primordial initiation and early development. This effect is considered to be a reflection of general growth inhibition rather than a specific effect on leaf development. In adult leaves BUDR and 2-TU caused modification of shape at the base of the primary leaves.

Application of 2,4-D to the apex of imbibed seedling axis altered the pattern of apical development, so that a ring structure resulting from the concrescence of the leaf and stipular primordia developed at the trifoliate leaf position. Treatment led to production of entire leaves at the first trifoliate leaf position. In some cases similar leaf shapes were produced at both primary and trifoliate leaf positions. 2,4-D inhibited apical growth mainly by inhibiting cell division. Leaf primordia grew relatively more in treated than in control apices; this is in contrast to growth of the apical dome which was almost totally inhibited by 2,4-D. Treatment with IAA or NAA did not modify early primordial development, althouth application of TIBA delayed this.

GA_3 treatment did not affect primordial initiation, but modified the shape of trifoliate leaves which were longer and narrower than the controls. This effect was dependent on the developmental stage of the apex at the time of treatment and was visible 96 hours after treatment. It is suggested that sensitivity of the apex and primordia to GA_3 treatment decreases with time.

CHAPTER I

INTRODUCTION

Despite the enormous amount of work that has been carried out on morphogenesis at the shoot apex some very important questions are still without answer. These include, for example: (a) What determines the site at which a new leaf primordium is to be initiated? (b) What are the changes that occur at the apex directly concerned with the initiation of new primordia? (c) To what extent are growth substances involved in these processes? Following primordial initiation a related question still requiring answer is what factors determine the final form of a leaf.

The classical observation that a localized increase in cell division rate is the first event for leaf initiation finds support in some recent work, as for example, the results of Hussey (1971) working with apices of tomato where a higher rate of cell division was observed and related to primordial initiation. These findings were confirmed for excised pea apices (Hussey, 1972). In *Xanthium* it has been shown that periclinal divisions in the second and third layers of the stem apex are the earliest indication of leaf inception (Maksymowych, 1973). However this view requires some modification, since there is evidence that the initiation of a leaf primordium at the stem apex is the result of a change in shape caused mainly by changes in the direction of growth there. Lyndon (1968) found that rates of growth and increase in cell number were

greater at the primordium than that at any other part of the apex. However he did not find marked differences in cell division rates at the site of primordial initiation throughout the plastochron (Lyndon, 1970a). The initiation of a leaf primordium was associated to a change in the polarity of growth in this same region of the apex (Lyndon, 1970b). Supporting evidence for the view that cell division may not be of crucial importance in primordial initiation is found from the work of Foard (1971) who showed that inhibition of cell division in shoot apical meristem of wheat by ionizing radiation did not prevent primordial initiation. Cell division was important for primordial growth but not for its inception.

There is good evidence that leaf form is determined very early in the course of primordial development. By the time the axis is a few hundred micrometres in length the form of the leaf may already be determined, as it was shown, for example, in Oryza sativa (Kaufman, 1959), Trifolium wormskioldii (Lersten, 1965). This involves the establishment of sub-axes in the case of compound or lobed leaves; and the proliferation of cells at the lateral margins of the axis to commence lamina formation (Dale, 1976; Esau, 1977).

The mechanisms for determination of leaf form have been interpreted by using two different approaches. The first of these relates leaf form to size of the subtending apex, this being influenced by a range of environmental and other factors (see below). The other interpretation invokes effects of growth substances in the control of leaf form. These explanations are of course not mutually exclusive.

It is well known that size and shape of the apex is no guide to the size or shape of leaves (Wardlaw, 1956). Nevertheless work of Allsopp (1953a; b; 1965; 1967) with the fern Marsilea has indicated that, in this species, leaf form may be related to apical size. He has concluded that a primordium develops into a more or less complex leaf form depending on nutritional conditions. High carbohydrate or nitrogen nutrition allows development of a larger apex and this is capable of initiating and developing leaves of a more complex form than a small apex.

Cellular aspects of determination of leaf form have been studied by Haber and Foard (1963, 1964a, b). They showed that leaf form could change naturally during development (in tobacco) or be changed by gibberellic acid or colchicine treatment (in wheat) without affecting or involving cell division. However it has been suggested (Dale, 1976) that the material they studied was already out of the early primordial phase so that shape was already determined.

It is well known that leaf initiation is usually highly predictable with respect to position at the apex, and to a lesser extent with respect to time. Considerable work has been carried out to ascertain what controls the pattern of primordial initiation (Richards, 1948; 1951; 1956; 1969). The field theory of primordial origin (Richards, 1948; Wardlaw, 1949) presupposes the involvement of chemical morphogens inhibiting or promoting outgrowth at the apex to form primordia. This idea has recently been revived with computer simulations of apical development by Hellendoorn and Lindenmayer (1974), Thornley

(1975, 1976), Veen and Lindenmayer (1977). This work will be discussed in more detail in Chapter VII. At this point it is worth noting that the existence of morphogens concerned with primordial initiation is uncertain.

The effects of a very large range of natural and synthetic growth regulators on leaf growth, form and shape in a large number of species have been examined. Auxins have not often been associated with effects on leaf growth and form, but it has been shown in some cases that auxin treatment modifies the final form of leaves. Ball (1944) showed that in Tropaeolum application of lAA or NAA led to production of multiple leaves. 2,4-D was also shown to cause modified leaf development (Watson, 1948; Gifford, 1953; Furuya and Osaki, 1955; Soma, 1968). TIBA, known as an anti-auxin, has been shown to affect primordial development resulting in many cases in the formation of almost leafless stems (Gorter, 1949 , 1951, Wardlaw, 1953). Gibberellins have been shown to produce changes in leaf shape in many species, leading usually to development of narrower leaves (Felippe, 1967; Engelke et al., 1973; Maksymowych et al., 1976). GA effects were particularly studied in plants which show a heteroblastic leaf sequence. In these plants gibberellin is generally associated with the juvenile phase of the plants (Robbins, 1957; 1960; Njoku, 1958; Feldman and Cutter, 1970a; b; Rogler and Hackett, 1975). Mention may also be made of other substances which affect leaf form, shape and growth as, for example, CCC, known as an inhibitor of gibberellin synthesis (Felippe, 1967; Njoku, 1971); kinetin (Purohit and Verma, 1970); Abscisic acid, usually associated

with the development of scales instead of normal leaves in the formation of winter buds (Eagles and Wareing, 1963; El-Antably et al., 1967) and phenylboric acid (Mathan, 1965).

At this stage an arbitrary distinction will be made between leaf form and leaf shape. By leaf form we mean the morphological description of the leaf, as for example, simple or compound; trifoliate or unifoliate; this can be extended to contrast juvenile and adult leaves when this involves gross modifications. By leaf shape we mean differences in leaves of a common form. Thus leaves of ovate form may differ in shape from another by being longer, or narrower, or broader. So, form relates to gross modifications whilst shape is associated with more minor differences. This arbitrary distinction is employed throughout this thesis.

After the initiation of a leaf primordium the apical meristem comprises a number of components. This leads to an important problem which is not always easy to resolve, namely the difficulty in deciding whether a particular response to growth substances endogenous or exogenously applied, results from a general effect on development of the apex as a whole, or from a differential effect on a part of the apex. A general effect on the apex as a whole could lead to a change in the number of primordia carried at the apex. Such a response might not affect leaf shape unless rapid extension of the apex frees the primordium from constraints to its growth which originate there (Williams and Rijven, 1965; 1970) so that activity in the leaf meristems is modified and thus form or shape altered by some mechanism as yet unknown (Cutter, 1965a).

It might be expected that growth substances, if they act at all to control leaf shape, do so by acting differentially on a part of the apex, or on the primordium itself.

Protein and nucleic acid synthesis are obviously important metabolic activities in the inception of primordium, and during leaf initiation and further development. However the involvement of these processes directly in determination of leaf form or shape is more uncertain. In fact there is not much work in this field. White (1963; 1966) suggested that protein synthesis might be involved in control of leaf form in Marsilea, since he found that adding 2-TU to the culture medium caused delay in appearance of the quadrifid adult leaf. Such evidence is however not very convincing since the effect could be a general one on growth and not specific to leaf form.

From what was described it is obvious that although a large amount of work has been done knowledge of the control of leaf form is still rudimentary. The present work had as its objective to try to obtain further information on leaf form control. For this purpose Phaseolus vulgaris was chosen since: (a) it could be easily grown in controlled environment; (b) from previous studies there is much information already available; (c) it has dimorphic leaves. In practical terms the problem was to determine whether leaf form could be varied experimentally and whether it was possible to change the form of uni or trifoliate leaves? To try to answer this question different approaches were used. Firstly, attempts were made to modify protein and nucleic acid synthesis at the apex by using inhibitors such as BUDR, 2-TU and cycloheximide. Secondly,

using growth substances such as GA_3, known to affect leaf shape, and 2,4-D known to affect form in *Phaseolus vulgaris.*

CHAPTER II

MATERIAL AND METHODS

1. MATERIAL

1.1 Foliar organization in *Phaseolus vulgaris*

Foliar organization in *Phaseolus vulgaris* comprises a pair of cotyledons at the lowest node, a pair of opposite simple leaves (primary leaves - P) at the second node, decussate to the cotyledons, then the compound trifoliate leaf at the third node (first trifoliate leaf - T_1) in superposition to one of the cotyledons. The successive trifoliate leaves (T_2, T_3, etc.) are uniform in shape and are arranged with distichous phyllotaxis.

1.2 Choice of cultivar

In the embryonic axis of fully mature seeds, development of the lamina of the primary leaves is far advanced and their stipules are also present. For the mature seeds of cultivar Canadian Wonder, Dale (1964) and Murray (1968) described the apex as being bare and without the primordium of the first trifoliate leaf. However preliminary analysis showed that in, at least, some seeds this primordium was present. Because of this discrepancy with earlier work a comparison of the apical structure of the embryonic axis on the dry seed was made in several cultivars.

For this study 5 readily identifiable stages, based on the development of the first trifoliate leaf were defined. In

brief, stage 0 is that when there is no visible primordium on the apex and stage 4 when lateral leaflets and stipular initials are visible on the flank of the apical dome. These stages are described in full detail subsequently.

The embryonic axis in the dry seed is brittle and in order to dissect this, it was necessary to imbibe seed samples without affecting the developmental status of the apex. For this, the embryonic axis was excised and imbibed in fixative solution (ethanol 50% - glacial acetic acid 9:1) for 24 hours. After this, apices were dissected and examined under a Zeiss stereo-microscope.

Examination of a total of 10 cultivars (with two different samples of 2 of them) showed that in all cases at least 50 per cent of the embryonic axis carried the primordium of the first trifoliate leaf, present at least at stage 1 (that is as a discernible bump on the flank of the apex). This was so even for the two samples of Canadian Wonder examined, and it is concluded that the earlier results of Dale (1964) and Murray (1968) are not of general application to this cultivar. Batch to batch variation can clearly be substantial within the same cultivar. Despite the substantial differences observed between the cultivars examined in no case was a primordium in stage 4 observed.

To characterize apical development a Mean Developmental Stage (MDS) was defined for the primordium of the first trifoliate leaf as follows:

$$MDS = \frac{\Sigma n x_n}{N}$$

where n is the developmental stage of the primordium, x is the number of apices at each stage and N is the total number of apices

examined. A high value of MDS reflects an advanced apical development.

The most uniform cultivar (table II. 1) was Mont D'Or Golden Butter; the majority of the embryonic axis apices analysed showed the same development (stage 1, table II. 1). For this reason this cultivar seemed to be very convenient for further work. Another and no less important reason was that this cultivar showed one of the lowest MDS of all those examined. This is important since there is good evidence (discussed by Dale, 1976) that leaf form is determined very early in the course of primordial development and it seemed reasonable to use a cultivar in which the first trifoliate leaf primordium was at an early developmental stage, since experimental treatment would be then more likely to be effective in this case. For these reasons the cultivar Mont D'Or Golden Butter was chosen for this project.

1.3 Choice of seed size

The size of seeds in Mont D'Or Golden Butter is not uniform. Apical development of seeds of different sizes was examined in order to determine whether seeds of any size could be used for further work or if it was necessary to use seeds of any particular size or a size range. Dry seeds were weighed and divided into 0.05g interval classes and the frequency of each class was determined. Samples of 25 seeds of each class were taken to examine the developmental stage of embryonic axis in the dry seed.

The MDS was found to increase with the increasing size of seeds (table II. 2). If the largest and smallest seeds were

TABLE II. 1. Developmental stage of the first trifoliate leaf primordium in the dry seed of different cultivars of *P. vulgaris*.
(Experiment I - 10 seeds samples; Experiment II - 50 seeds samples).

Cultivar	Experiment I						Experiment II					
	% of apices in each stage					MDS	% of apices in each stage					MDS
	0	1	2	3	4		0	1	2	3	4	
Canadian Wonder (A)	50	50	0	0	0	0.5	42	58	0	0	0	0.58
Canadian Wonder (B)	0	50	40	10	0	1.6	0	34	56	10	0	1.76
Mont D'Or Golden Butter	0	100	0	0	0	1.0	10	86	4	0	0	0.94
The Prince	30	50	20	0	0	0.9	14	58	28	0	0	0.86
Cordon	20	70	10	0	0	0.9	24	64	12	0	0	0.88
Earligreen	10	80	10	0	0	1.0	16	78	6	0	0	0.90
Glamis	10	50	40	0	0	1.3						
Masterpiece (A)	0	20	60	20	0	2.0						
Masterpiece (B)	10	10	60	20	0	1.9						
Tendergreen	10	60	30	0	0	1.2						
Flair	10	30	60	0	0	1.5						
Kinghorn Wax	0	70	30	0	0	1.3						

(A) and (B) - Different batches of seeds.

TABLE II. 2. Developmental status of the first trifoliate leaf on seeds of different weights.

seed weight (g)	frequency as % of total seed number	MDS
0.16 - 0.20	12.5	0.80
0.21 - 0.25	29.6	0.88
0.26 - 0.30	44.9	0.96
0.31 - 0.35	10.7	0.96
0.36 - 0.40	2.3	1.00

discarded all the others could safely be used as uniform material with respect to initial apical status. As the frequency of the extreme size classes was very low most of the seeds could be used.

2. METHODS

2.1 Cultural Details

Seeds of *Phaseolus vulgaris* cv. Mont D'Or Golden Butter within the weight range 0.20 - 0.35g were used. Very small and very large seeds were discarded. Seeds were imbibed in washed river sand at 25°C in a growth cabinet with 12 hour days and irradiance 180 - 200 $\mu E/m^2/s$. The light source was warm white fluorescent tubes.

After treatment, in short term experiments, seeds were put in Petri-dishes and kept in darkness at 25°C, in the growth cabinet. In long term experiments, when plants were allowed to develop beyond 96 hours they were transplanted to John Innes Compost and grown on in a glasshouse under conditons of natural daylight and temperature not less than 18°C. Plants were watered as necessary.

2.2 Developmental Analysis

2.2.1 Examination of the apices

Routine examination of the apices was carried out using a Zeiss stereo-microscope. Apices were also examined

using a Cambridge Mark II Scanning Electron Microscope operated at 3 kv. For such examination fresh material should ideally be used (Falk, Gifford and Cutter, 1971) and, in some cases, this was possible. However we have found rapid desiccation to be a serious problem with young apices. Critical point drying methods have proved unsatisfactory for us and the method adopted for this work involved fixing material in FAA - 50, followed by slow rehydration in a graded ethanol-water series to water alone. The specimen was then examined with no further processing; distortion due to desiccation, and charging up, became serious after 6 to 8 minutes in the SEM.

2.2.2 Apical Developmental Stages

Based on the development of the primordium of the first trifoliate leaf five developmental stages were described. Stage 0 (figure II. 1.A) is defined when the primordium is not yet visible. The primordium is in stage 1 when it is present on the apex as a small and barely discernible bump (figure II. 1.B). Subsequently the primordium enlarges to have a more definite, but still unipartite form and its tip is still below the apical dome, this was designated as stage 2 (figure II. 1.C). In most apices development of stipules starts on the flanks of the primordium at this stage. Further enlargement of the primordium occurs to result in it overtopping the dome (stage 3 - figure II. 1.D) and soon afterwards development of the lateral leaflets occurs (stage 4 - figure II. 1.E).

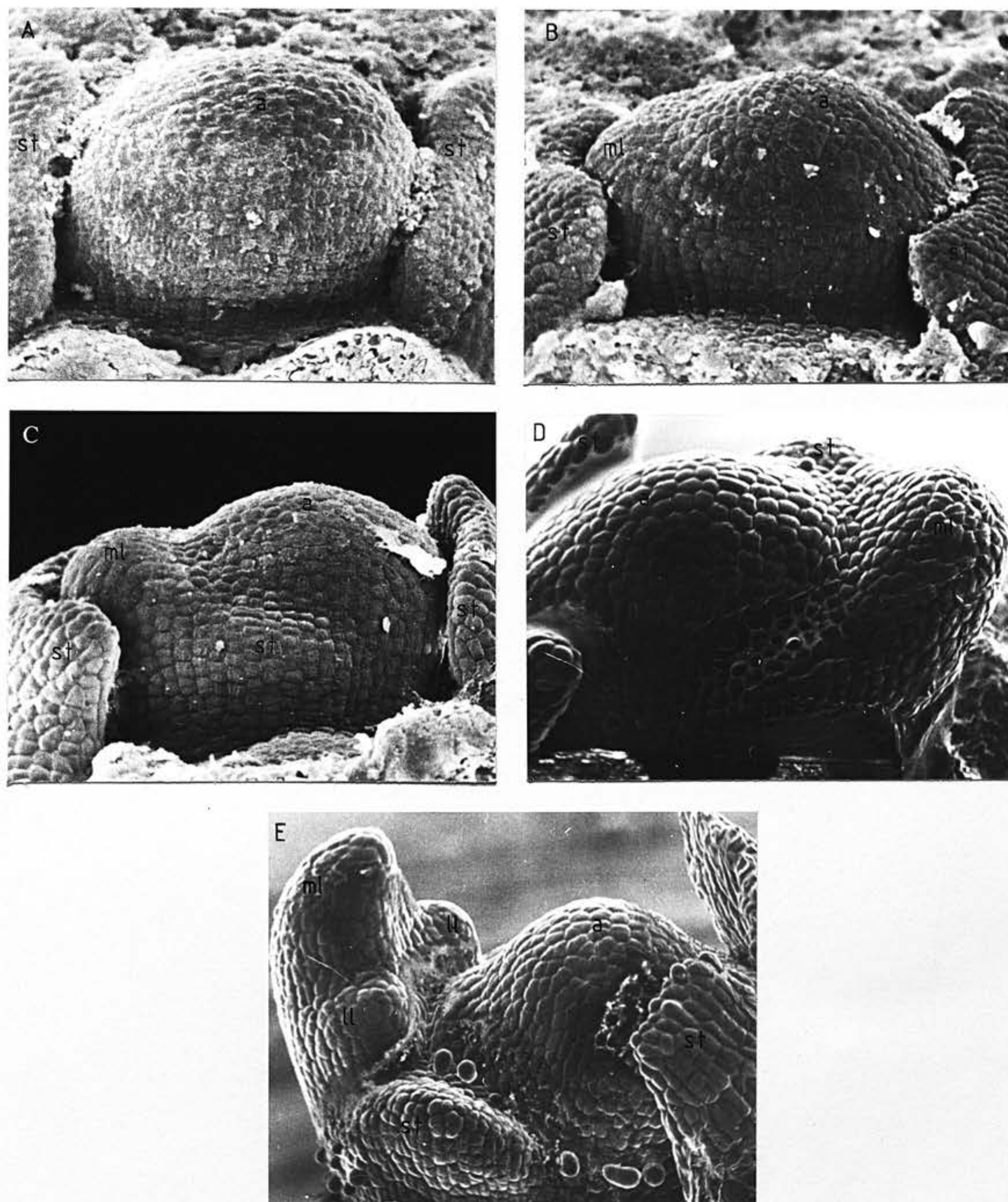


Figure II. 1. SEM micrographs of stem apices illustrating the sequential development of the first trifoliate leaf primordium.

A - stage 0 B - stage 1
C - stage 2 D - stage 3
E - stage 4
a - apical dome st - stipule
ml - middle leaflet ll - lateral leaflet

2.2.3 Histological Preparations

Stem apices were prepared for microscopic analysis by fixing in FAA-50 (formalin-glacial acetic acid-ethanol 50%::1:1:18) for at least 24 hours. After dehydration in a graded ethanol-water series apices were embedded in paraplast and serial longitudinal sections were cut at 10 µm using a "Beck" microtome. Sections were stained with haematoxylin and fast-green and preserved as permanent mounts (Purvis, Collier & Walls, 1964).

The apex was studied in two different aspects. Firstly as a whole. For this purpose the serial sections of each apex were drawn in outline on paper with the aid of a camera lucida. Secondly the apex was studied in its individual parts, that is, leaf primordia, axial regions related to the primordia and apical dome. To identify these parts the apex was divided into regions (figure II. 2) based on the apical division used by Lyndon (1968).

2.2.4 Estimation of Apical Volume

Knowing the magnification used in drawing the outlines, the thickness of the sections and the weight of the outline drawings of the sections, compared with that of a known area of the same paper, the volume of the different regions was determined.

2.2.5 Estimation of cell number

In the early phase of this investigation attempts were made to count cells using either the squash technique described

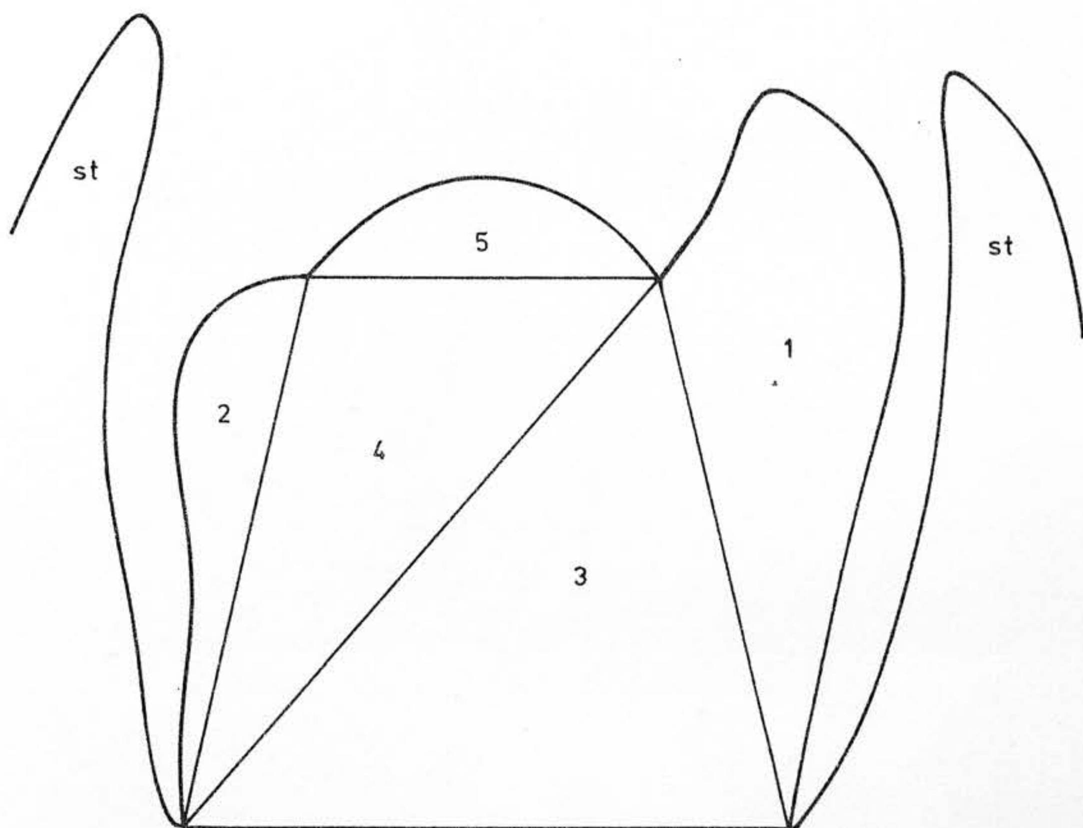


Figure II. 2. Outline of a median section of a stem apex.
1 - first trifoliate leaf primordium
2 - second trifoliate leaf primordium
3 - axial region related to the first leaf
4 - axial region related to the second leaf
5 - apical dome and next primordia
st - stipule

by Sunderland and Brown (1956) or suspension of cells prepared by ultrasonic sonication. Neither technique was satisfactory. With the squash technique it was not possible to spread the apex so as to give a uniform preparation of finite area within which cells could be identified and counted in a single layer. With ultrasonic sonication some cells remained clumped together whilst the treatment resulted in disintegration of others. Another technique for estimation of cell number was then adopted. This involved counting the number of cells in the median section of each apex, which volume was known, and by extrapolation calculating the number in the whole apex. The accuracy of this extrapolation was verified by counting cells in all the sections of an apex and comparing these values with those found by counting only the cells in the median section. The difference between the two values was never over 10%.

2.2.6 Estimation of cell volume

Mean cell volume for each region was estimated using the data for volume and cell number.

2.2.7 Comparative growth rate (COM)

To examine the contribution of each region to the increase in total apical volume during a certain time interval a comparative growth rate (COM) was defined as follows:

$$COM = \frac{v_2 - v_1}{V_2 - V_1}$$

where, $v_2 - v_1$ is the increase in volume of one region during a certain time interval and $V_2 - V_1$ is the increase in volume of the whole apex for the same period of time. This parameter permits analysis of the contribution of each region to the total growth of the apex.

In order to examine the effect of a treatment upon any of the regions the following ratio (Z) was defined:

$$Z = \frac{COM_t}{COM_c}$$

where COM_t is the comparative growth rate of a certain region of a treated apex for a determined time interval and COM_c the comparative growth rate of the same region of a control apex during the same time interval.

These ratios defined for increase in volume were calculated also for increase in cell number.

2.3 Estimation of leaf area

For area determinations an outline of the leaf was traced onto paper of known weight, cut out and weighed, leaf area was determined from the ratio of weight of tracing to that of a known area of paper.

2.4 Determination of leaf shape

2.4.1 Leaf shape index

Leaf shape index was determined following the method

described by Melville (1937) and used for *Phaseolus* by Felippe (1967). In this procedure the outline of a leaf was traced and its length divided into ten units from apex (0) to the base (10). Leaf width was determined at 1/10 intervals along the length of the lamina (figure II. 3). Comparison of shape of leaves with different sizes was possible by expressing the values of width at different positions along the length as values of w'_n determined by the ratio:

$$w'_n = \frac{w_n \cdot 10}{L}$$

where w_n is the width of the leaf at the $n/10$ of the length and L is the leaf length. The ratio between width (w') of treated and control leaves for the same position along the leaf length defines the index of leaf shape (I) for that position.

2.4.2 Leaf types

In some experiments shape of adult leaves was determined qualitatively by comparing the leaves with the leaf types outlined in figure II. 4. Leaf types were worked out from the most common leaf shapes obtained in several experiments. When this method was used results were expressed as percentage of leaves included in a determined type.

2.5 Chemical Treatments

2.5.1 Treatments applied to mature seeds

Treatments were usually made to seeds imbibed for

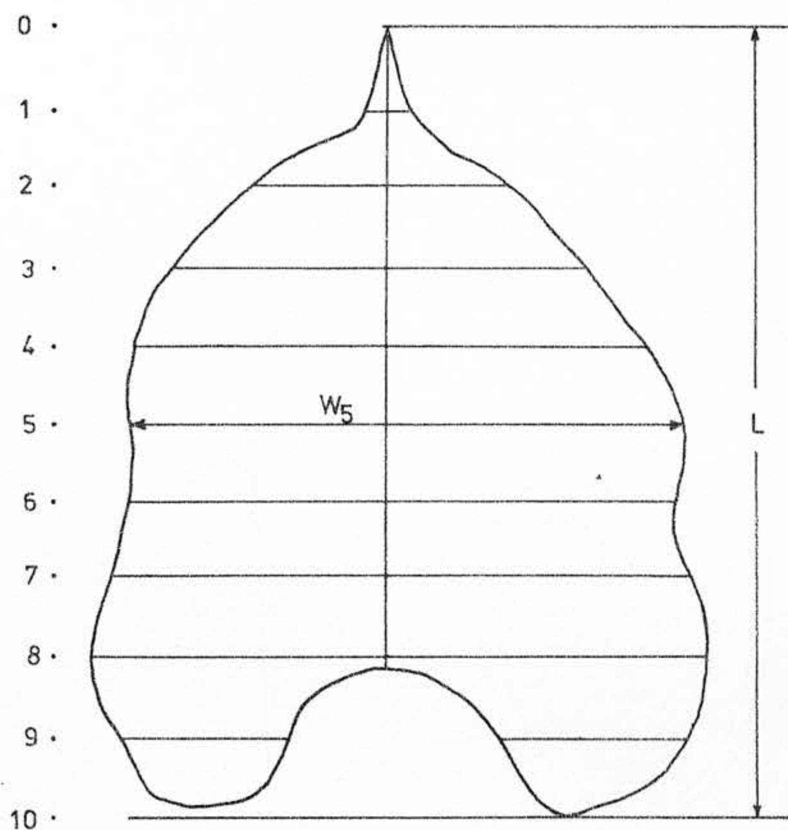


Figure II. 3. Outline of a primary leaf to illustrate the determination of the leaf shape index.
e.g.:
w_5 - width of the lamina at 5/10 of leaf length
L - leaf length

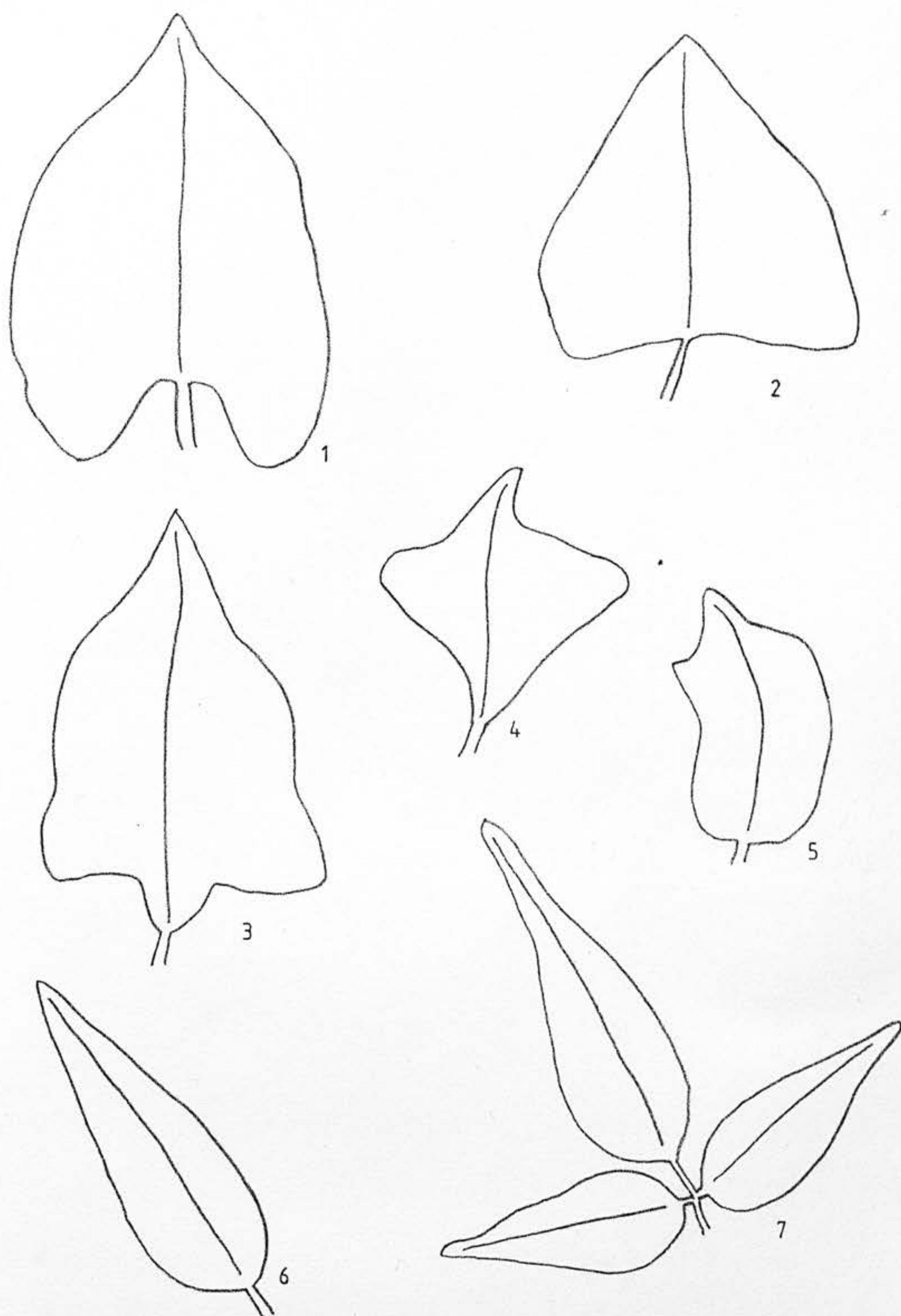


Figure II. 4. Outlines of leaf types.
1 - normal primary leaf; 7 - normal trifoliate leaf; 2 to 6 - observed leaf types as a result of treatment.

24 hours. One cotyledon was removed and, in some cases, the apices were bared by removing the primary leaves, in a way that treatments could be made directly on the apex; in others the apical region was left intact and chemicals were applied on the base of primary leaves which cover the apex.

Chemical compounds were applied in form of aqueous solution, using 0.01% tween 80 as wetter, or in lanolin. When in aqueous solution droplets of 0.6 to 5.0 $\mu\ell$ were applied using a micrometer syringe. Where application was made in lanolin a 200 or 2000 ppm paste was applied although the exact amount supplied was not known.

2.5.2 Treatments applied to immature seeds

In some experiments immature seeds were treated with chemical compounds. The first method used to do this was application of the solution by means of injections to the pods between the developing seeds. In spite of the great care taken to avoid touching the seeds with the needle, when harvested many of them were found to be damaged.

A different method was then adopted which involved abrasion of the pod wall with sandpaper and then brushing the pod with the solution. The volume applied was approximately 0.4 ml per pod.

In some cases immature seeds were harvested and left to dry to constant weight and then used either for germination studies or for treatment with growth regulators.

2.6 Replication

Values of cell number, cell volume and apical volume are the average of 5 apices. In the majority of other experiments samples of 10 seeds or plants were used. When a different sample size was used this is stated in the description of the experiment.

CHAPTER III

PRELIMINARY RESULTS

1. INTRODUCTION

Some preliminary experiments were designed to examine three important points before the main part of the work could be done. The first of them concerned the batch to batch variation shown in the description of the material (Section 1.2 - Chapter II). It was suggested that differences in apical development between different batches of seeds could be the result of different growing conditions. An experiment was designed to examine this point.

The second aspect investigated concerned the pretreatments necessary for application of chemicals to the apex of the embryonic axis of mature and immature seeds. For direct application to the apex to be achieved one cotyledon has to be removed and sometimes the primary leaves are removed as well. An experiment was designed to examine how the removal of these organs affected early primordial development and subsequent growth of the plants.

Since part of this work involved experiments with immature seeds and embryos it seemed important to investigate and quantify their normal pattern of growth. The third point examined in this section deals with the development of flowers, fruits, seeds and embryos.

2. GROWING CONDITIONS AND PLANT DEVELOPMENT

Ten days after sowing in sand plants were divided into two groups; one of these received full strength nutrient solution twice a week and was placed on a bench in the glasshouse so as to be exposed to direct natural daylight conditions. The other group received 1/10 strength nutrient solution once a week and was put under the bench, so that light intensity was greatly reduced to about 10 percent of that experienced by the high nutrient control set. The flowering of these plants was followed by daily tagging and the flowers were numbered for later examination of pod growth. After maturation of pods, 45 days after anthesis, seeds were harvested and the apical development of the seedling axis was examined on the dry seed and after different periods of imbibition.

The start of flowering was delayed by poor nutritional conditions but the most striking effect was on the number of flowers produced. This was very much reduced and whereas the high nutrient plants produced about 24 flowers per plant, those in the low nutrient set produced about 4 flowers per plant (figure III. 1). Subsequent work showed that when grown in compost plants could produce substantially more than 24 flowers per plant (see figure III. 4). The reduction in flower production was probably due to abortion and shedding of the flower buds although a reduction in bud initiation cannot be ruled out.

The poor growing conditions also reduced the number of seeds per pod but seed weight was not affected (table III. 1).

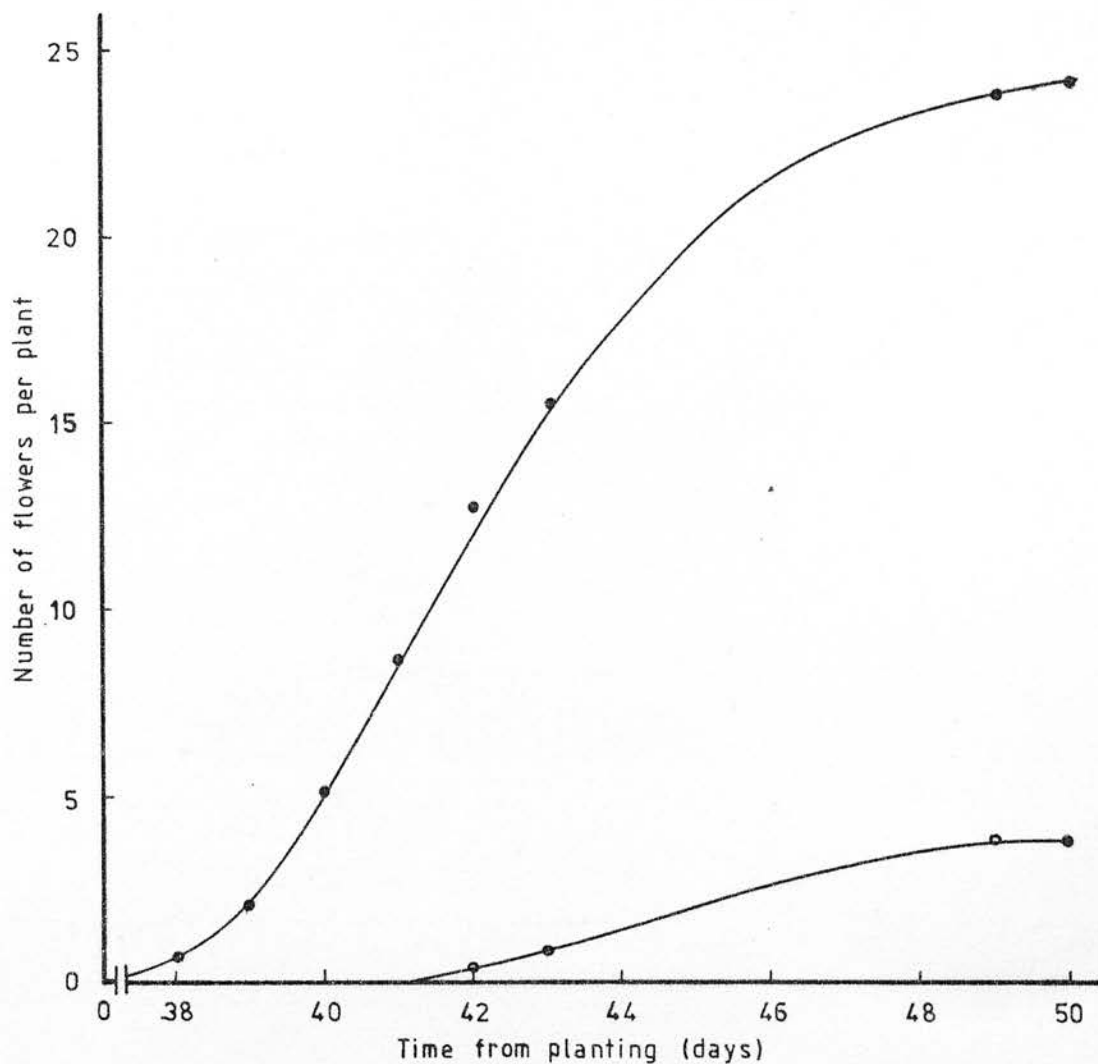


Figure III. 1 Effect of growing conditions on flower production.
● - high nutrient : high light
o - low nutrient : low light

TABLE III. 1. Effect of growing conditions on seed development.

Treatment	Number of seeds per pod	seed weight (g)
high nutrient: high light	4.8 ± 0.57 (1)	0.36 ± 0.01
low nutrient: low light	1.8 ± 0.64	0.39 ± 0.04

(1) 95% confidence limits are quoted

The data suggest that growing conditions can constitute a limiting factor for the number of seeds produced but do not affect the size of these seeds.

Apical development of the seedling axis of these seeds was not affected nor was the number of primordia developed up to 72 hours following seed imbibition. Confidence limits were calculated and no significant difference was found (table III. 2).

The conclusion from this investigation is that despite large variation in growing conditions the apical development of the progeny was not affected. In view of this the batch to batch variation found in Canadian Wonder may not be attributable to environmental conditions under which it was produced but may be associated with genetic variation in this cultivar which is known to exibit substantial variation according to its provenance. It may also be pointed out that over this study several batches of seeds of Mont D'Or Golden Butter have been used and variation between them has been found to be slight.

3. REMOVAL OF ONE COTYLEDON AND THE PRIMARY LEAVES

To determine the effects of leaf and cotyledon removal seeds which were imbibed for 24 hours at 25°C were treated as follows:

1. One cotyledon removed (1C + 2L).
2. One cotyledon and the primary leaves removed (1C + 0L).
3. Intact seeds (2C + 2L) which were used as control.

Early development of the seedling axis in these seeds

TABLE III. 2. Effect of growing conditions on apical development of seedling axis (C = high nutrient: high light; T = low nutrient: low light).

time of imbibition (h)	treatment	Number of primordia	MDS of each primordium				
			1	2	3	4	5
0	C	1.0	1.2	0.0			
	T	1.0	1.7	0.0			
24	C	1.0	1.8	0.0			
	T	1.0	2.8	0.0			
48	C	2.0	4.0	1.8	0.0		
	T	1.8	4.0	1.2	0.0		
72	C	4.0	4.0	4.0	2.5	1.5	0.0
	T	4.0	4.0	4.0	2.5	1.0	0.0

was measured firstly by examination of the development of the primordia of the first and second trifoliate leaves up to 96 hours after treatment. Secondly, the growth of the primary leaves and stem-roots axis was examined by determination of dry weight during the same period of time.

Further leaf growth was measured by determining the area of fully expanded leaves of plants grown for 20 days under glasshouse conditions.

Flower production was observed in another experiment where from a group of seeds one cotyledon and the primary leaves were removed (1C + 0L) with an intact set (2C + 2L) as control. These plants were maintained in the glasshouse up to the end of the flowering period.

Figure III. 2 (A and B) shows the development of the primordium of the first and second trifoliate leaves. It is evident that the early development of, at least, the two first trifoliate leaves is not affected by the removal of either one cotyledon or one cotyledon and the two primary leaves. From these results it seems either that presence of the primary leaves does not affect development of the subsequent primordia or, alternatively, that its effect has been exerted by the time of primordium initiation.

The reduction of nutrient source caused by the removal of one cotyledon did not alter primordial development. This suggests that at this early stage of development only one cotyledon is sufficient to support the first phase of apical growth and development, since, it was observed that in the absence of both cotyledons the seedling axis failed to grow.

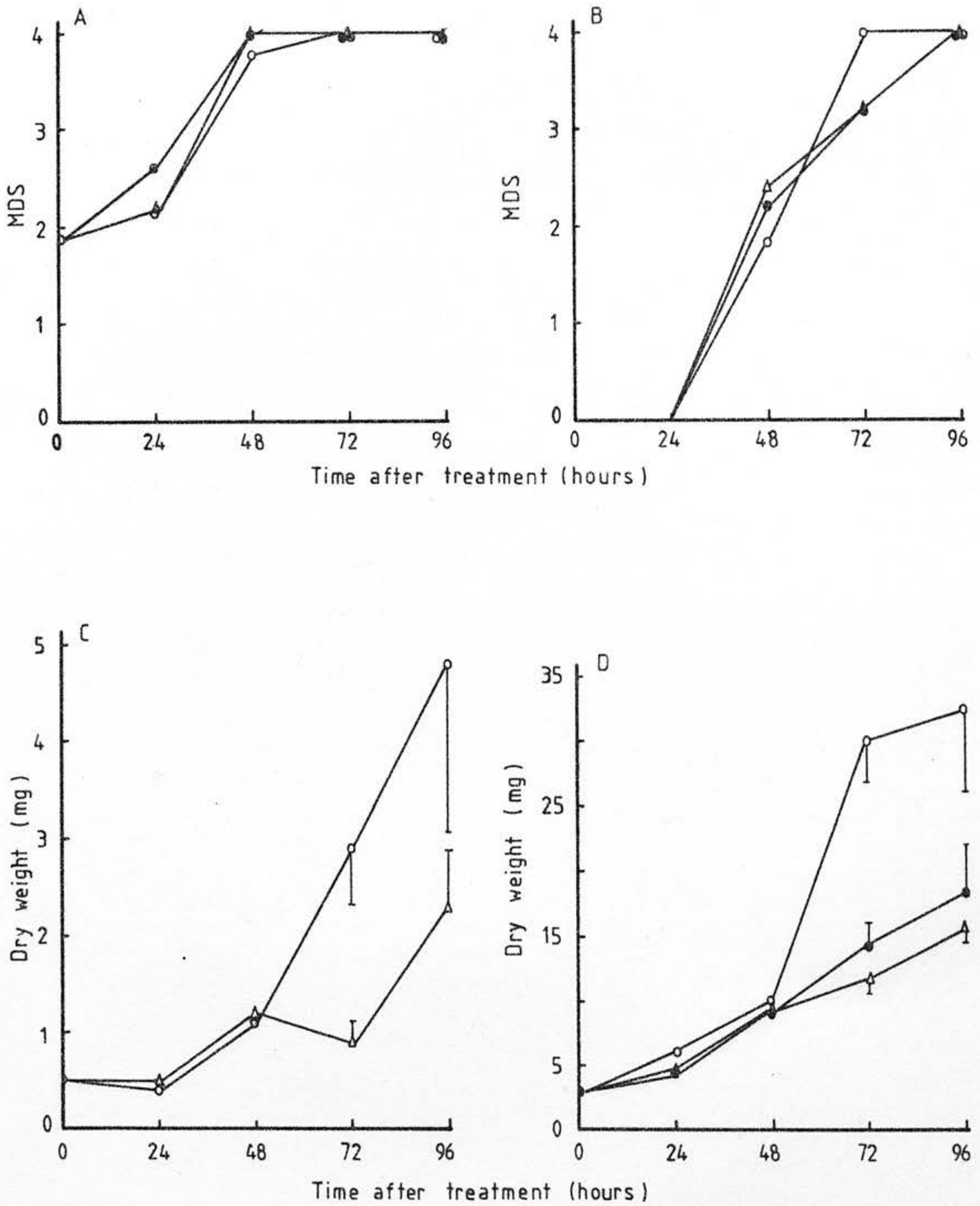


Figure III. 2. Effect of removal of one cotyledon and primary leaves on primordial development and seedling axis growth.
A - first trifoliate leaf primordium
B - second trifoliate leaf primordium
C - primary leaves dry weight
D - stem-roots dry weight
o - 2C+2L ▵ - 1C+2L ● - 1C+0L

Removal of primary leaves did not affect growth of the seedling axis, and there was no significant difference in growth in seeds with or without primary leaves (figure III. 2.D). However removal of one cotyledon did reduce the growth of the seedling axis and the primary leaves, 72 hours later (figure III. 2. C; D), implying that by this stage one cotyledon alone was inadequate to meet the requirements of the growing seedling.

Final area of the primary leaves was reduced by the removal of one cotyledon (table III. 3) and area of the first trifoliate leaf was smaller in plants developed without one cotyledon. Removal of the primary leaves affected growth of the first trifoliate leaf to a much greater extent (table III. 3). This is explicable on the hypothesis that growth of a leaf is determined by contributions from the older leaf below it (Dale, 1976).

The growth of the petiole of the middle leaflet of the first trifoliate leaf was reduced by the removal of the primary leaves but the leaflet length was much more affected. This was reflected in the ratio between petiole and leaflet length which increased significantly when primary leaves were removed (table III. 4). These data suggest that the presence of the primary leaves does not affect the growth of the first trifoliate leaf in a uniform way, leaf lamina being more affected than petiole.

From these results it can be concluded that although early development of trifoliate leaf primordia is not affected by removal of primary leaves the same does not occur when further growth of the first trifoliate leaf is considered.

TABLE III. 3. Effect of removal of one cotyledon and primary leaves on maximum leaf area (cm^2) of primary leaves and the first trifoliate leaf.

	Treatments		
	2C + 2L	1C + 2L	1C + 0L
Primary leaves	61.4 ± 7.9 [1]	49.6 ± 3.3	-
First trifoliate leaf	166.3 ± 16.3	136.9 ± 10.4	36.7 ± 9.5

(1) 95% confidence limits are quoted

TABLE III. 4. Effect of removal of one cotyledon and primary leaves on the length of the petiole and lamina of the middle leaflet of the fully expanded first trifoliate leaf.

length (cm)	Treatments		
	2C + 2L	1C + 2L	1C + 0L
Petiole (P)	2.7 ± 0.36 [1]	2.4 ± 0.36	1.8 ± 0.27
Leaflet (L)	14.9 ± 0.78	13.8 ± 0.53	7.2 ± 1.31
P/L	0.18 ± 0.02	0.17 ± 0.03	0.26 ± 0.05

(1) 95% confidence limits are quoted

Data from an experiment on flowering are now considered. The start of flowering was delayed and the number of flowers produced was reduced in plants in which one cotyledon and primary leaves were removed (figure III. 3. A), but pod growth was not affected by treatment (figure III. 3. B).

Plants of this cultivar normally produce 4 and sometimes 5 trifoliate leaves on the main stem; after this flower primordia are initiated. The leaf primordia are initiated between 96 and 120 hours after planting. Murray (1968) described the appearance of the first flower primordium on day 6, for cultivar "Canadian Wonder".

These facts lead us to think that flower primordia in cultivar "Mont D'Or Golden Butter" are initiated very early during the plant development. This may explain why removal of one cotyledon and primary leaves at an early stage of development could have such a marked effect on flowering. It may well be that as a result of treatment early initiation of flower buds was inhibited. To ascertain this a much more detailed analysis would be necessary. Such an analysis could also answer the question: "Is the observed effect on flowering due to removal of the cotyledon, the primary leaves or to both". Clearly removal of the cotyledon could affect early floral initiation, but leaf removal might be expected to have more pronounced effects.

4. REPRODUCTIVE DEVELOPMENT

In this section a number of aspects of development of control, untreated plants was examined. For this purpose plants

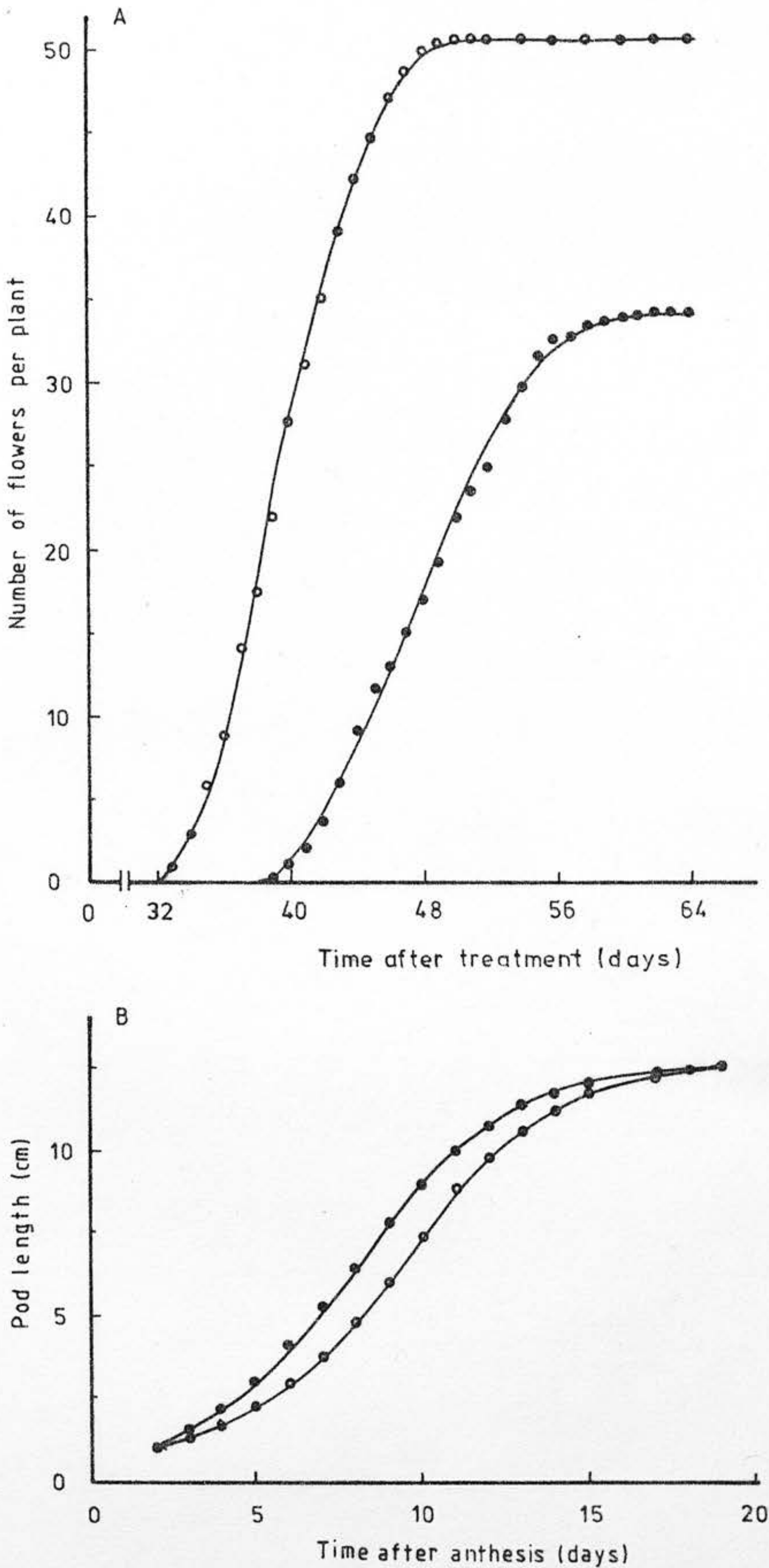


Figure III. 3. Effect of removal of one cotyledon and primary leaves on flower production (A) and pod growth (B).
o - 2C+2L • - 1C+0L

were grown under glasshouse conditions up to the maturation of the seeds, 45 days after anthesis.

Flowers were numbered and tagged for analysis of pod growth which was followed daily.

Samples of pods were harvested daily for the analysis of seed, cotyledon and embryo growth. Measurements of length were usually made using an ocular micrometer on a Zeiss stereo-microscope.

Dry weight was determined by oven drying at $80^{o}C$ to a constant weight. This usually required about 48 hours.

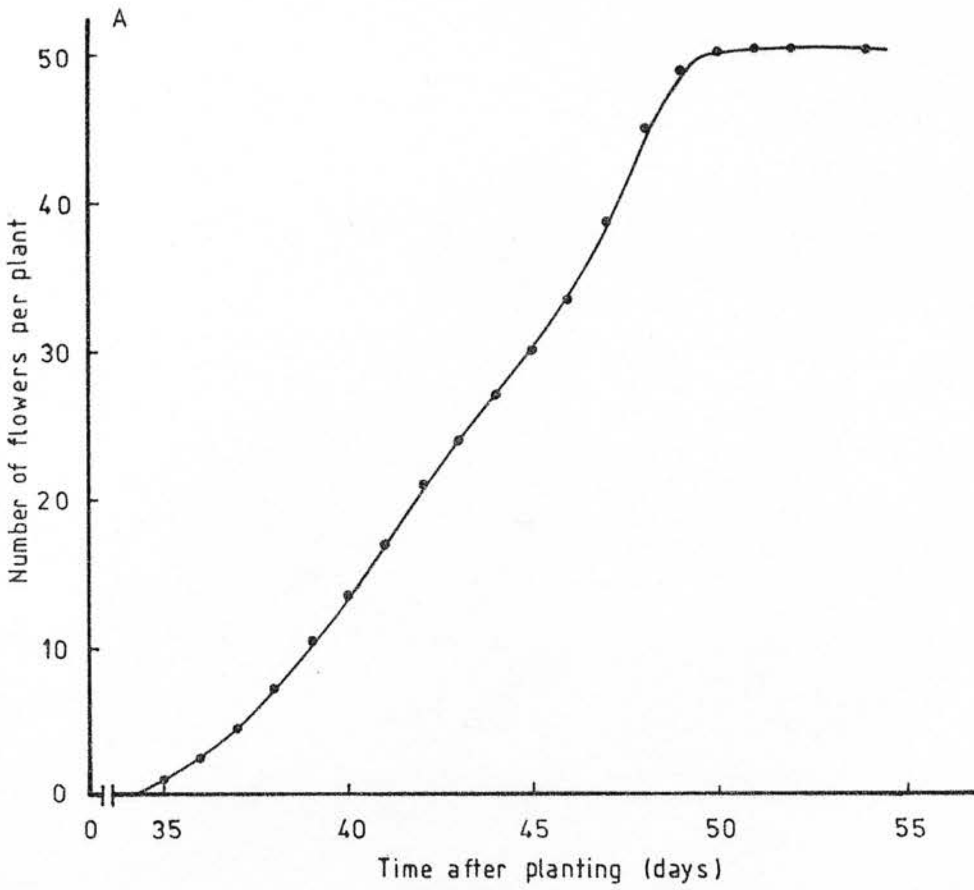
Measurements of apical development were made in embedded and sectioned apices. Photomicrographs were taken using a Zeiss photomicroscope to illustrate the development of the apex. Development of the primary leaves is shown by means of photographs taken using an Olympus stereo-microscope with an attached camera.

Germination of immature dry seeds was studied in a growth cabinet at $25^{o}C$ in darkness.

Flowering commenced on day 35 and the number of flowers per plant increased at a steady rate until about day 49 (figure III. 4. A). This means that flower production lasted about two weeks.

Increase in pod length followed a typical sigmoid pattern of growth. The growth starts immediately after fertilization and pods reach their maximum length within about 15 days. After day 40 length declined due to dehydration of the pod (figure III. 4. B).

Seed and cotyledon growth showed clearly a diauxic



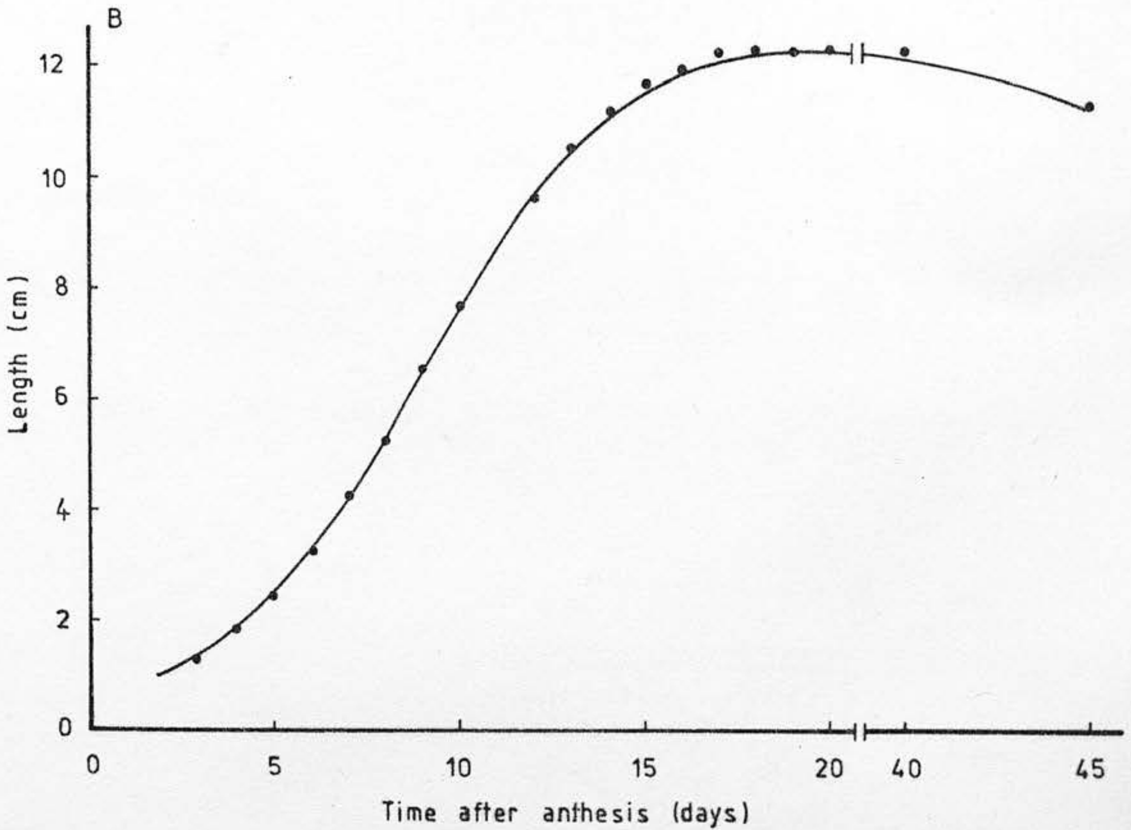


Figure III. 4. Flower production (A) and pod growth in length (B) in control plants.

growth curve (figure III. 5). Cotyledon and whole seed present a similar shape of growth curve after day 20. Up to this age cotyledons are significantly smaller than the seed. In order to simplify the presentation of the data the confidence limits are shown only for some points in figure III. 5. From day 20 on there is no space between the seed coat and the cotyledons.

For seed and cotyledons the first phase of rapid growth ended about 25 days from anthesis (figure III. 5). Growth rate was then very reduced up to about day 30 when a new phase of rapid growth started and continued up to about day 40, when dehydration started and consequently seed and cotyledon size declined.

Growth of embryo indicated by changes in length (figure III. 6. A) showed a reduction in rate between day 25 and 30. After that there was a second period of rapid growth which was followed by decrease in length after day 40. Although a diauxic curve was traced a sigmoid pattern can not be ruled out because of the variation of the data. When increase in dry weight was considered (figure III. 6. B) the precise pattern of growth was not very clear, although it seems more likely that increase in dry weight follows a sigmoid pattern. Walbot et al., (1972) did not find a diauxic pattern of growth in embryo dry weight.

Primary leaves also showed a diauxic growth pattern in length during seed development (figure III. 7). Primordia of primary leaves were present on the embryo on day 12 after anthesis (figure III. 8. A). But it was only about day 16 that lamina differentiation was initiated (figure III. 8. B). On day 19

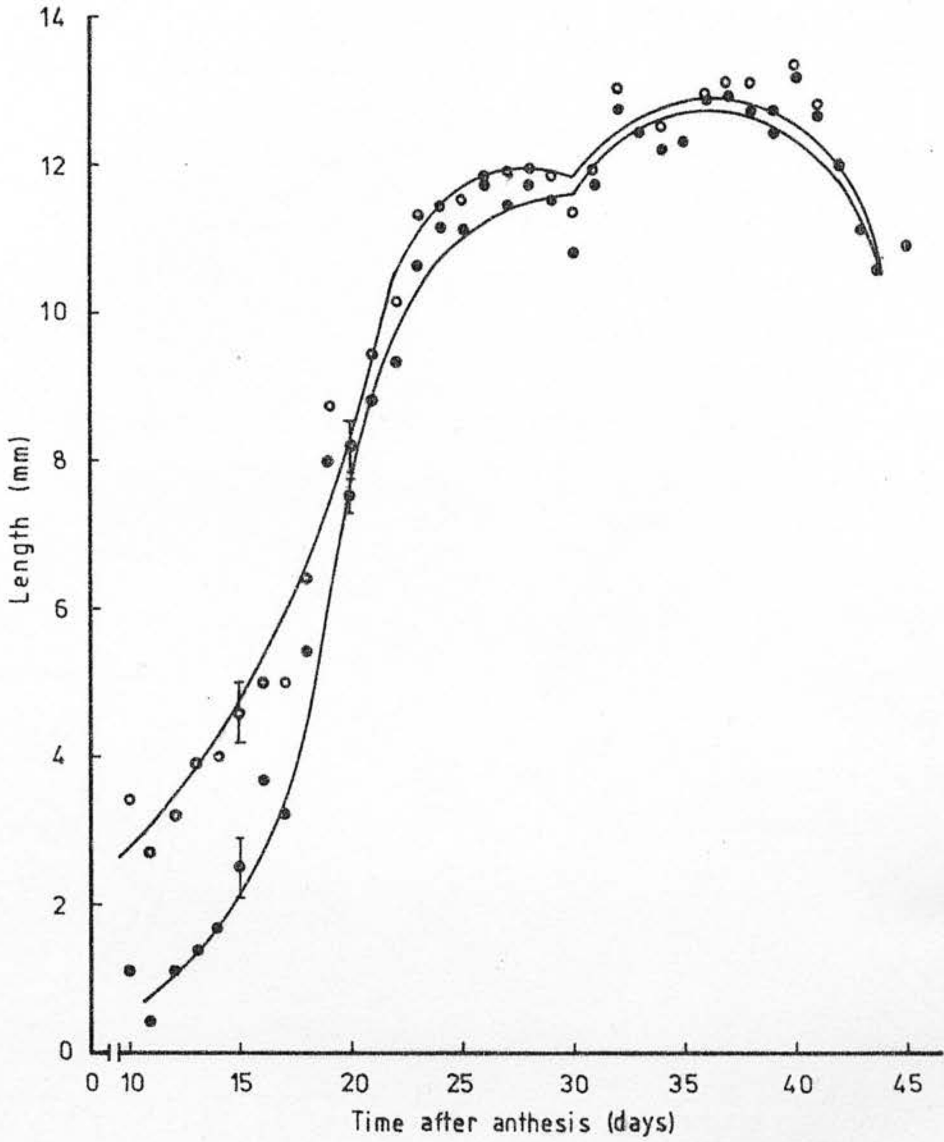
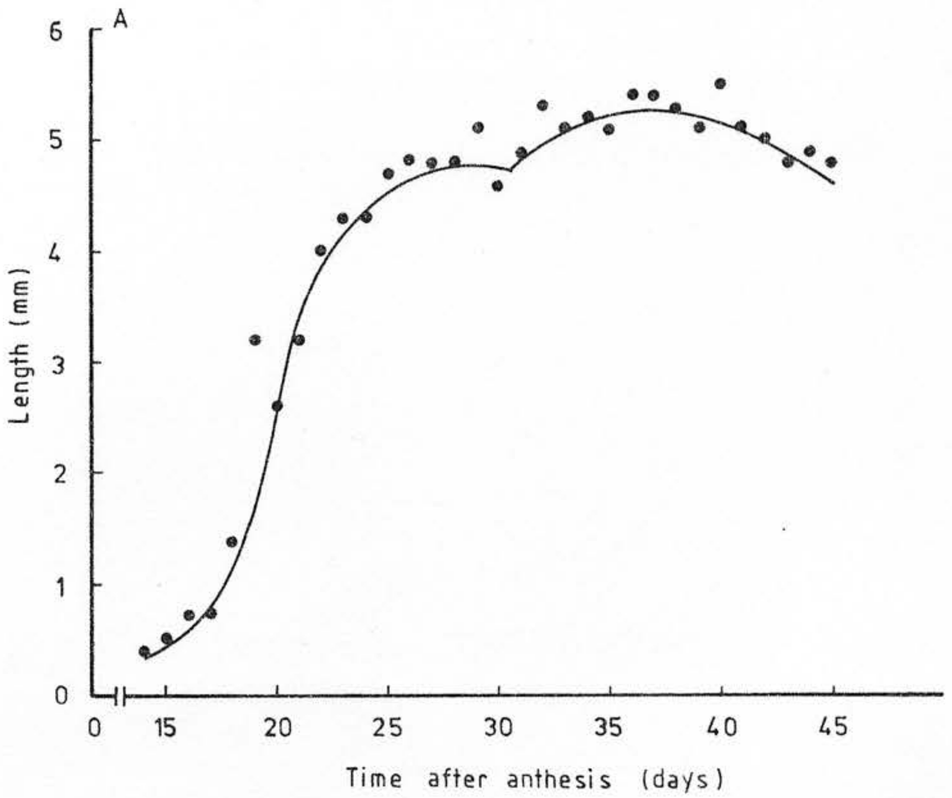


Figure III. 5. Seed (o) and cotyledon (●) growth in control plants.



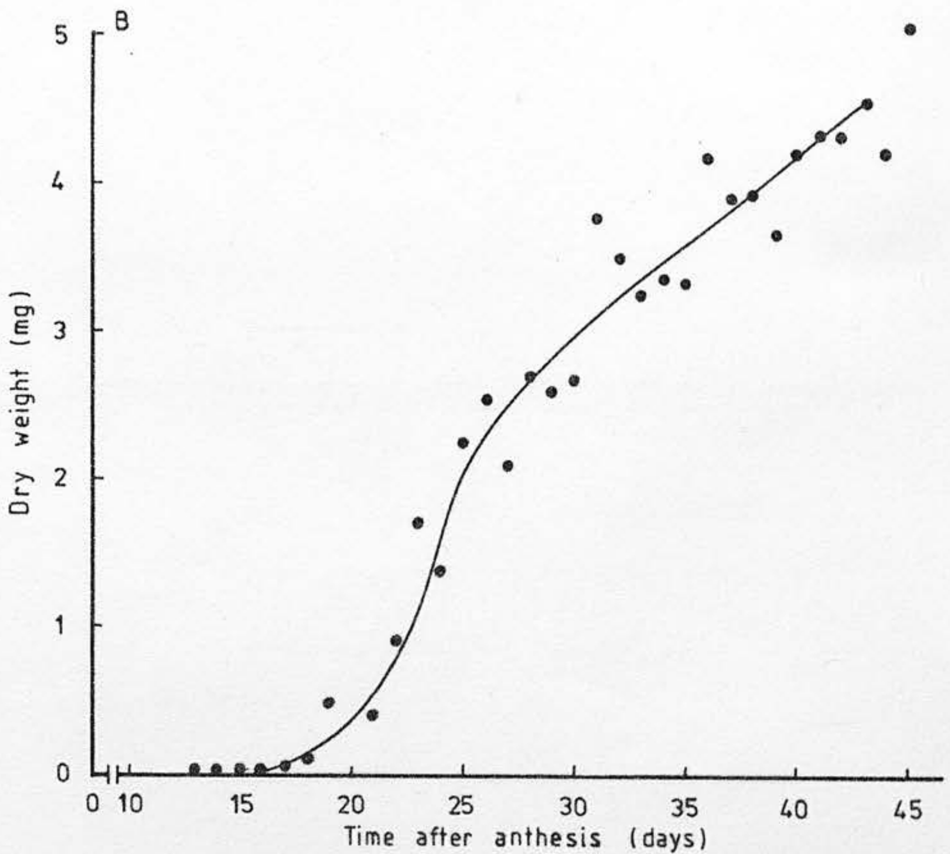


Figure III. 6. Embryo growth in length (A) and dry weight (B).

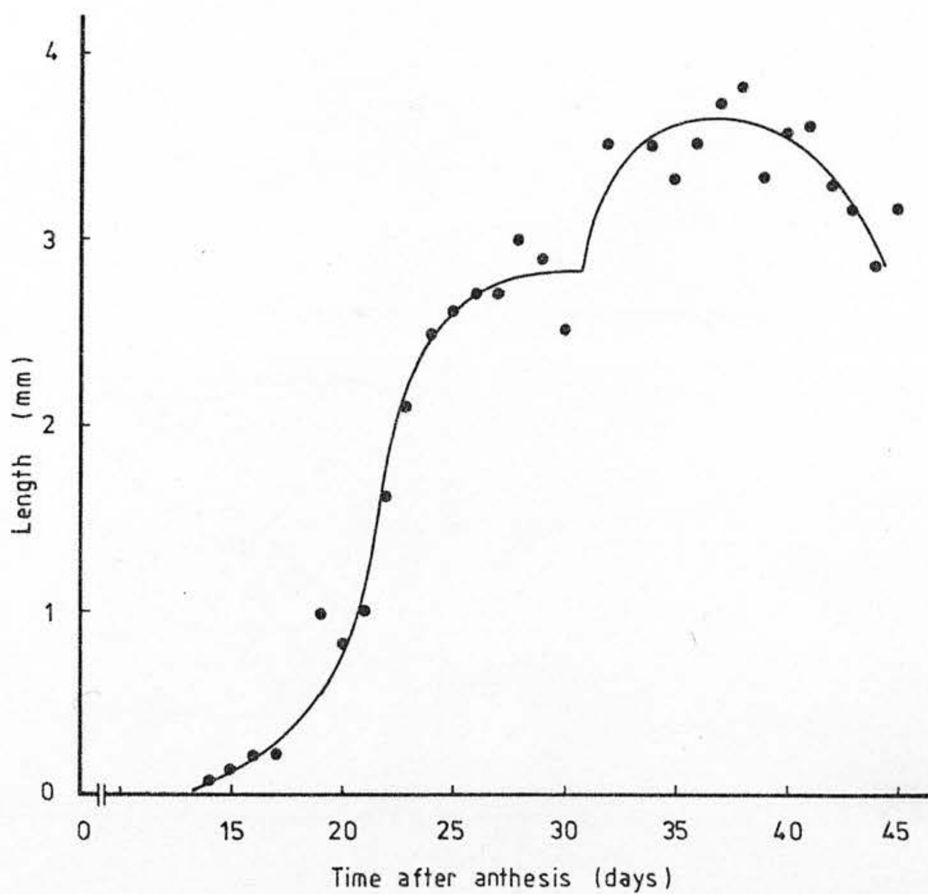


Figure III. 7. Growth of embryonic primary leaves during seed development.

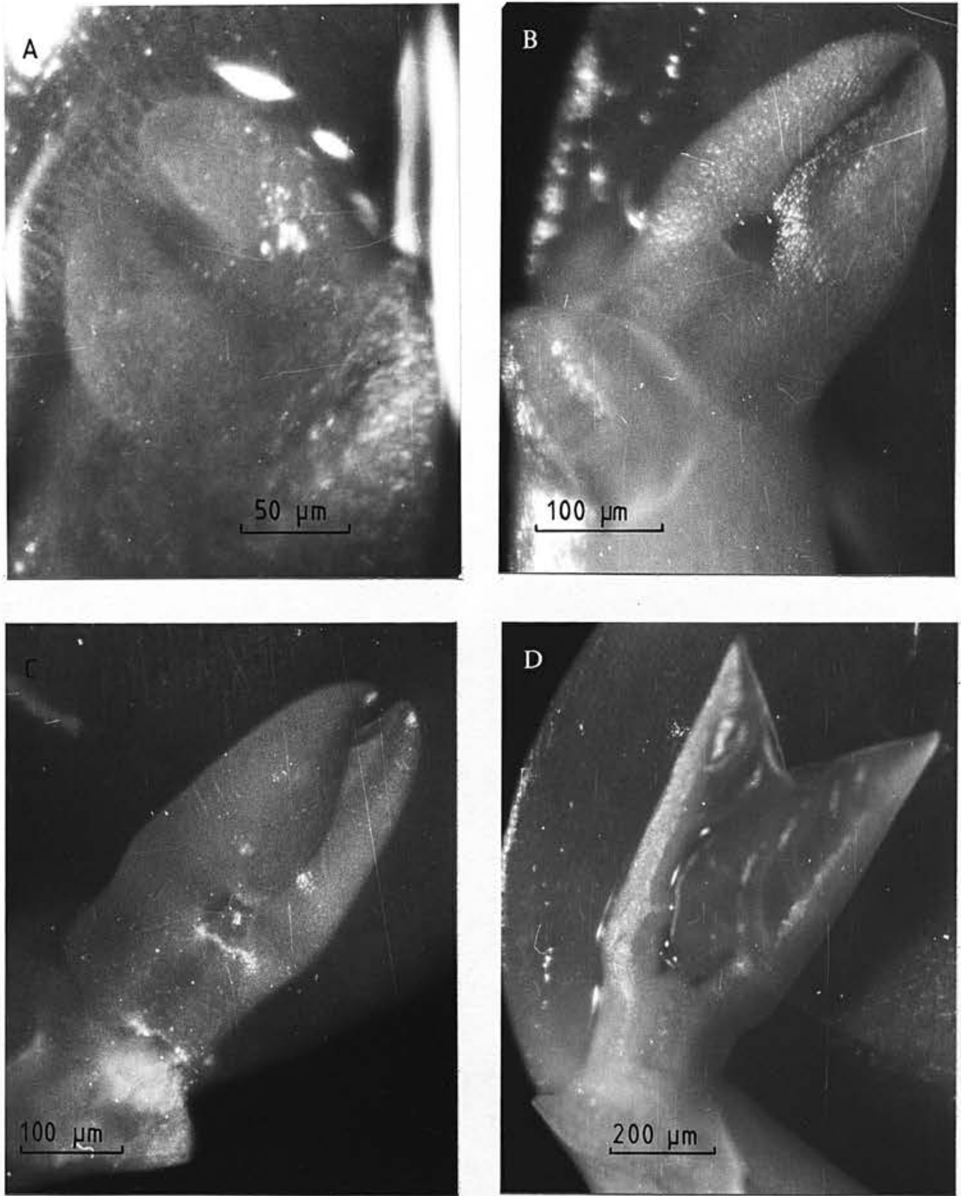


Figure III. 8. Primary leaf development.
Time after anthesis (days):
A - 12 B - 16 C - 19 D - 21

the development of the lamina was well advanced (figure III. 8. C) but it was only by day 21 that the veins were noticeable (figure III. 8. D). By this time changes in leaf morphology appeared to be completed although its growth continued until the maximum size, in the seed, was reached; this was by day 40 (figure III. 7).

These data show that within the first 9-10 days of primordial development the major early differentiation of the primary leaves was completed. Leaf shape was defined within a very short period from its initiation.

Examination of sectioned material showed that the increase in apical volume during seed development did not show the pattern of growth with a lag phase at about days 25-30 found for other parameters of embryo and seed growth (figure III. 9). Growth rate seems to be steady with a good linear fit ($r = 0.996$) up to day 40, when apical volume started decreasing as a result of dehydration. Unfortunately precise conclusions about the pattern of growth are not possible, in this case due to the long interval between relatively few observations.

The apical dome was hardly noticeable on day 10 (figure III. 10. A) of embryo development, although it was very well developed by day 15 (figure III. 10. B). Figure III. 10. C shows an apex 25 days from anthesis still bare of the trifoliate leaf primordium, although this primordium was initiated by day 30 (figure III. 10. D). The initiated primordium is densely stained (figure III. 10. D) and although the second trifoliate leaf primordium is not visible there is a

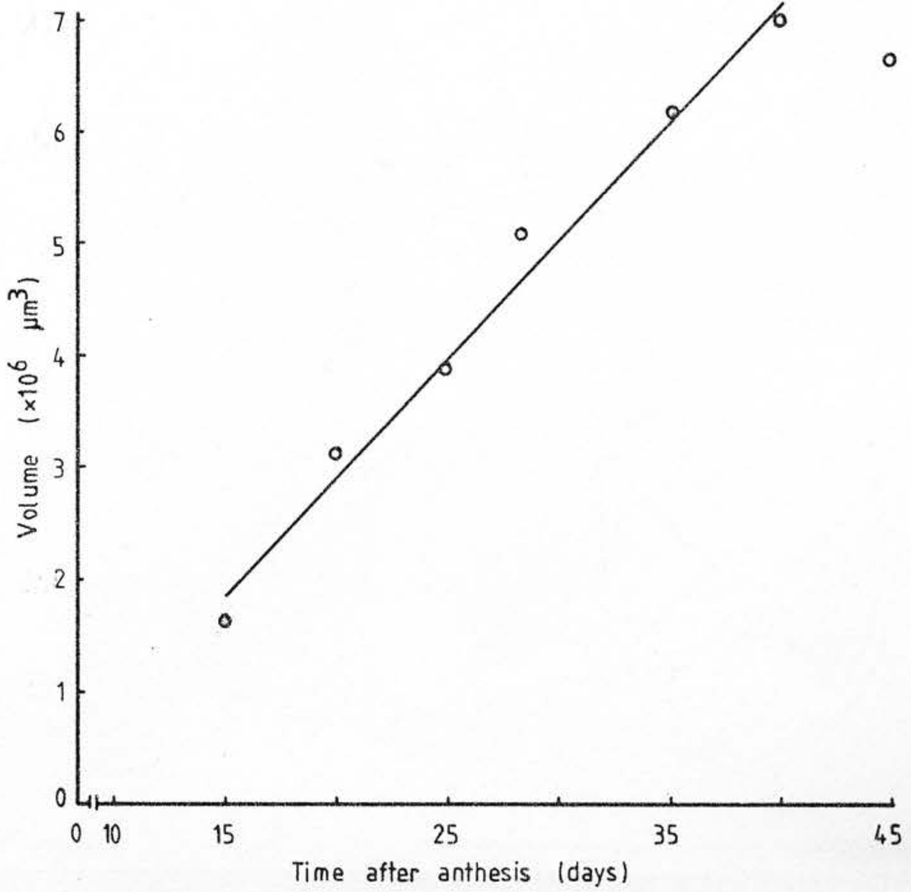


Figure III. 9. Changes in apical volume during embryo development.
Equation of the regression line ($r=0.996$):
$y = 6.40358 + 0.04679x$

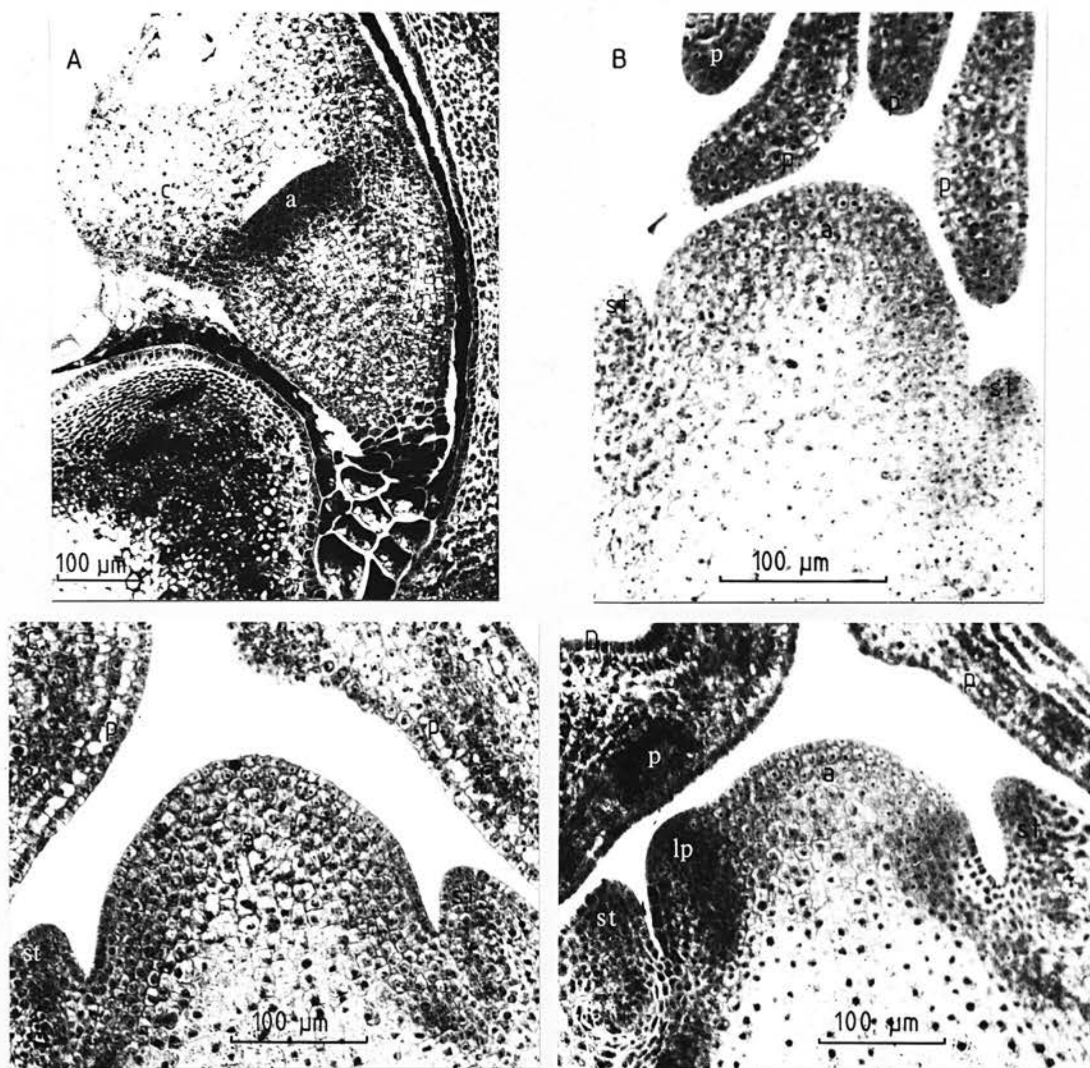


Figure III. 10. Development of the embryonic apex.
Time after anthesis (days):
A - 10 B - 15 C - 25 D - 30
a - apical dome
c - cotyledon
p - primary leaf
lp - first trifoliate leaf primordium
st - stipule

region of dense staining coinciding with its ultimate position. This may mean that the second trifoliate leaf primordium is incipient by this time although it is only visible much later, at 48 hours after imbibition.

There is no expression of the trifoliate leaf shape during development within the seed, and, as already stated, in the mature dry seed the only trifoliate leaf primordium present is in stage 1.

Comparing the development of primordia of the primary leaves and the first trifoliate leaf it is noticeable that in the case of primary leaves, leaf shape is well defined in the dry seed but that nothing can be said about final shape of the first trifoliate leaf by examining the primordium in the dry seed. Another great difference between both types of leaves is that development of primary leaves is very rapid within the seed and it is very slow in the case of the first trifoliate leaf.

Germination of immature seeds was also examined. After being allowed to dry seeds 25 days from anthesis or older, but not younger, were able to germinate in Petri-dishes with moist filter paper (table III. 5). There was no difference in the germination percentage of immature seeds, from 25 days on, and mature seeds (45 days).

5. DISCUSSION

Concerning the effect of nutritional conditions it seems that while they are important to growth and flowering they do not govern apical development of the progeny during seed

TABLE III. 5. Germination of immature seeds after 6 days (%)

Seeds age (time after anthesis) (days)						
15	20	25	30	35	40	45
0	0	85	80	100	70	95

growth as was initially thought.

The hypothesis that nutritional conditions are an important factor for heteroblastic leaf sequence (Allsopp, 1953a; b; 1967) seem not to be supported in this case, since neither at primordial nor at adult stage was the formation of a trifoliate leaf prevented or delayed.

One cotyledon seems to be sufficient to support early primordial development in the seedling but both are necessary to ensure high growth rates of the seedling axis and primary leaves. Early removal of cotyledons was shown to reduce leaf area of primary leaves in P. vulgaris cv. Canadian Wonder (Wheeler, 1966).

The presence of primary leaves is not essential for early primordial development, although the final area of the first leaf above them is much affected by their presence, suggesting that the primary leaves support growth of the first leaf above them. These data are in agreement of the hypothesis that growth of a leaf is determined by contributions from the older leaf below it (Dale, 1976). This effect was not found to be uniform, since the growth of the lamina is reduced proportionally more than that of the petiole.

In the early stages analysed the presence or absence of the primary leaves did not affect growth of the stem-root axis. These data support the results of Valio and Schwabe (1978) who found that partial or total removal of the primary leaves does not affect hypocotyl and epicotyl growth, although it stimulates stem elongation of one-week old seedlings.

Flowering is inhibited either by poor growing conditions

or removal of one cotyledon and primary leaves. A more detailed analysis is necessary to examine whether this inhibition results through a similar mechanism, for example by flower bud shedding or whether different processes, such as reduced bud formation and/or shedding, are involved so that coincidentally the result is the same.

Seed and embryo growth both show a diauxic pattern. This pattern of growth was previously described by Carr and Skene (1961) for seed growth of the cultivar Hawkesbury Wonder of Phaseolus vulgaris and more recently Walbot et al.(1972) also described it for seeds of cultivar Taylor's Horticultural of the same species and for seeds of Phaseolus coccineus. Some important metabolic changes during seed development of P. vulgaris cv. Mont D'Or Golden Butter must be occurring about day 25 after anthesis, since at that time growth rate is very reduced only to increase again about 5 days later. It is also at this age that seeds are competent to germinate and the first trifoliate leaf primordium is initiated. Extensive differentiation of the primary leaves has occurred shortly before this stage is reached. Data of Oliker et al. (1978) for increase in seed dry weight of P. vulgaris cultivar Tenderette showed the lag phase to exist between 29 and 32 days after anthesis.

The observation made by Haskell et al. (1962) for embryos of Acer saccharinum and by Gregory and Romberger (1972) in Picea abies seedlings that the shoot apex becomes visibly organised before the initiation of the cotyledons does not seem to be true for Phaseolus vulgaris, since at day 10 from anthesis the

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apical dome is barely noticeable and the development of cotyledons is already well advanced.

The hypothesis that more complex leaves need a longer period of meristematic growth for development (Allsopp, 1954), supported by studies made by Hageman (mentioned in Allsopp, 1967) with Adiantum in which the primordia of the primary leaves are not only smaller but develop into mature tissue within a few days as opposed to the several months required by adult leaves, is also supported by our results. The primordia of the entire primary leaves are initiated at day 12 and at day 20 the major features of leaf shape have been established. The primordium of the first trifoliate leaf is initiated at about day 30 of embryo development and its trifoliate shape cannot be visualized before 48 hours of imbibition of the mature seed.

Another aspect thought to be important for a complex leaf to be initiated is the size of the apex (Allsopp, 1965; 1967). Our data show that at the time of the initiation of the primordia of the primary leaves the apex is substantially smaller than at the time of the initiation of the trifoliate leaf primordium. However more detailed studies are necessary to conclude whether these features are related.

Seeds are capable of germinating before maturation. This ability is attained at about day 25 after anthesis and from this age onwards there is no difference between mature and immature seeds with respect to germination. Germination capacity of immature embryos of soybean cultured in vitro has been shown to increase during the process of maturation and this was related to endogenous levels of ABA (Quebedeaux et al., 1976). Slightly

different results were found in *P. vulgaris* cv. Taylor's Horticultural. Immature embryos cultured in vitro are able to germinate at a very early stage of embryo development, although this ability is suppressed during the normal embryogeny (Walbot et al., 1972). Morris (1978) found similar results for cultivar Masterpiece of the same species and associated this variation with changes in endogenous levels of a growth inhibitor with properties similar to abscisic acid.

CHAPTER IV

EFFECTS OF INHIBITORS OF NUCLEIC ACID AND PROTEIN SYNTHESIS ON PRIMORDIAL INITIATION AND DEVELOPMENT

1. INTRODUCTION

During the growth of primordia cell division and cell elongation occur. It is obvious that both protein and nucleic acid synthesis must occur during these processes. What has not been shown is the involvement of these processes in the generation of leaf form.

The involvement of a substance in a developmental process can be demonstrated either by addition of the substance, or by specific inhibition of its synthesis in the developing system. If the specific inhibition of nucleic acid and protein synthesis brings about a change in leaf form then it can be said that these processes are involved in the generation of form of leaves.

Specific metabolic inhibitors have been shown to interfere in processes of differentiation and development. Mention should be made to some of them as, for example, 5 - bromodeoxyuridine, which is a thymidine analogue that is incorporated into DNA in many different systems. Studies made mainly in animal systems have shown that nontoxic concentrations of BUDR inhibit differentiation or development (Holliday and Pugh, 1975). Such effects include the irreversible inhibition of the formation of myotubes and synthesis of myosin in chick embryo cells (Stockdale et al., 1964).

Other studies have shown abnormalities of development to be produced by exposing sea urchin eggs to BUDR (Gontcharoff and Mazia, 1967). In culture of mesenchyme cells of chick-limb buds chondrogenic differentiation was irreversibly inhibited when BUDR was added during the first 48 hours (Levitt and Dorfman, 1972).

Other inhibitors to be mentioned include 5 - fluorouracil and 2 - thiouracil which are also pyrimidine analogues, but are known to inhibit RNA synthesis (Mahler and Cordes, 1967). Cycloheximide is an antibiotic which has been widely used as a protein synthesis inhibitor. This was shown, for example in yeast (Fukuhara, 1965). In _Neurospora crassa_ it inhibited synthesis of ribosomal RNA (Fiala and Davis, 1965). Siegel and Sisler (1964) concluded that in _Saccharomyces pastorians_ the primary action of cycloheximide is the inhibition of protein synthesis and that other alterations in treated cells are the reflection of disrupted protein synthesis.

Much less work has been done with these substances using plant systems. Raghavan (1964) studied the effect of various purine and pyrimidine analogues on the differentiation in fern gametophytes. He observed that the analogues of RNA bases, 5-FU and 2-TU completely inhibited two dimensional differentiation in the gametophytes. Thymine analogues like 5-FUDR or 5-BUDR showed a nonspecific growth inhibition.

Working with _Marsilea_ Allsopp (1963) suggested that protein synthesis in the apex may be a major factor determining the heteroblastic leaf sequence in this fern. In fact, the development of quadrifid leaves in _Marsilea_ is delayed when

2-TU and 5-FU is added to the culture medium (White, 1963). In some cases not only the number of leaflets (heteroblastic leaf sequence) is affected but the number of leaves is also much reduced. Higher concentrations affected strongly the differentiation of mesophyll cells (White, 1966). The author relates these effects to inhibition of protein synthesis.

In higher plants very little work has been done with this group of substances, most studies examining their effects on the reproductive development. Thus Brown (1962) found significant reduction of flowering in *Arabidopsis thaliana* treated with thymidine analogues as FUDR and IUDR. 5-Fluorouracil was shown to inhibit photoperiodic induction in *Xanthium* by inhibiting the synthesis of RNA in the bud during the first part of the inductive dark period (Bonner and Zeevart, 1962). In *Pharbitis nil* the inhibition of flowering by 5-fluorouracil and 5-fluorodeoxyuridine seems to be due to suppression of DNA synthesis in the shoot apex. The inhibited process is more likely to be involved in the initiation of flower primordia rather than in the translocation of photoperiodic stimulus to the apex (Zeevart, 1962). Flowering is partly inhibited in the short-day plant *Cannabis sativa* by treatment with 2-TU (Heslop-Harrison, 1960). Besides effects on flowering other observations were made for this species by treatment of fully expanded leaves with 2-TU. Failure of differentiation in the mesophyll tissues was observed and palisade cells expanded almost isodiametrically (Heslop-Harrison, 1960; 1962). Cell division was totally inhibited in the younger leaf primordia in treated plants. Loss of apical dominance and changes in phyllotaxis were also observed

(Heslop-Harrison, 1962). Studies using labelled 2-TU showed that the substance is incorporated into RNA, suggesting the involvement of nucleic acid metabolism on the morphogenetic effects observed (Heslop-Harrison, 1960).

Very few references have been found in relation to the effect of these inhibitors on leaf development, and even fewer mentioning primordial development. Here we have been concerned with the effects of BUDR, 2-TU and cycloheximide on leaf development with special attention to the development of leaf primordia.

2. EFFECTS OF 5-BROMODEOXYURIDINE (BUDR) ON PRIMORDIAL AND FURTHER DEVELOPMENT

2.1 Effects on primordial development

This series of experiments was designed to determine whether BUDR affected primordia initiation and development and if so the most effective concentration to produce such effects. For this purpose aqueous solutions of BUDR were applied directly to the apex as described previously (Section 2.1, Chapter II). The primary leaves were removed in these cases.

Table IV. 1 shows the effects of BUDR on primordial development obtained with low volume (0.2 $\mu\ell$ per apex) and concentrations varying from 3.26×10^{-9}M to 3.26×10^{-4}M. Up to 48 hours after treatment BUDR at low volume and low concentrations did not affect either the number of primordia initiated per apex or the development of these primordia.

TABLE IV. 1. Effect of BUDR on development of leaf primordia 48 hours after treatment.

BUDR Concent. (M)	Number of Primordia	D.S. of each primordium				
		1	2	3	4	5
0	3.1	4.0	4.0	2.7	0.1	0
3.26×10^{-9}	2.9	4.0	3.9	2.2	0.0	0
3.26×10^{-8}	3.0	4.0	3.9	2.7	0.0	0
3.26×10^{-7}	3.2	4.0	3.8	2.9	0.2	0
3.26×10^{-6}	3.1	4.0	4.0	2.6	0.1	0
3.26×10^{-5}	2.9	4.0	4.0	2.8	0.1	0
3.26×10^{-4}	2.9	4.0	4.0	2.6	0.1	0

When the applied volume was increased to 0.6 $\mu\ell$ per apex a significant reduction in the number of primordia initiated was found for both concentrations of BUDR used, at 72 hours after treatment but not at 48 hours (table IV. 2).

Primordial development was delayed significantly by the highest concentration of BUDR (3.26×10^{-3}M) 72 hours after treatment from the second primordium on. Although not significant this tendency to delay primordial development could already be observed 48 hours after treatment (Table IV. 2).

This result indicated that BUDR could affect primordium development and thus next experiment attempted to analyse this effect in more detail. The same volume (0.6 $\mu\ell$ per apex) as previously used with the concentration of BUDR 3.26×10^{-3}M was used. Observations were made each 6 hours up to 72 hours after treatment.

The number of primordia initiated followed a stepwise increase in the control set through all the period of time in which apices were analysed in this experiment. Initially this stepwise pattern was closely followed by the treated set. After 48 hours there was a departure, in that the primordium of T_4 was initiated in all the apices of the treated set only when the primordium of T_5 was starting to be initiated in the control set. Initiation of the previous primordia was not affected (figure IV. 1). It was only in the initiation of the primordia of T_4 and T_5 that BUDR delayed the process, although only the primordium of T_1 was present at the time of treatment.

The development of primordia from T_3 onwards, in control apices was much faster than in treated ones. The

TABLE IV. 2. Effect of different concentrations of BUDR on development of leaf primordia 48 h and 72 h after treatment.

time after treatment	BUDR concert. (M)	Number of Primordia	D.S. of each primordium				
			1	2	3	4	5
	0	3.0	4.0	4.0	2.0	0.0	0.0
48 h	3.26×10^{-4}	3.0	4.0	3.8	1.8	0.0	0.0
	3.26×10^{-3}	2.4	4.0	3.2	0.6	0.0	0.0
	0	4.5	4.0	4.0	3.6	2.8	0.6
72 h	3.26×10^{-4}	4.0*	4.0	4.0	3.7	2.5	0.0*
	3.26×10^{-3}	3.0*	3.8	3.4*	1.7*	0.9*	0.0*

* - significant at 5% level

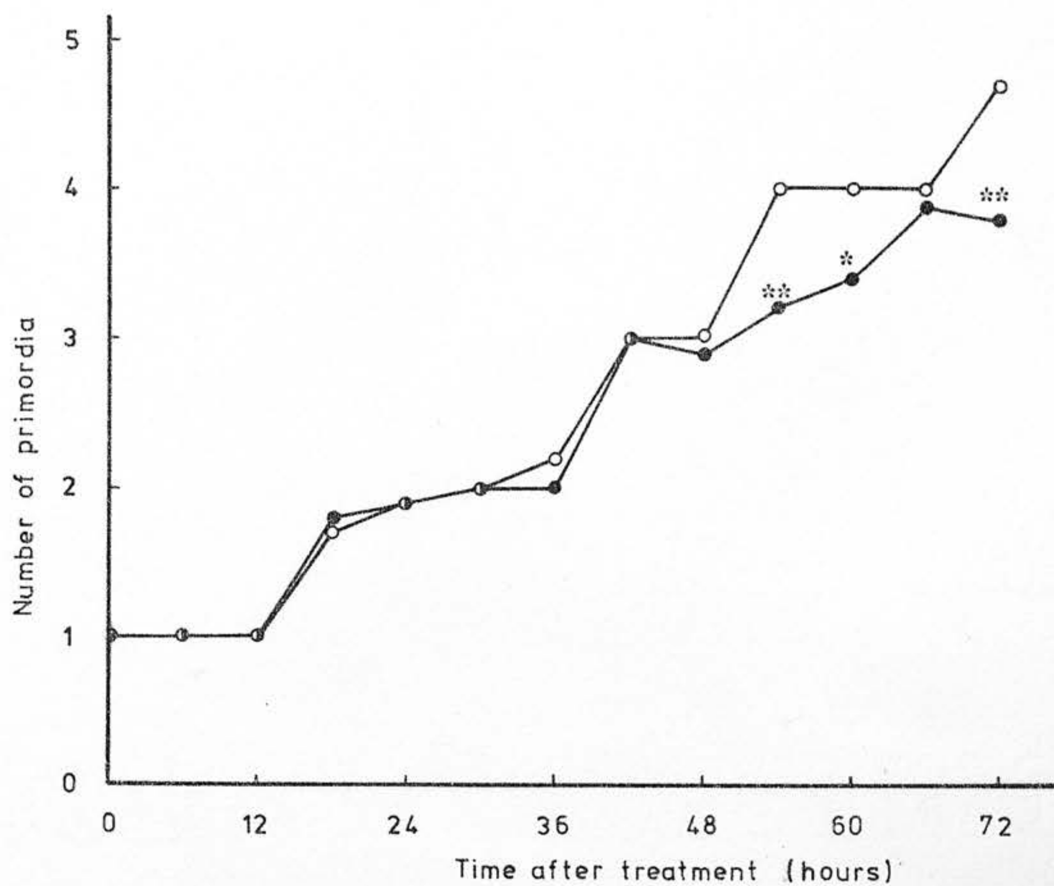


Figure IV. 1. Effect of BUDR on the number of primordia.
o - control ● - BUDR
* - significant at 5% level
** - significant at 1% level

development of primordia of T_1 and T_2 was not significantly affected, although there was some delay in the development of T_2 in treated apices between 30 and 48 hours, when compared with the control apices (figure IV. 2).

Although BUDR modified primordial development at the apex it did not prevent the formation of trifoliate primordia for leaves T_1 and T_2 at least.

In the next experiments the volume and concentration of BUDR were increased to examine whether effects on leaf form could be demonstrated. For this purpose two different treatments were made. In the first a droplet of 2.0 $\mu\ell$ of BUDR 10^{-3}M was applied twice on the base of the primary leaves, which were left intact. The first application was done 24 hours from planting and the second 24 hours later. Observations were made 48 and 72 hours after the first application (experiment I). The second treatment consisted in applying even larger volume of BUDR in a single application of 5 $\mu\ell$ at 10^{-3}M to the base of the primary leaves. Observations were made 72 hours after treatment (experiment II).

The same sort of effect was observed in these experiments as previously. 72 hours after the first application the number of primordia was reduced and the development of these primordia was significantly delayed when treated apices were compared to control ones (table IV. 3 - experiment I). In experiment II (table IV. 3) these results were confirmed. Although the amount of BUDR applied was greatly increased its effect was not markedly different and transition to developmental stage 4, characteristic of the trifoliate primordium still occurred (table IV. 3 - experiment I and II).

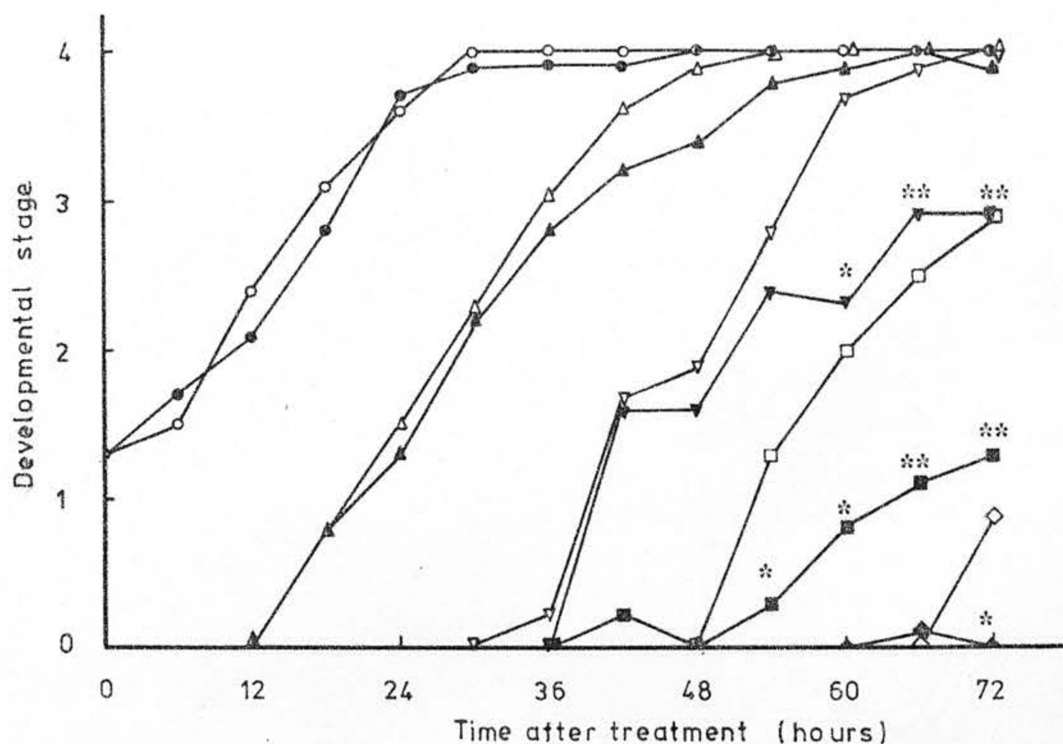


Figure IV. 2. Effect of BUDR on primordial development.
o - first trifoliate leaf primordium
Δ - second trifoliate leaf primordium
∇ - third trifoliate leaf primordium
□ - fourth trifoliate leaf primordium
◇ - fifth trifoliate leaf primordium
open symbols - control
closed symbols - BUDR
* - significant at 5% level
** - significant at 1% level

TABLE IV. 3. Effect of BUDR 10^{-3}M on apical development.

Treatment	Number of Primordia	D.S. of each primordium				
		1	2	3	4	5
Experiment I : 2 applications of 2.0 $\mu\ell$ each						
48 hours after treatment						
H_2O	2.6	4.0	2.6	0.6	0.0	0.0
BUDR	2.5	3.9	2.5	0.5	0.0	0.0
72 hours after treatment						
H_2O	4.0	4.0	4.0	3.6	2.0	0.0
BUDR	3.0*	4.0	3.7	2.1*	0.1**	0.0
Experiment II : 1 application of 5.0 $\mu\ell$						
72 hours after treatment						
H_2O	4.0	4.0	4.0	3.4	1.6	0.0
BUDR	3.0*	4.0	3.4*	2.2*	0.0*	0.0

* - significant at 5% level

** - significant at 1% level

An important point to examine was if the delay in primordial development caused by BUDR was only one aspect of a generalised growth inhibition or if it was a specific effect on leaf development.

To answer this question an experiment was designed where the seedling apex of seeds imbibed for 24 h received a droplet of 5.0 $\mu\ell$ of BUDR 10^{-3}M at the base of the primary leaves, as in the previous experiment. After 72 hours the seedlings were harvested and dry weight of the primary leaves, the stem-root axis and the one cotyledon left, was determined (table IV. 4).

Although no statistically significant differences were found a tendency towards slower growth was noticed for both the stem-root axis and the primary leaves of treated seedlings. The cotyledon dry weight was higher in treated seedlings, representing about 84% of the total dry weight, than in control where it was only about 77% of the total dry weight (table IV. 4).

The results suggest that BUDR treatment may lead to a slower translocation of material from cotyledons to support the seedling growth. This effect seems to result in a general growth inhibition rather than a specific effect on leaf development.

2.2 Effects on further development

Experiments were designed to examine whether the effect of BUDR on seedling growth would persist in adult plants and whether any long term effects on leaf shape would result. For this purpose two different treatments were made. The first

TABLE IV. 4. Dry weight (mg) of different parts of the seedling and cotyledon 72 h after treatment with BUDR

	H_2O	BUDR
Cotyledon	82.3 ± 9.7[1]	93.7 ± 16.4
Stem-roots	22.5 ± 5.1	16.4 ± 2.5
Primary leaves	2.3 ± 1.1	1.9 ± 0.3

(1) -95% confidence limits are quoted

consisted of three applications of 1.0 μℓ each of BUDR 10^{-3}M to the base of the primary leaves. In the second BUDR was applied in lanolin, at the concentrations of 200 and 2000 ppm, so that it would remain in contact with the apex for a longer period of time. Leaf area and shape were examined 20 days after planting. In this case leaf shape was determined by comparing the leaves with the leaf types outlined in figure II. 4 (Section 2.4.2, Chapter II).

BUDR applied as a solution did not affect either area or shape of the primary leaves or of the first trifoliate leaf. When applied in lanolin leaf area was not significantly affected (as determined by calculating the confidence limits) either in the primary leaves or in the first trifoliate leaf. Shape was slightly modified in the primary leaves of a low percentage of the treated plants. This modification occurred at the base of the primary leaves which in control (type 1) developed 2 lobes. These two lobes were missing in the modified leaves (type 2). The shape of the first trifoliate leaf was not affected, all the plants developing normally (table IV. 5).

3. EFFECTS OF 2-THIOURACIL (2-TU) ON LEAF DEVELOPMENT

2-TU was found by Heslop-Harrison (1960, 1962) to have large effects on differentiation of leaf tissues in Cannabis sativa. Experiments were designed to examine the effects of this substance on leaf development in Phaseolus vulgaris either at the primordial stage or when fully expanded. Two experiments were done. In the first of these to examine effects at

TABLE IV. 5. Effect of BUDR on leaf area and shape.

		leaf area (cm^2)	leaf type[1] (%) 1	2	7
BUDR applied as a solution 10^{-3}M : 3 $\mu\ell$ per apex					
P	H_2O	65.0	100	-	-
	BUDR	58.0	100	-	-
T_1	H_2O	89.5	-	-	100
	BUDR	92.0	-	-	100
BUDR applied in lanolin : 200 and 2000 ppm					
	lan.	49.5	100	-	-
P	BUDR 200	43.2	67	33	-
	BUDR 2000	41.5	75	25	-
	lan.	71.0	-	-	100
T_1	BUDR 200	68.2	-	-	100
	BUDR 2000	55.2	-	-	100

(1) - see figure II. 4 (Section 2.4.2 - Chapter II).

primordial initiation a droplet of 5 $\mu\ell$ of 2-TU 10^{-3}M was applied to the base of the primary leaves in imbibed seeds. Observation of apices was made 24, 48 and 72 hours after treatment. The second experiment was designed to determine effects of 2-TU on leaf area and shape of the primary leaves and of the first trifoliate leaf. 2-TU at the concentration of 10^{-3}M was applied three times as a droplet of 2.0 $\mu\ell$ per apex, each time. The first application was made after 24 hours of imbibition and subsequently at 24 hour intervals. 24 h after the last application the seedlings were transferred to pots with John Innes Compost and kept in the glasshouse for 20 days. Leaf shape was determined by comparing the leaves with the leaf types outlined in figure II. 4 (Section 2.4.2, Chapter II).

Treatment with 2-TU delayed significantly but did not stop completely primordial initiation up to 48 hours after treatment. After that time the difference between the number of primordia produced in control and treated apices was not significant (table IV. 6).

As shown by the developmental stage data, the development of these primordia was greatly inhibited by 2-TU during the first 48 hours after treatment, but subsequently the observed differences were not significant (table IV. 6).

In spite of the inhibition of primordial development this substance, as with BUDR, did not prevent the formation of a trifoliate primordium.

Leaf area of fully expanded leaves was substantially reduced in both the primary leaves and the first trifoliate leaf following treatment with 2-TU. The shape of the primary leaves was modified to type 2 by 2-TU in about two-thirds of

TABLE IV. 6. Effect of 2-TU on development of leaf primordia different periods after treatment.

Time after treatment		Number of Primordia	D.S. of each primordium				
			1	2	3	4	5
24 h	H_2O	1.9	3.9	1.1	0.0	0.0	0.0
	2-TU	1.0*	2.9*	0.0*	0.0	0.0	0.0
48 h	H_2O	3.0	4.0	3.3	1.8	0.0	0.0
	2-TU	2.3*	3.5*	1.7*	0.5*	0.0	0.0
72 h	H_2O	4.0	4.0	4.0	3.0	1.6	0.0
	2-TU	3.5	3.9	3.5	2.4	0.9	0.0

* - significant at 5% level

the treated plants. In all treated plants the first trifoliate leaf developed normally (table IV. 7).

The main difference between leaves type 1 and type 2 (see figure II. 4 in Section 2.4.2, Chapter II) is at the base. Type 1 develops two lobes at the base which are not formed in type 2. The data suggest that the modification of the primary leaf shape caused by 2-TU occurred at the site of application.

4. EFFECT OF CYCLOHEXIMIDE ON LEAF DEVELOPMENT

Protein synthesis in the apex has been mentioned as a major factor in the determinaton of heteroblastic leaf sequence in *Marsilea* (Allsopp, 1963), although no detailed work has been recorded showing the importance of protein synthesis in the early primordial development. Experiments were designed to examine how application of cycloheximide, a known inhibitor of protein synthesis, would affect primordial initiation and early development.

Cycloheximide was applied as a solution at 4 different concentrations directly to the seedling axis apex after the removal of the primary leaves. A volume of 1.0 $\mu\ell$ was applied per apex. The results are shown in table IV. 8.

Concentrations of cycloheximide as low as 10 ppm were effective in delaying the initiation of leaf primordia 24 and 48 h after treatment. After longer periods apices overcame the effect of treatment and there was no significant difference in the number of primordia initiated in treated or control apices. Higher concentrations were able to maintain this inhibition, at

TABLE IV. 7. Effect of 2-TU on leaf shape and area.

	Treatment	leaf area (cm^2)	leaf type[1] (%)		
			1	2	7
P	H20	65.0	100	-	-
	2-TU	31.3**	33	67	-
T_1	H20	89.5	-	-	100
	2-TU	47.8	-	-	100

** - significant at 1% level

(1) - see figure II. 4. (Section 2.4.2 - Chapter II).

TABLE IV. 8. Effect of different concentrations of cycloheximide on development of leaf primordia.

Time after treatment	Cycloheximide concent. (ppm)	Number of Primordia	D.S. of each primordium				
			1	2	3	4	5
24 h	0	1.5	2.3	0.5	0.0	0.0	0.0
	10	1.0*	1.6	0.0*	0.0	0.0	0.0
	25	1.0*	1.7	0.0*	0.0	0.0	0.0
	50	1.0*	1.5*	0.0*	0.0	0.0	0.0
	100	1.0*	1.3*	0.0*	0.0	0.0	0.0
48 h	0	2.6	4.0	3.1	0.7	0.0	0.0
	10	2.1*	3.4*	1.9*	0.2*	0.0	0.0
	25	1.7*	3.3**	1.0**	0.0*	0.0	0.0
	50	1.5*	2.6**	0.6**	0.0*	0.0	0.0
	100	1.7*	3.0**	1.2**	0.0*	0.0	0.0
72 h	0	3.7	4.0	4.0	3.0	1.0	0.0
	10	3.4	4.0	4.0	3.0	0.6	0.0
	25	3.2*	4.0	3.5*	1.6**	0.2*	0.0
	50	3.0*	4.0	3.4*	1.3**	0.0*	0.0
	100	2.2*	3.8	2.9**	0.5**	0.0*	0.0
96 h	0	4.9	4.0	4.0	3.9	2.7	1.2
	25	3.9**	4.0	4.0	3.1	1.3*	0.0**
	50	3.9**	4.0	4.0	2.9*	1.2*	0.0**
120 h	0	4.8	4.0	4.0	4.0	3.7	1.5
	25	4.0**	4.0	4.0	4.0	2.9	0.0**
	50	4.0**	4.0	4.0	3.9	2.6	0.0**

* - significant at 5% level

** - significant at 1% level

least, up to 120 hours after treatment (table IV. 8).

Cycloheximide at concentration of 10 ppm was most effective 48 h after treatment, when all the primordia initiated had their development inhibited. Earlier than this only the last-formed primordium was inhibited and later than 48 hours cycloheximide at this concentration was ineffective in delaying development of primordia. Higher concentrations were effective for longer periods of time, especially considering development of the later-formed primordia. The first primordium overcame the effect of treatment as soon as 72 h after treatment and the second 24 hours later. 120 hours after treatment only the development of the fifth primordium was significantly inhibited (table IV. 8).

The data show that although the primordia developed at a slower rate in treated than in control apices, they eventually overcame the effect of treatment. The duration of the effect increased with the increase of concentration of cycloheximide, at least, within the range used in this experiment. But apices treated even with the highest concentration did not fail to develop trifoliate primordia.

5. DISCUSSION

When dealing with small systems such as a seedling apex, chemical treatments involve small amounts of substances and the problem of penetration must always be taken into account. Of the amount of substance supplied to the apex we do not have any idea as to how much penetrated into the cells. However it is

clear that a certain amount did penetrate, since, all of the substances applied were effective, on some aspect of development; BUDR 54 h after treatment and both 2-TU and cycloheximide 24 h after application.

The inhibitory effect of BUDR on leaf development is ephemeral, since it was not carried through to the adult leaves, and it seems to be one aspect of a general growth inhibition in initial development of the seedling.

2-TU had an inhibitory effect somewhat different from that found in apices treated with BUDR, since, 2-TU inhibits primordial development during the first 48 hours from treatment whereas BUDR inhibits the process after this period. This difference can be interpreted as indicating either different rates of penetration or different rates of incorporation of the different analogues into nucleic acids.

Considering adult plants the shape of the trifoliate leaves was not affected by 2-TU and three points can be advanced to explain this lack of effect. Firstly, it could be explained by an absence of translocation, since the shape of primary leaves is modified on the site of 2-TU application. However this is not very likely to occur, since the initial development of trifoliate leaf primordia is affected by treatment. Secondly, it might be that the incorporation of the analogue inactivates the RNA and this happens after a period of time which is early enough for normal trifoliate leaves to be formed, but is not early enough for primary leaves not to be affected. This seems to be quite possible since the trifoliate leaf primordia soon overcome the inhibitory effect of 2-TU. However the effect on the primary

leaves can be noticed much later in development. Thirdly, it is possible that 2-TU does not have the potential to affect shape of trifoliate leaves, and the delay caused on leaf primordia early development is a reflection of a general growth inhibition.

The effect of cycloheximide was noticeable very soon after its application. In this respect it was very similar to 2-TU, but the inhibition by cycloheximide persisted for a much longer period of time. Increase in the concentration of cycloheximide caused an increase of the inhibitory effect on both initiation and early development of leaf primordia, at least up to 100 ppm. This was not what happened with BUDR, where after the concentration had reached an effective level its increase did not modify its effect.

All the substances used showed inhibition on the initiation and early development of trifoliate leaf primordia, but in all cases sooner or later a trifoliate primordium was formed. The data obtained suggest that inhibition of nucleic acid or protein synthesis will affect both primordia initiation and early leaf development but these effects are more likely to be on general growth of the seedling than specific to mechanisms concerned with primordia initiation and growth.

CHAPTER V

EFFECTS OF GIBBERELLIC ACID (GA_3) ON LEAF DEVELOPMENT

1. INTRODUCTION

In plants which exibit heteroblastic development the role of gibberellins on leaf morphogenesis has been interpreted as maintaining the juvenile condition. Allsopp (1962) denies a specific function of a juvenility hormone for gibberellins, although, at least in Marsilea they have the ability to maintain a low level of plant differentiation by preventing the development of land forms.

GA_3 prevents the development of the adult lobed leaf in Centaurea solstitialis in plants developed in sterile culture (Feldman and Cutter, 1970a) and in excised leaf primordia (Feldman and Cutter, 1970b).

Sparks and Postlethwait (1967) showed a more striking effect of GA_3 in Cyanopsis tetragonoloba. Here the first leaves formed are simple and after development of a certain number of these, trifoliate leaves are developed. Environmental conditions such as short days regulate the number of simple leaves formed. GA_3, as short days, was found to favour the formation of simple leaves.

The ability of GA_3 to maintain the juvenile phase was also shown in Ipomoea caerulea by Njoku (1958) and the same effect was described in Hedera (Robbins, 1957; 1960; Rogler and Hackett, 1975), with the difference that in Hedera the juvenile leaf is lobed

and the adult leaf is simple. Another example showing the efficiency of GA_3 in maintaining the juvenile phase of a plant is given by Williams (1961) for Humulus lupulus, where GA_3 leads to production of the juvenile type of leaf over a prolonged period. In the palm Caryota mitis, Fisher (1976) induced the persistence of the juvenile leaf form for longer periods by spraying young plants with GA_3 solution.

Although a great amount of evidence suggests that GA_3 is able to maintain the juvenile form of leaves this is still a controversial area, since in some cases opposite results have been found. For example, in Proserpinaca palustris under conditions which favour the development of the juvenile type of leaf, plants treated with GA_3 produce adult-like leaves (Davis, 1967). In Marsilea GA_3 increased the rate of heteroblastic development, so that adult leaves were produced at an earlier node (Allsopp, 1959). A similar effect of GA_3 was found on Eucalyptus (Scurfield and Moore, 1958).

Other effects of GA_3 on leaf development have been reported. Most of this work shows GA_3 treatment to result in the development of narrow leaves. This effect was first mentioned for different species by Brian et al., (1954) and soon afterwards for many other species by Gray (1957). Later the same effect was shown by Felippe (1967) in Phaseolus where, as a result of treatment with GA_3 primary and trifoliate leaves were narrower and longer than control.

GA_3 accelerated the rate of leaf initiation and affected leaf morphology in Xanthium pennsylvanicum by inducing the development of lanceolate leaves instead of the normal

deltoid ones, with reduction of leaf area and length (Maksymowych and Maksymowych, 1973 and Maksymowych et al., 1976), although later formed leaves reverted to the normal deltoid type (Maksymowych and Maksymowych, 1973). The increase in the rate of leaf initiation was associated with enlargement of the apical dome and the reduction of leaf area was correlated to inhibition of area of epidermal cells (Maksymowych et al., 1976).

Modification in phyllotaxis was reported for *Hydrocharis* (Cutter, 1963; 1965b) and *Xanthium* (Maksymowych et al., 1976; Maksymowych and Erickson, 1977).

In sterile culture of callus of *Nicotiana tabacum* the induction of narrow or rounded leaves depended on the ratio of gibberellin to cytokinin. High ratios resulted in tall shoots with narrow leaves and in presence of low ratios rounded leaves were developed (Engelke et al., 1973). Another interesting example of gibberellins and cytokinins in the control of leaf shape was shown by Mauseth and Halperin (1975) and Mauseth (1976) working with the cactus *Opuntia polyacantha*. Leaf primordia would develop as spines if GA_3 was added to the culture medium, although the formation of photosynthetic leaves would occur in the presence of a cytokinin.

With this background in mind experiments were designed to examine the effects of GA_3 on leaf growth and development from primordium initiation to full lamina expansion. For this purpose GA_3 was applied at different stages of primordial development during seed imbibition or at different stages of the embryo development in the immature seed.

2. EFFECTS OF TREATMENT APPLIED TO MATURE SEEDS

Initial experiments examined whether application of GA_3 either directly on the apex after removal of the primary leaves, or to the base of the primary leaves, would affect the rate of primordial initiation and the subsequent rate of primordial development.

After imbibition of the seeds for 24 hours a droplet of 1.0 $\mu\ell$ of GA_3 at three different concentrations was applied on the apex of the seedling axis. Treatment was applied directly on the apex after removal of the primary leaves. Observations were made 24, 48 and 72 h after treatment.

Under the conditions of this experiment the initiation of primordia was not affected by treatment. Although the differences were not significant the data suggest that the development of the later-formed primordia was rather more rapid in treated than in control apices, especially when the highest concentrations are considered (table V. 1).

In the next experiment only the highest concentration of GA_3 (10^{-3}M) was used. A droplet of 5.0 $\mu\ell$ was applied on the base of the primary leaves which, in this case, were left intact.

Even with a much greater amount of GA_3 primordial initiation was not affected (table V. 2).

As was suggested in the previous experiment the later-formed primordia developed more rapidly in treated apices than in control. The differences are significant 72 hours after treatment (table V. 2).

The effect of GA_3 on the length of the middle leaflet

TABLE V. 1. Effect of GA_3 at different concentrations on the development of leaf primordia.

time after treatment	GA_3 concent. (M)	Number of Primordia	D.S. of each primordium				
			1	2	3	4	5
24 h	0	1.9	3.0	1.0	0.0	0.0	0.0
	10^{-5}	1.9	2.8	0.9	0.0	0.0	0.0
	10^{-4}	1.6	2.4	0.6	0.0	0.0	0.0
	10^{-3}	2.0	3.1	1.1	0.0	0.0	0.0
48 h	0	3.1	4.0	3.6	1.3	0.1	0.0
	10^{-5}	2.8	3.9	3.0	0.9	0.1	0.0
	10^{-4}	2.9	4.0	3.5	1.4	0.0	0.0
	10^{-3}	3.0	4.0	3.5	1.4	0.1	0.0
72 h	0	4.0	4.0	4.0	3.0	1.6	0.0
	10^{-5}	4.0	4.0	4.0	3.2	1.6	0.0
	10^{-4}	4.0	4.0	4.0	3.3	2.0	0.0
	10^{-3}	3.9	4.0	4.0	3.5	2.6	0.0

TABLE V. 2. Effect of GA_3 on primordial development different times after treatment.

time after treatment	treatment	Number of Primordia	D.S. of each primordium				
			1	2	3	4	5
24 h	H_2O	2.0	4.0	1.8	0.0	0.0	0.0
	GA_3	2.0	4.0	2.0	0.0	0.0	0.0
48 h	H_2O	3.2	4.0	3.5	1.9	0.2	0.0
	GA_3	3.0	4.0	3.9	2.1	0.0	0.0
72 h	H_2O	4.0	4.0	4.0	2.8	1.5	0.2
	GA_3	4.4	4.0	4.0	3.8*	2.6*	0.4

* - significant at 5% level

of the first trifoliate leaf was examined in detail for seedlings treated with 5 $\mu\ell$ of GA_3 10^{-3}M applied on the base of the primary leaves after different periods of imbibition.

GA_3 applied at 12, 24 or 48 h from planting led to an increase in length of the middle leaflet of the first trifoliate leaf detectable by 96 hours from planting and was still noticeable 24 hours later (table V. 3). This effect of GA_3 treatment in the early growth of the leaflet was independent of the stage when treatment was applied, since no difference was found in the sensitivity of apices, at least, in the three different stages used in this experiment (table V. 3).

The higher values found for control sets 96 hours from planting are probably due to the fact that this was an independent experiment, using a different seed batch.

Analysis of fully developed trifoliate leaves showed a somewhat different result of GA_3 treatment. Application of GA_3 in lanolin at the concentration of 200 ppm on the base of primary leaves 12 hours from planting led to a significant increase in both final length and area of fully expanded trifoliate leaves; but the effect of treatment at 24 and 48 h from planting was not significant (table V. 4).

When primary leaves were considered, the effect of GA_3 was not the same. Treatment 12 or 24 h from planting did not affect significantly the final length or area of the primary leaves. But when applied 48 h from planting GA_3 treatment significantly reduced the length and area of these leaves (table V. 4).

Leaf shape of fully expanded leaves was expressed quantitatively using the method previously described (see

TABLE V. 3. Effect of GA_3 on the length of the middle leaflet of T_1 (mm).

Age (hours)	treatment	Time of treatment		
		12h	24h	48h
72	H_2O	0.16 ± 0.09 [1]	0.31 ± 0.05	0.31 ± 0.03
	GA_3	0.20 ± 0.13	0.33 ± 0.17	0.29 ± 0.09
96	H_2O	0.63 ± 0.06	0.67 ± 0.09	0.70 ± 0.07
	GA_3	0.83 ± 0.06	0.89 ± 0.07	0.99 ± 0.08
120	H_2O	0.52 ± 0.08	0.50 ± 0.06	0.51 ± 0.13
	GA_3	0.80 ± 0.01	0.83 ± 0.05	0.87 ± 0.08

(1) 95% confidence limits are quoted

TABLE V. 4. Effect of GA_3, applied in lanolin, on the length and area of fully expanded leaves.

time of imbibition		treatment	leaf area (cm^2)	leaf length (cm)
12 h	P	lanolin	62.3 ± 8.7 (1)	11.5 ± 1.0
		GA_3	60.5 ± 10.3	11.7 ± 1.8
	T_1	lanolin	44.0 ± 18.5	7.3 ± 1.7
		GA_3	83.0 ± 20.1	11.5 ± 1.3
24 h	P	lanolin	55.8 ± 13.7	11.3 ± 1.5
		GA_3	46.6 ± 6.0	10.3 ± 0.8
	T_1	lanolin	43.0 ± 31.2	7.9 ± 2.8
		GA_3	57.8 ± 18.5	10.4 ± 1.2
48 h	P	lanolin	64.0 ± 4.0	12.0 ± 0.5
		GA_3	50.8 ± 9.1	10.6 ± 0.8
	T_1	lanolin	50.2 ± 29.2	7.8 ± 2.7
		GA_3	44.8 ± 16.4	8.7 ± 1.6

(1) 95% confidence limits are quoted

section 2.4.1, Chapter II). In this method leaf width is determined at 1/10 intervals along the lamina and corrected in relation to leaf length. The ratio of these values for treated and control leaves gives an estimate of the effect of treatment on shape, values less than 1 indicating a narrowing of the treated leaf.

Treatment with GA_3 led to a narrowing of the middle leaflet irrespective of the time of application, although this effect appeared to be slightly greater when GA_3 was applied 12 or 24 h than at 48 h from planting. Considering development along the length of the lamina, apical region was slightly more affected by GA_3 than the basal region of the leaflet (figure V. 1).

The shape of primary leaves was not significantly modified by GA_3 treatment as it is shown in figure V. 2.

3. EFFECT OF TREATMENT APPLIED TO IMMATURE SEEDS

Experiments described in the previous section showed some minor changes in leaf shape caused by GA_3. In some cases the effect of GA_3 depended on the stage of the apex at the time of treatment. It is then conceivable that, going back earlier during embryo development and leaf initiation, major effects of GA_3 on leaf shape might be detected. For this purpose experiments were designed to examine the effect of this growth substance on the development of leaves either at the primordial stage or when fully expanded, when applied to the developing pod at different stages. Treatments were given to pods shortly after the primordia of the primary leaves have been initiated

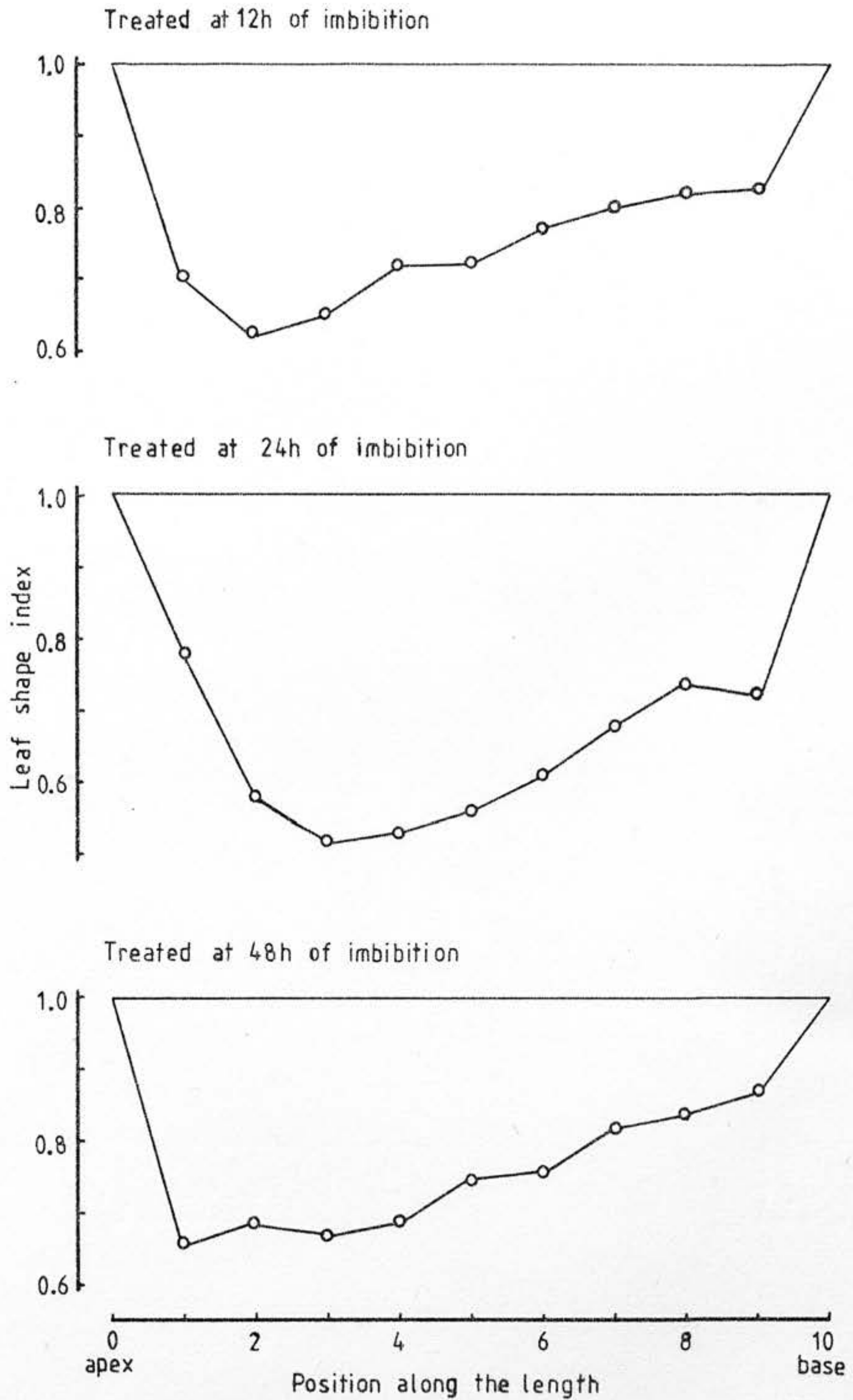


Figure V. 1. Effect of GA_3 on the final shape of the middle leaflet of the first trifoliate leaf when treatment was applied after different periods of imbibition.

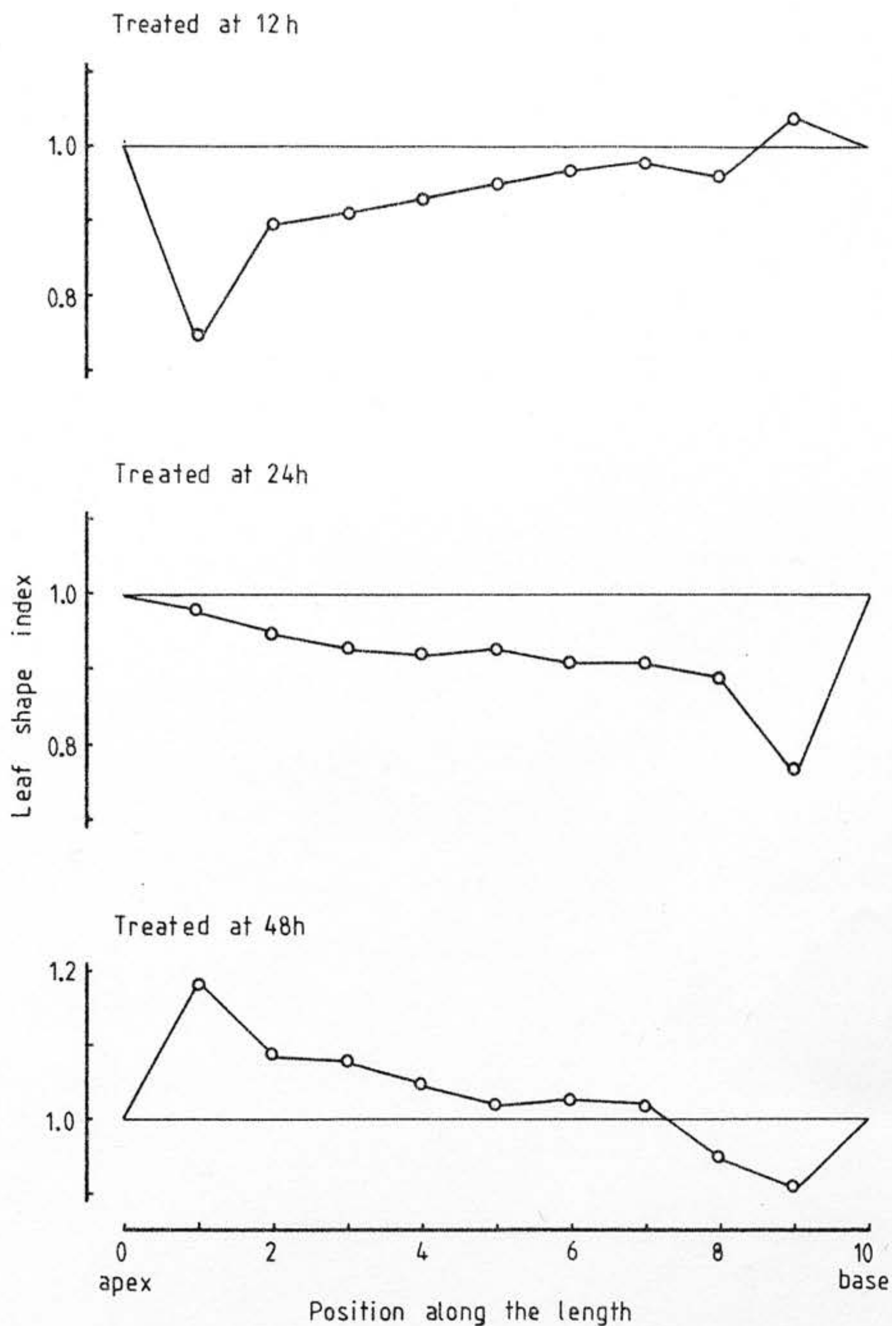


Figure V. 2. Effect of GA_3 on the final shape of the primary leaves when treatment was applied after different periods of imbibition.

(15 days from anthesis) or before the initiation of the primordium of the first trifoliate leaf (20 days after anthesis). GA_3 at the concentration of $10^{-3}M$, was applied as a solution brushed on the developing pod wall after gentle abrasion with sandpaper. An average volume of 0.4 mℓ per pod was applied. This was equivalent to about 140 µg of GA_3 per pod.

Neither the number nor the final size of the seeds produced under these conditions was significantly affected by treatment (table V. 5).

GA_3 applied to the immature seed had no effect in modifying either the early primordial development of seedlings which were examined 72 hours from planting (table V. 6) or in the further development of the primary leaves and the first trifoliate leaf examined 20 days after planting (table V. 7).

The shape of these leaves was examined and the results are shown in figure V. 3. For this parameter, as in the others examined, no effect of GA_3 treatment could be detected.

GA_3 applied to immature seeds at 15 or 20 days from anthesis did not modify either the growth of the seeds or the leaf development of the plants into which these seeds developed. Even the minor effects as narrowing of leaflets caused by GA_3 when applied to mature seeds could not be detected as a result of treatment of immature seeds.

4. DISCUSSION

The increase on the rate of leaf initiation described in Xanthium as an effect of GA_3 (Maksymowych and Maksymowych,

TABLE V. 5. Effect of GA_3 on seed development when applied to the developing pod.

pod age (days)	treatment	seed final weight (g)	number of seeds per pod
15	H_2O	0.34 ± 0.01 (1)	5.8 ± 0.4
	GA_3	0.36 ± 0.02	5.6 ± 0.8
20	H_2O	0.31 ± 0.01	5.9 ± 0.7
	GA_3	0.34 ± 0.02	5.1 ± 0.7

(1) 95% confidence limits are quoted

TABLE V. 6. Effect of GA_3 on primordial development when applied on the developing pod.

pod age (days)	treatment	Number of Primordia	D.S. of each primordium				
			1	2	3	4	5
15	H_2O	4.0	4.0	4.0	2.8	1.1	0.0
	GA_3	4.0	4.0	4.0	2.5	1.0	0.0
20	H_2O	4.0	4.0	4.0	2.2	1.0	0.0
	GA_3	4.0	4.0	4.0	2.7	1.1	0.0

TABLE V. 7. Effect of GA_3 on leaf length and area when applied to the developing pod.

pod age (days)	treatment		leaf area (cm^2)	leaf length (cm)
15 days	P	H_2O	83.8 ± 4.8[1]	11.5 ± 1.4
		GA_3	80.6 ± 6.3	12.7 ± 0.6
	T_1	H_2O	40.7 ± 16.5	5.9 ± 3.0
		GA_3	27.2 ± 9.7	6.6 ± 1.1
20 days	P	H_2O	84.6 ± 5.8	13.2 ± 0.4
		GA_3	87.1 ± 6.7	13.5 ± 0.6
	T_1	H_2O	41.8 ± 12.0	8.5 ± 1.0
		GA_3	40.8 ± 10.5	8.2 ± 1.1

(1) 95% confidence limits are quoted

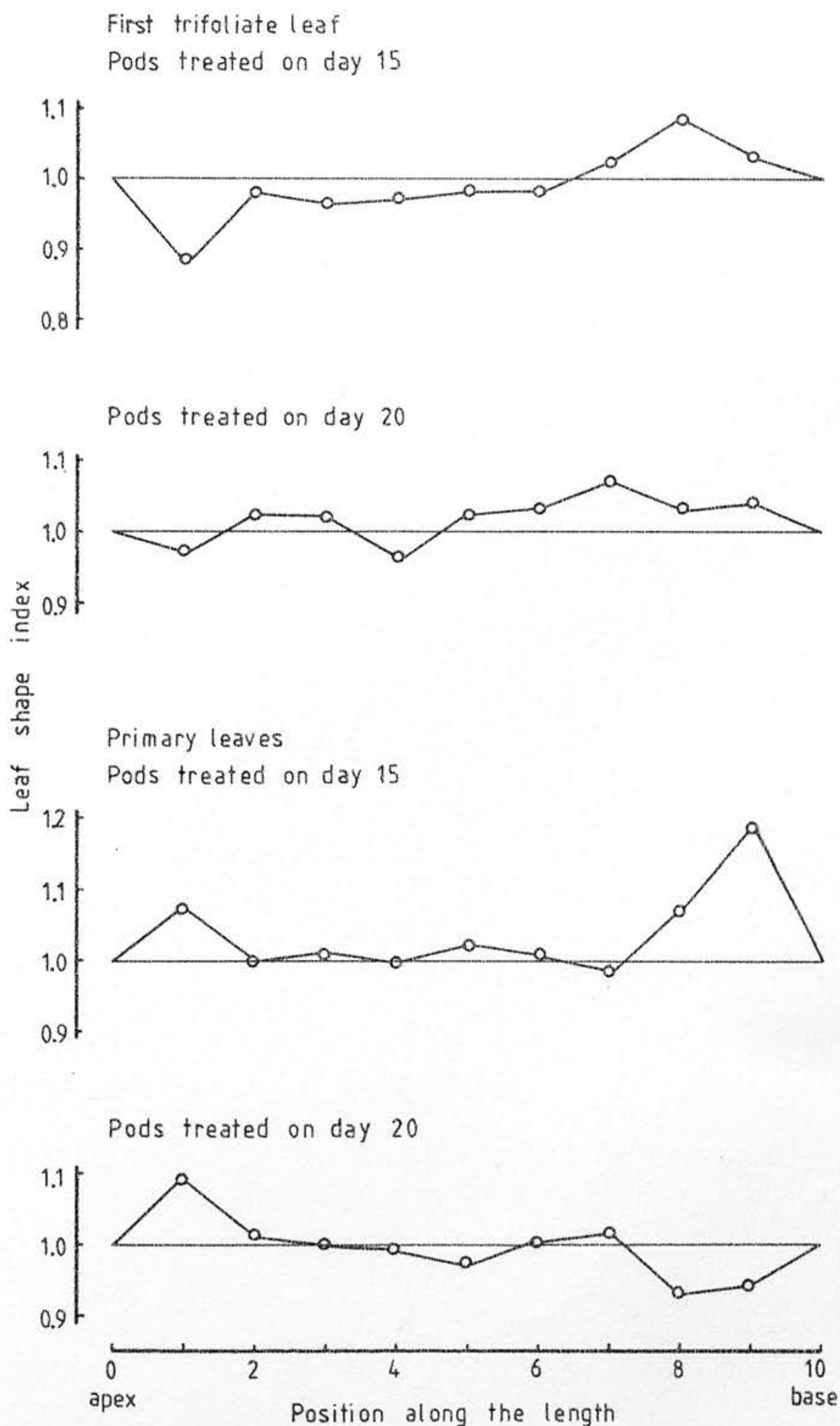


Figure V. 3. Effect of GA_3 on the final shape of the primary leaves and the middle leaflet of of the first trifoliate leaf when immature pods were treated.

1973; Maksymowych et al., 1976) was not found in Phaseolus vulgaris either when GA_3 was applied to immature seeds or to imbibed mature seeds.

It is clear, from the results, that the effects of GA_3 on the growth of the middle leaflet of the first trifoliate leaf are dependent on the stage when the treatment is applied. Although ephemeral effects on the initial increase in length of this leaflet were shown as results of treatments at 24 and 48 hours from planting, the effect on length persisted up to the full development of this leaf only when treatment was applied as early as 12 hours from planting. It seems that as the primordium ages it becomes less sensitive to GA_3 treatment.

Treatment applied at very early stages of primordial development resulted in the development of a narrower adult leaf. The same effect was shown for this species when GA_3 was applied as late as 7 days from planting (Felippe, 1967). However there were quantitative differences in the response. Treatment applied at early stages had more or less uniformily large effects over the whole length of the blade. If a difference is to be considered we would say that there is a tendency for larger effects on the apical region than in the basal region of the leaflet. Treatment on day 7, as shown by Felippe (1967) led to a greatest effect on the leaf base which is morphologically the youngest part of the leaf. This suggests that as the primordium increases in length, older regions become less sensitive to GA_3 treatment.

The data suggest that ageing reduces sensitivity to GA_3 either when the growth of the whole leaflet is considered

after treatment at different stages or considering growth along the primordium length whereby apical and basal sensitivity differs.

If primary leaves are considered as a characteristic of a juvenile phase in Phaseolus vulgaris GA_3 does not show any effect on maintaining this phase for longer periods, since in no case was the formation of trifoliate leaves prevented or delayed.

Treatment applied to immature seeds was totally ineffective in modifying leaf shape. As the substance was applied to the pod wall it is possible that it was not effective because did not reach the embryo. The applied GA_3 might have been involved in the formation of a complex by binding to other substance in the cotyledons. Mature bean seeds have been shown to have very low gibberellin levels (Skene and Carr, 1961; Dale, 1969; Skene, 1970). This low level in dry seeds has usually been suggested to be the result of the formation of bound-gibberellins (Dale and Felippe, 1968; Dale, 1969; Barendse et al., 1968), since immature bean seeds can show at certain phases of development high levels of endogenous gibberellins (Skene and Carr, 1961; Skene, 1970).

Another possibility to explain the inefficiency of GA_3 applied to the immature pod is that it might have not even reached the seed, being metabolised before in the tissues of the pod.

CHAPTER VI

EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D), OTHER AUXINS AND 2,3,5-TRIIODOBENZOIC ACID (TIBA) ON LEAF DEVELOPMENT

1. INTRODUCTION

Studies on the effects of 2,4-D on plant development span a period of more than 30 years and during this time much work has been done with this synthetic auxin, although the details of the nature of its effects are far from being understood. Many effects of 2,4-D altering leaf development have been described. Leaf shape and form can be drastically affected by 2,4-D and this can be caused either in the leaves whose primordia were present at the time of treatment (Watson, 1948; Eames, 1951), or for leaf primordia which formed after treatment (Gifford, 1953; McIlrath and Ergle, 1953).

The degree of response seems to depend mainly on the species and the stage of development when treatment is applied. Application of 2,4-D to an expanding leaf does not affect its form but treatment at an early stage can change a compound leaf to a simple one as has been shown for *Erodium cicutarium* (Linser and Kirchner, mentioned in Andel et al., 1976). 2,4-D caused fusion of leaflets of leaves of *Vicia faba* which were initiated after treatment, leaves already present at the apex in the primordial stage by the time of treatment developed narrower and smaller but otherwise normal compound leaves (Geranmayeh, in Andel et al., 1976). Soma (1968) added 2,4-D in lanolin to the apex of imbibed seeds of the cv.

Masterpiece of _P. vulgaris_ at different stages during the development of the first and second trifoliate leaf primordia. He found the material to be maximally sensitive to treatment at about the time when lateral leaflet and stipular primordia were being initiated for the first trifoliate leaf and shortly before the appearance of the next leaf primordia. Treatment caused the generation of bizarre leaf shapes and forms varying from unifoliate to double or multiple. Formation of bizarre leaf shapes was observed in cotton when 2,4-D was applied to fully expanded cotyledons (Gifford, 1953).

Furuya and Osaki (1955) showed the formation of unifoliate leaves at the first trifoliate leaf position when dry seeds of _Phaseolus vulgaris_ received prolonged treatment with vapour of the methyl esther of 2,4-D. Upper nodes of these plants developed leaves with more than 3 leaflets.

Gross modifications have been described also for the primary leaves of _P. vulgaris_ as a result of 2,4-D treatment. Great reduction in size with development of a thick and veiny blade and the formation of a dark green ruffled band around most of the blade margin have been frequently observed (Eames, 1951; Furuya and Osaki, 1955; Soma, 1968).

The most common types of abnormalities induced in leaves by 2,4-D were reviewed by Andel et al. (1976). Fusion of leaflets of compound leaves is one of the modifications caused by 2,4-D. This was clearly shown in leaves of _Erodium cicutarium_ sprayed with 2,4-D at different stages of development. The degree of fusion was dependent on the stage when treatment was applied (Linser and Kirchner, in Andel et al., 1976). The

development of funnel-shaped or tubular leaves may arise from the fusion, at an early stage, of two or more primordia. Development of funnel leaflets were found in P. vulgaris when very young seedlings were treated with 2,4-D (Furuya, 1958); in lupin the same phenomenon was shown by Steveninck (1956).

Changes in phyllotaxis have frequently been described as one of the results of treatment with 2,4-D. Soma (1968) reported the change from distichous phyllotaxis to almost opposite in treated plants of P. vulgaris.

Some of the modifications described as a result of treatment with 2,4-D were also found by treating plants with the anti-auxin TIBA.

The most severe effect of TIBA on the apical development was the total inhibition of leaf primordia initiation, described for tomato plants, although flower primordia were developed. This resulted in a stem without leaves bearing an inflorescence at the tip (Gorter, 1951; Bedesem, 1958). Wardlaw (1953) studied in detail these tomato shoot apices and concluded that treatment caused cessation of growth in the most distal cells of the apex but did not inhibit totally the growth in the subjacent regions.

Heteroblastic leaf segmentation was found to be affected by TIBA treatment in Marsilea. In control cultures the typical adult leaves with 4 segments were developed whilst in cultures with TIBA an average of 1.5 segments per leaf was obtained (Allsopp and Rao, 1968).

TIBA was found to cause modifications in phyllotaxis in some species. Cutter (1965b) showed this effect of TIBA in

Hydrocharis. Dramatic effects in phyllotaxis were described by Schwabe (1971) in Chrysanthemum as a result of TIBA treatment. In this species the first TIBA effect is mentioned to be on the shape of the apex. Some narrowing in diameter of the incipient stem below the bare apical dome and a greater vertical separation of the primordial insertions are described.

Other auxins are more rarely found to affect leaf development so drastically. This does not mean that they are ineffective, since Ball (1944) in his classical studies with Tropaeolum found IAA application to result in formation of leaf primordia out of their normal positions, this leading to production of multiple leaves. This effect of IAA altering normal position of leaf primordia was recently confirmed for Lupinus albus (Varnell and Vasil, 1978).

Since so many abnormalities could be caused in leaves, especially by 2,4-D, in such a range of species, this substance seems to be an interesting tool to attempt to answer some important questions on the mechanisms of control of leaf form in the dimorphic species Phaseolus vulgaris. Some of the questions we will attempt to answer deal with the involvement of auxins in the determination of leaf form. If they are involved, the questions arise as to how and when their effects are first shown.

Experiments were designed to examine the effect of 2,4-D on leaf development at both primordial and adult stage. Apical development was examined in the SEM and in some more detail in histological preparations. The effect of other auxins and TIBA was examined on primordial development.

2. EFFECTS OF 2,4-D ON LEAF DEVELOPMENT AT DIFFERENT STAGES

2.1 Effects of 2,4-D on adult leaves

Most of the effects found to be caused by 2,4-D seems to vary with the species and the stage when treatment is applied. Striking effects of 2,4-D applied directly to the seedling apex at different developmental stages were shown by Soma (1968) working with *Phaseolus vulgaris* cv. Masterpiece. This first set of experiments was designed to examine whether cv. Mont D'Or Golden Butter of *P. vulgaris* would also respond to 2,4-D treatment.

2,4-D was applied in lanolin at the concentration of 2000 ppm on the base of the primary leaves after seed imbibition for different periods of time so that treatment was applied at different apical developmental stages.

In this experiment only qualitative observations were made and they are summarized on table VI. 1.

There were no substantial differences in the response of apices treated at different developmental stages, even when treatment was applied after 48 h of seed imbibition. By that time the lateral leaflets of the primordium of the first trifoliate leaf had already been initiated (MDS = 4.0) (table VI. 1).

Changes in phyllotaxis were frequent on the third node of treated plants. First and second trifoliate leaves developed opposite to each other in many plants, instead of the normal distichous disposition.

TABLE VI. 1. Effect of 2,4-D applied in lanolin to mature seedlings after different periods of imbibition (+ = presence of the character in all plants; – = absence of character in all plants; ± = presence of the character in some plants and absence in others).

	Time of imbibition (h)			
	18	24	36	48
M.D.S.	1.2	1.2	2.5	4.0
P modified	+	+	+	+
T_1	entire	entire	entire	entire
T_2 with more than 3 leaflets	±	–	+	+
Phyllotaxis changed in the 3rd. node	+	+	+	±

The modifications in shape described by Furuya and Osaki (1955) for primary leaves of plants of Phaseolus vulgaris cv. Masterpiece treated with 2,4-D were also found for the cv. Mont D'Or Golden Butter either when seedlings were treated as early as 18 h or as late as 48 h from planting (table VI. 1).

At the first trifoliate leaf position entire leaves were formed in all cases, even when treatment was applied after the lateral leaflets had been initiated. These leaves often developed a long and narrow blade, so that leaf area was apparently reduced.

Leaves at the second trifoliate leaf position tended to develop more than three leaflets especially when treated at later developmental stages (36 and 48 h from planting) (table VI. 1).

In the next experiment an attempt at quantifying the effects of 2,4-D on leaf development was made. Leaf shape was determined by comparison with the leaf types shown in figure II. 4 (Section 2.4.2 - Chapter II). Leaf area was determined for both primary and the first trifoliate leaves. 2,4-D was applied as a solution at concentration of 10^{-3}M on the base of the primary leaves. Three applications of 2.0 $\mu\ell$ each were made at 24 h intervals, the first being 24 h from planting.

Confirming the previous results, the normal trifoliate leaf (type 7) found in the control plants was not observed in any of the treated plants (table VI. 2). Shape of primary leaves was also affected by treatment. It is interesting to note that treatment led to abnormalities such that some similar shaped leaves were found in both primary and

TABLE VI. 2. Effect of 2,4-D on leaf area and shape when applied to the seedling apex after 24 h imbibition of the mature seed.

	treatment	leaf area (cm^2)	leaf type[1] (%) 1	2	3	4	5	7
P	H_2O	65.0 ± 8.6[2]	100	-	-	-	-	-
	2,4-D	15.3 ± 4.1	-	6	29	65	-	-
T_1	H_2O	89.5 ± 61.5	-	-	-	-	-	100
	2,4-D	13.9 ± 6.0	11	-	22	-	67	-

(1) See Figure II. 4. (Section 2.4.2, Chapter II)

(2) 95% confidence limits are quoted

trifoliate leaf positions (table VI. 2).

As was suggested in the previous experiment leaf area was significantly reduced by 2,4-D treatment in both primary leaves and the first trifoliate leaf (table VI. 2).

When seedlings were treated with 5 $\mu\ell$ of 2,4-D 10^{-3}M at 24 h of imbibition and the dry weight of different parts was determined 72 hours after treatment the effect of 2,4-D inhibiting leaf growth was already present. The primary leaves represented 2.1% of the total weight in control but it was less than 0.8% in treated seedlings. The growth of the stem-roots axis was not significantly affected at this stage (table VI. 3).

Treatment of mature seedlings led to effects on leaf development which were similar independently of the apical developmental stage at the time of treatment. Perhaps a difference in sensitivity of the apex to 2,4-D treatment could be detected going back earlier during the process of embryo development. For this purpose seeds 30, 35, 40 and 45 days after anthesis were harvested and left to dry and were then treated as described for mature seeds (Section 2.5.1 - Chapter II). In this experiment a droplet of 2.0 $\mu\ell$ of 2,4-D 10^{-3}M was applied 3 times on the base of the intact primary leaves after 24 hours of imbibition.

Independent of the stage of maturity of the seed leaf area was substantially reduced in both primary and first trifoliate leaves (table VI. 4). Leaf shape and form was also markedly affected by 2,4-D in both cases. Here again, similar leaves were found in primary and trifoliate positions. The

TABLE VI. 3. Effect of 2,4-D on seedling dry weight (mg) at 72 h after treatment.

	H_2O	2,4-D
cotyledon	82.3 ± 9.7[(1)]	95.9 ± 18.8
stem-root axis	22.5 ± 5.1	18.0 ± 4.3
primary leaves	2.3 ± 1.1	0.9 ± 0.2

(1) 95% confidence limits are quoted

TABLE VI. 4. Effect of 2,4-D on leaf area when applied directly to the immature seeds.

	seeds age (days)	leaf area (cm^2)	
		control	2,4-D
Primary leaves	30	41.6 ± 9.0[1]	15.2 ± 3.0
	35	70.8 ± 7.8	8.0 ± 8.4
	40	77.1 ± 9.5	8.7 ± 3.8
	45	81.7 ± 10.7	8.5 ± 1.8
First trifoliate leaf	30	221.5 ± 53.3	59.0 ± 75.3
	35	243.0 ± 17.6	19.0 ± 14.5
	40	246.3 ± 32.8	23.0 ± 15.4
	45	230.0 ± 49.3	16.0 ± 11.1

(1) 95% confidence limits are quoted

percentage of leaves of each different type was not found to vary with the age of the seed at the time of treatment (table VI. 5).

In the next experiment an attempt to treat even younger seeds was made. However as seeds younger than 25 days do not germinate (see Section 4, Chapter III) a different approach was tried in this case. Here 2,4-D 10^{-3}M was applied to the developing pod by brushing the pod wall with the solution after gentle abrasion. An average volume of 0.4 ml was applied to pods 15 and 20 days after anthesis. This was equivalent to 88 ug of 2,4-D per pod. After treatment the seeds were allowed to mature in the pod. They were harvested at the time of normal pod maturation (45 days after anthesis). Seeds were then planted in John Innes compost and examined 20 days later.

For pods treated either 20 or 15 days after anthesis there was evidence that 2,4-D reached the embryo and affected leaf development. When pods 15 days post-anthesis were treated the area of both primary and first trifoliate leaf was reduced, but only area of primary leaves was significantly affected when pods 20 days post-anthesis were treated (table VI. 6).

Leaf shape in this experiment was determined quantitatively by the method described in Chapter II, Section 2.4.1. The form of the first trifoliate leaf was not modified in any case and in all plants the normal trifoliate leaf was developed (figure VI. 1). Shape of primary leaves was significantly affected when pods were treated at day 20 post-anthesis but not in younger pods (figure VI. 2).

TABLE VI. 5. Effect of 2,4-D on leaf shape when applied directly to immature seeds.

seeds age (days)	treatment	leaf type[1] (%)					
		1	2	4	5	6	7
	Primary leaves (P)						
30	H_2O	100					
	2,4-D			100			
35	H_2O	100					
	2,4-D			100			
40	H_2O	100					
	2,4-D		29	71			
45	H_2O	100					
	2,4-D			100			
	First trifoliate leaf (T_1)						
30	H_2O						100
	2,4-D		50			50	
35	H_2O						100
	2,4-D					100	
40	H_2O						100
	2,4-D			20	20	60	
45	H_2O						100
	2,4-D					100	

(1) See Figure II. 4. (Section 2.4.2, Chapter II).

TABLE VI. 6. Effect of 2,4-D on leaf area (cm^2) when immature pods were treated.

	treatment	Time of treatment : days after anthesis	
		15	20
P	H_2O	83.8 ± 4.8 [1]	84.6 ± 5.8
	2,4-D	58.0 ± 4.8	63.0 ± 4.5
T_1	H_2O	40.7 ± 12.2	41.8 ± 12.0
	2,4-D	20.6 ± 7.4	38.8 ± 11.0

(1) 95% confidence limits are quoted

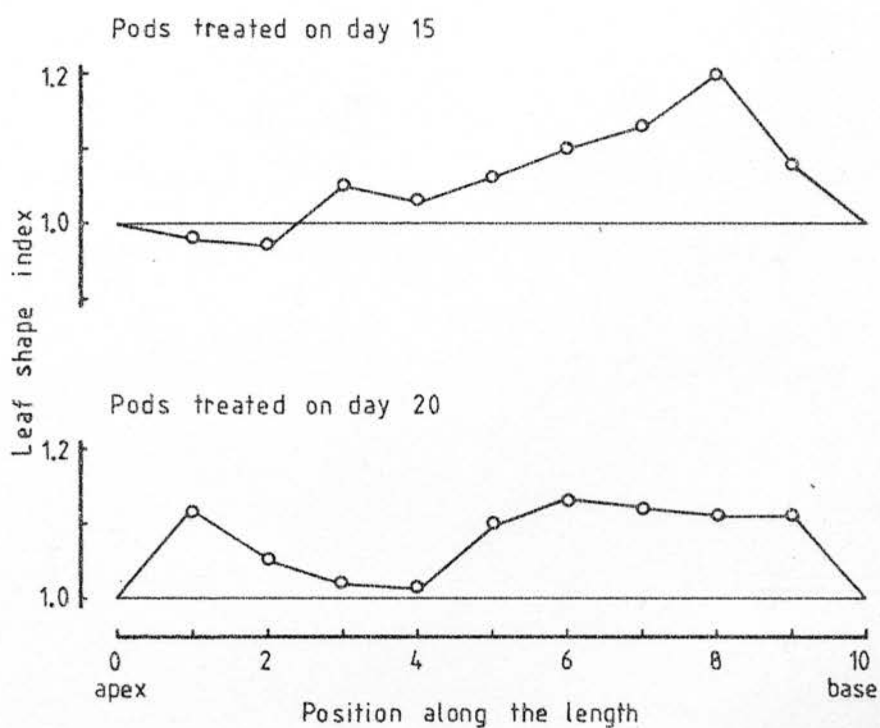


Figure VI. 1. Effect of 2,4-D on the final shape of the middle leaflet of the first trifoliate leaf when immature pods were treated.

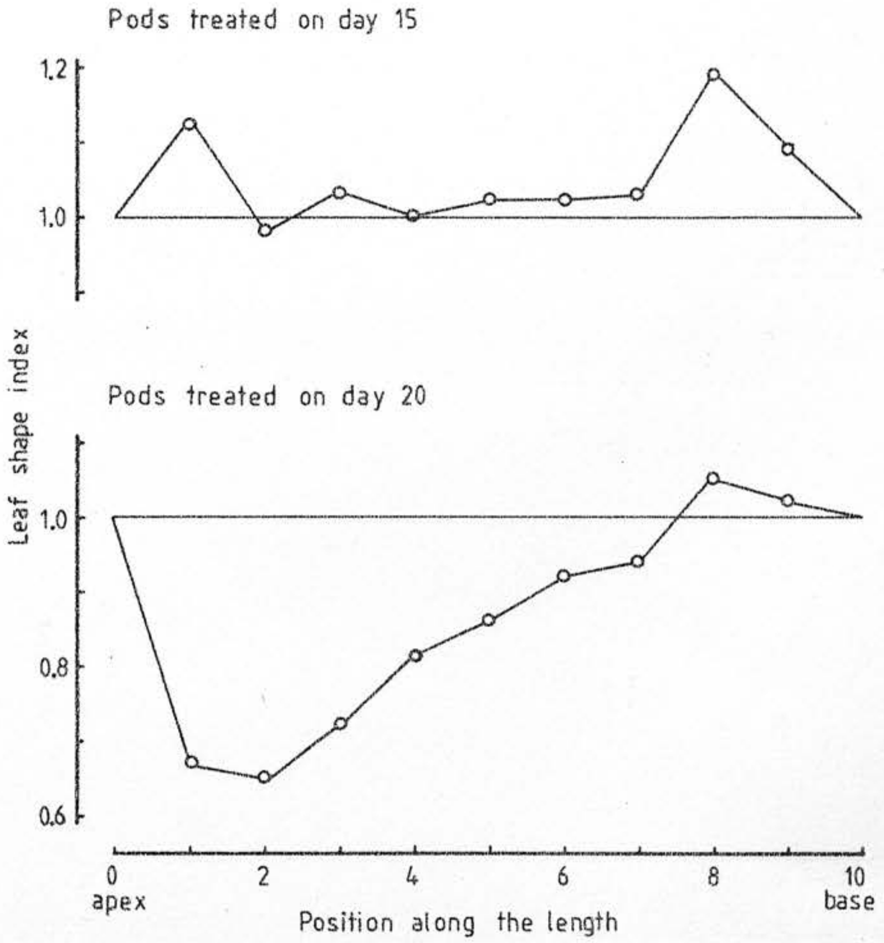


Figure VI. 2. Effect of 2,4-D on the final shape of the primary leaves when immature pods were treated.

It seems that different degrees of sensitivity can be distinguished in the immature seeds during embryo development, this sensitivity being hardly modified after the stage in which the seed, although immature, is able to germinate.

2.2 Effect of 2,4-D on primordial development

In the previous section evidence was presented that 2,4-D can modify leaf shape and form in *Phaseolus vulgaris* cv. Mont D'Or Golden Butter. When immature seeds were treated at very early stages such as 15 or 20 days after anthesis there were certain differences in 2,4-D effects. In the next experiment apical development was examined after 72 h of imbibition of seeds from pods which had been treated at day 15 or 20 after anthesis.

Rate of leaf initiation was reduced and the early development of trifoliate leaf primordia of seedlings from seeds treated at day 15 post-anthesis was delayed. Neither primordial initiation nor early development was affected when pods were treated at day 20 from anthesis (table VI. 7).

These data together with those from previously mentioned (2.1) suggest that this effect of 2,4-D on early leaf development is more likely to be a reflection or a consequence of a general growth inhibition than a specific effect on leaf development, since the growth in area of adult leaves was affected but not leaf form or shape in either primary or first trifoliate leaves.

When mature seeds were treated the shape and form of adult leaves was modified. The next set of experiments

TABLE VI. 7. Effect of 2,4-D on apical development of seedlings when immature pods were treated. Results 72 h from planting.

pod age (days)	treatment	Number of primordia	D.S. of each primordium				
			1	2	3	4	5
15	H_2O	4.0	4.0	4.0	2.8	1.1	0.0
	2,4-D	2.7**	3.8	2.3**	1.0**	0.0**	0.0
20	H_2O	4.0	4.0	4.0	2.2	1.0	0.0
	2,4-D	3.9	4.0	4.0	1.9	0.9	0.0

** - Significant at 1% level

attempted to determine when this effect is first shown during primordial development. For this purpose 2,4-D was applied directly to the seedling apex after seed imbibition for 24 hours. Four different concentrations were used with an applied volume of 1.0 $\mu\ell$ per apex. Primordial development was examined 72 hours after treatment.

The volume of 1.0 $\mu\ell$ at the concentration range from 10^{-6}M to 10^{-3}M was ineffective in modifying either the rate of leaf initiation or the early development of these primordia (table VI. 8).

In the next experiments the amount of 2,4-D applied was increased. Three different treatments were made. In the first, three applications of 2,4-D 10^{-3}M of 2.0 $\mu\ell$ each at 24 hours intervals, commencing 24 h from planting. In the second a single application of 2 $\mu\ell$ 2,4-D 10^{-3}M was made on the apex. The third treatment was the same as the second but a larger volume was applied. In this case 5.0 $\mu\ell$ were supplied per apex after 24 h of seed imbibition. Treatment was always made on the base of the intact primary leaves. For the three experiments similar results were obtained and only one set of data is presented. The treated apices were examined at intervals after treatment, using the SEM.

Figures VI. 3 to VI. 6 show the developmental sequence for control and treated apices. Comparison shows there to be little or no visible effect of treatment within 24 hours (Figure VI. 3 - A , B). However by 36 hours treated apices began to show formation of a sub-apical annulus extending equatorially from the flanks of the primordium which was still recognisable

TABLE VI. 8. Effect of 2,4-D on primordial development 72 hours after treatment.

2,4-D conc. (M)	Number of primordia	D.S. of each primordium				
		1	2	3	4	5
0	4.0	4.0	4.0	3.0	1.4	0.0
10^{-6}	4.0	4.0	4.0	2.8	1.2	0.0
10^{-5}	3.8	4.0	4.0	3.0	1.3	0.0
10^{-4}	3.7	4.0	3.9	2.7	0.7	0.0
10^{-3}	3.8	4.0	3.8	2.7	1.1	0.0

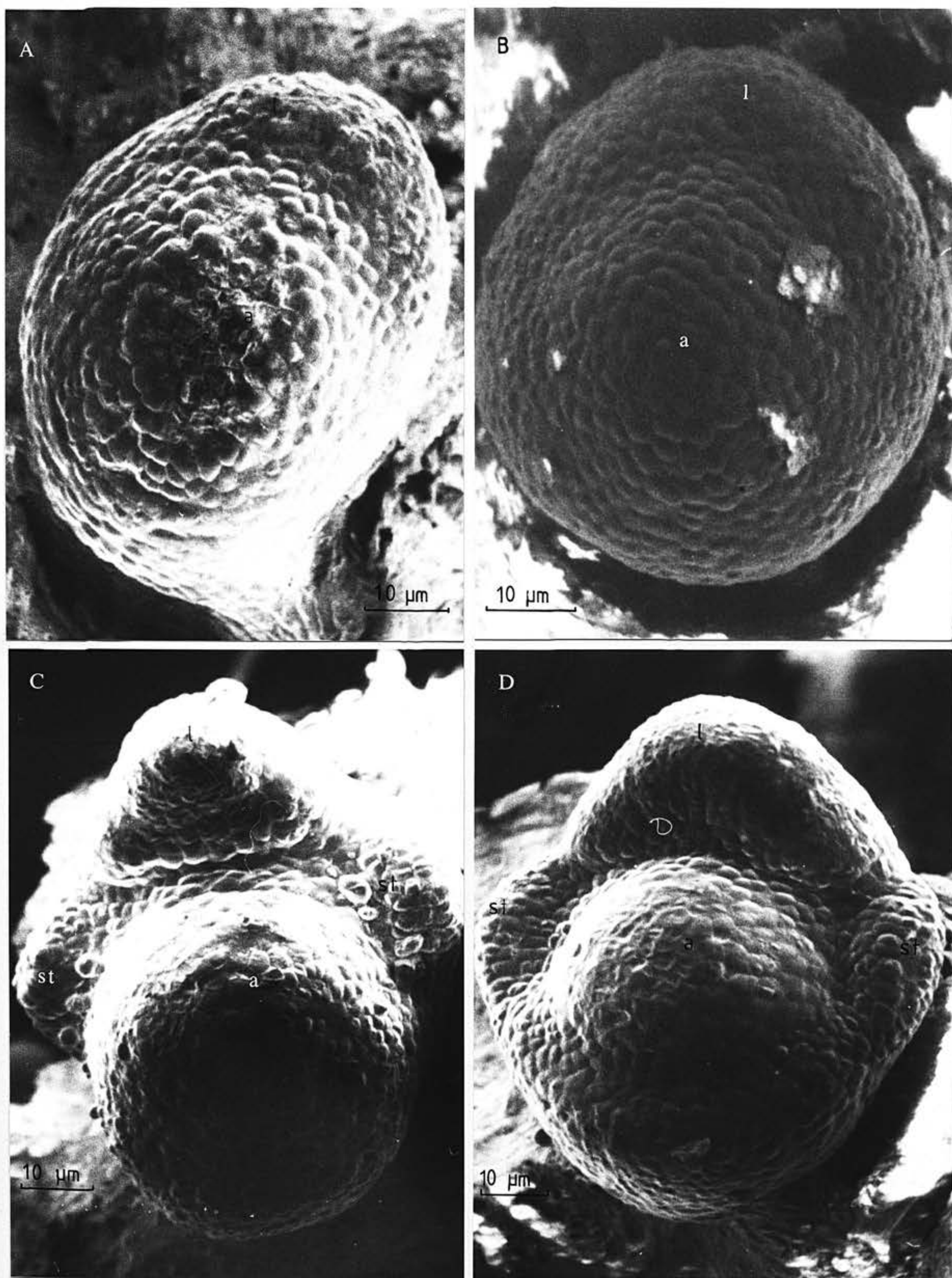
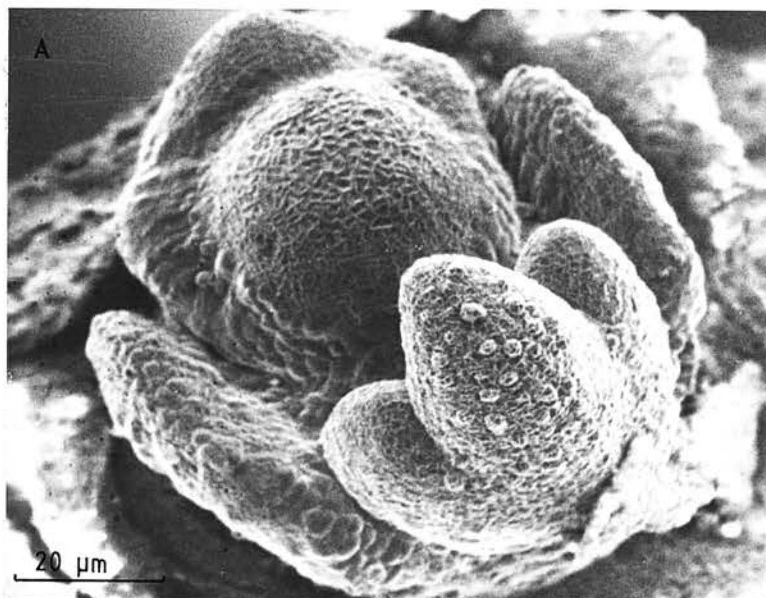


Figure VI. 3. Effect of 2,4-D on primordial development 24h (A and B) and 36h (C and D) after treatment.
A and C - control B and C - treated
a - apical dome st - stipule l - leaf primordium
For further description see text.



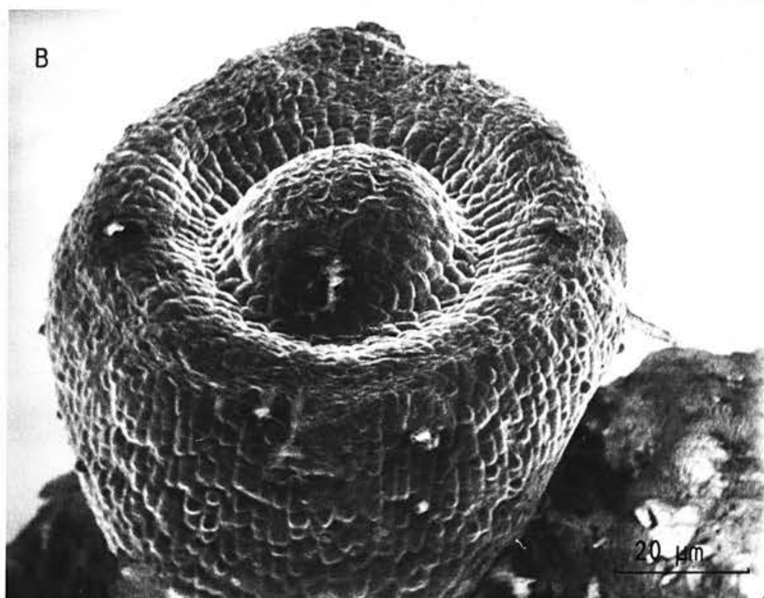
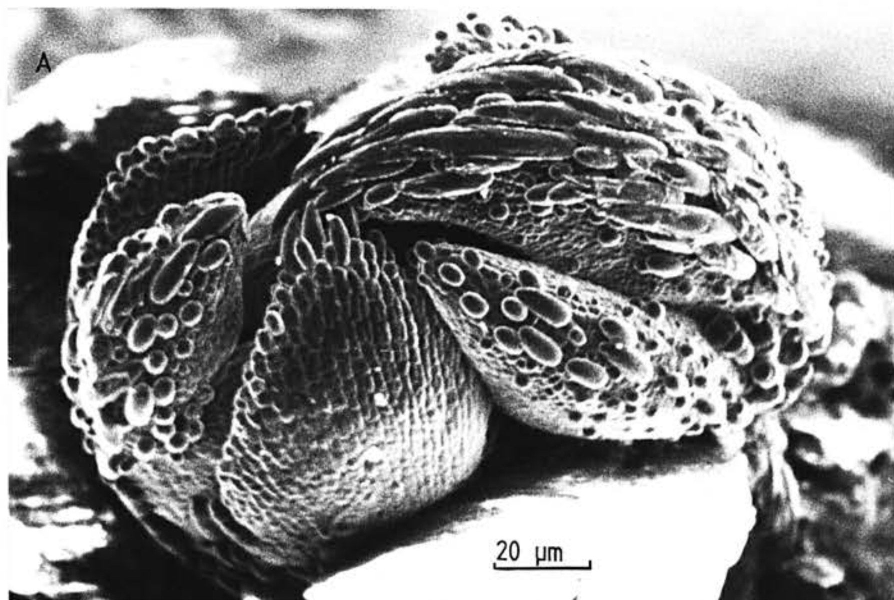


Figure VI. 4. Effect of 2,4-D on apical development 48h after treatment.
A - control B - treated
For further description see text.



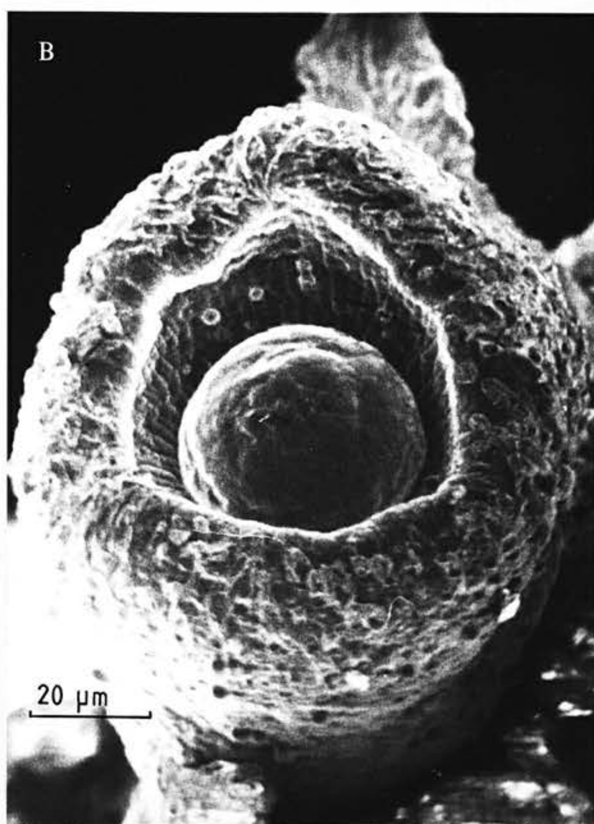
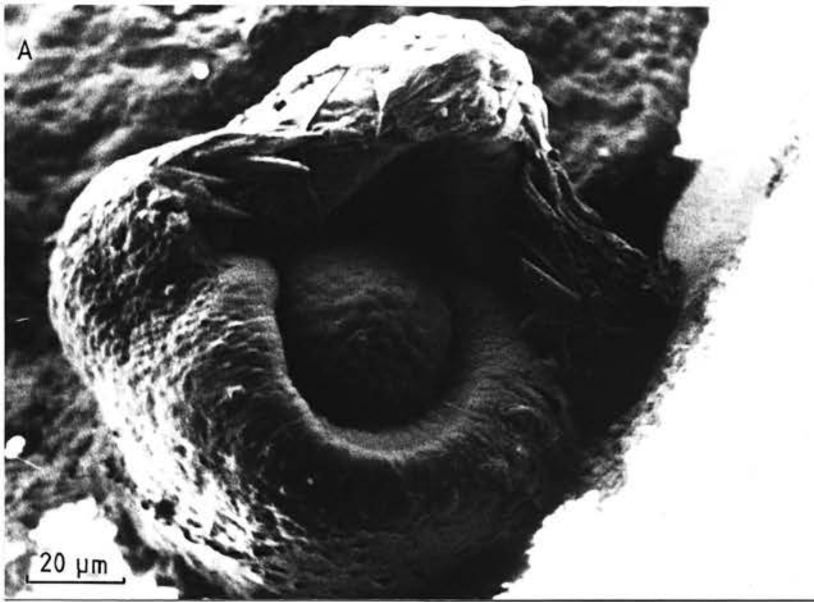


Figure VI. 5. Effect of 2,4-D on apical development 72h after treatment.
A - control B - treated
For further description see text.



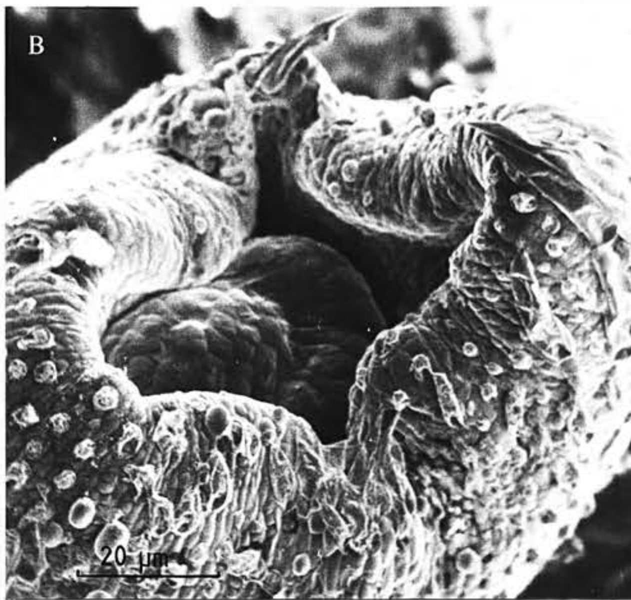


Figure VI. 6. Effect of 2,4-D on apical development 96h (A) and 120 (B) after treatment. For further description see text.

although not tripartite (figure VI. 3 - D).

The exact course of development in treated apices varied in detail but usually by 48 h after treatment ring formation was complete (figure VI. 4 - B) and sometimes it presented an eccentricity on one side corresponding to the primordium; this was in contrast to the control which reached stage 4 and beyond in the example shown here (Figure VI. 4 - A). After a further 24 h proliferation upwards of the ring led to a cup shaped structure with the apical dome being submerged below the rim of the cup (Figure VI. 5 - B). 24 Hours later, usually, the initial of the unifoliate blade which replaces the normal trifoliate was apparent (Figure VI. 6 - A). Occasionally a second ring developed inside the first (Figure VI. 6 - B). Examination of the intact apex without further dissection was not possible in control apices beyond 72 hours after treatment (Figure VI. 5 - A) and in treated apices 120 h after treatment (Figure VI. 6 - B) because of the extensive growth of hairs.

From the results presented it is clear that when seedlings are treated after 24 h of imbibition the 2,4-D effect is noticeable 36 hours later and well established 48 h after treatment. The next experiment was designed to examine whether early primordial development is differently affected when 2,4-D is applied to the apex at different developmental stages. For this purpose seeds were imbibed for 36 h and 48 h. A droplet of 5 $\mu\ell$ of 2,4-D 10^{-3}M was applied to the apex of the seedlings. Apices were examined after different periods of time using the SEM.

Results of this experiment are illustrated in figures VI. 7 to VI. 9. When seedlings were treated at 36 h from planting the 2,4-D effect started to show 12 hours later. No signs of lateral leaflets were visible in treated apices (Figure VI. 7 - A), although they were apparent in control apices by this time. The effect of treatment was still only slightly visible 12 hours later (at 60 hours from planting.) There was no difference at this stage, between apices treated at 36 or 48 hours from planting (Figure VI. 7 - B, C). Differences between the two treatments became apparent 72 hours from planting.

In seedlings treated at 36 h it was noticed by that time that the first trifoliate leaf primordium was involved in the ring formation (Figure VI. 8 -A). This ring was clearly visible 24 hours later (Figure VI. 9 - A). When treatment was applied to seeds imbibed for 48 h after another 24 hours it was noticeable that the first trifoliate leaf primordium was not going to be included in the ring structure which, by this time, was being initiated (Figure VI. 8 - B). This fact was very clear in seedlings 96 h from planting. The sub-apical annulus developed but it did not include the first trifoliate leaf primordium. However the shape of this primordium was modified, since, the lateral leaflets failed to develop (Figure VI. 9 - B).

The results presented do not show marked difference between the effect caused by 2,4-D treatment on primordial development of seedlings treated either at 24 h or 36 h from planting. In both cases a sub-apical annulus was developed which included the first trifoliate leaf primordium.

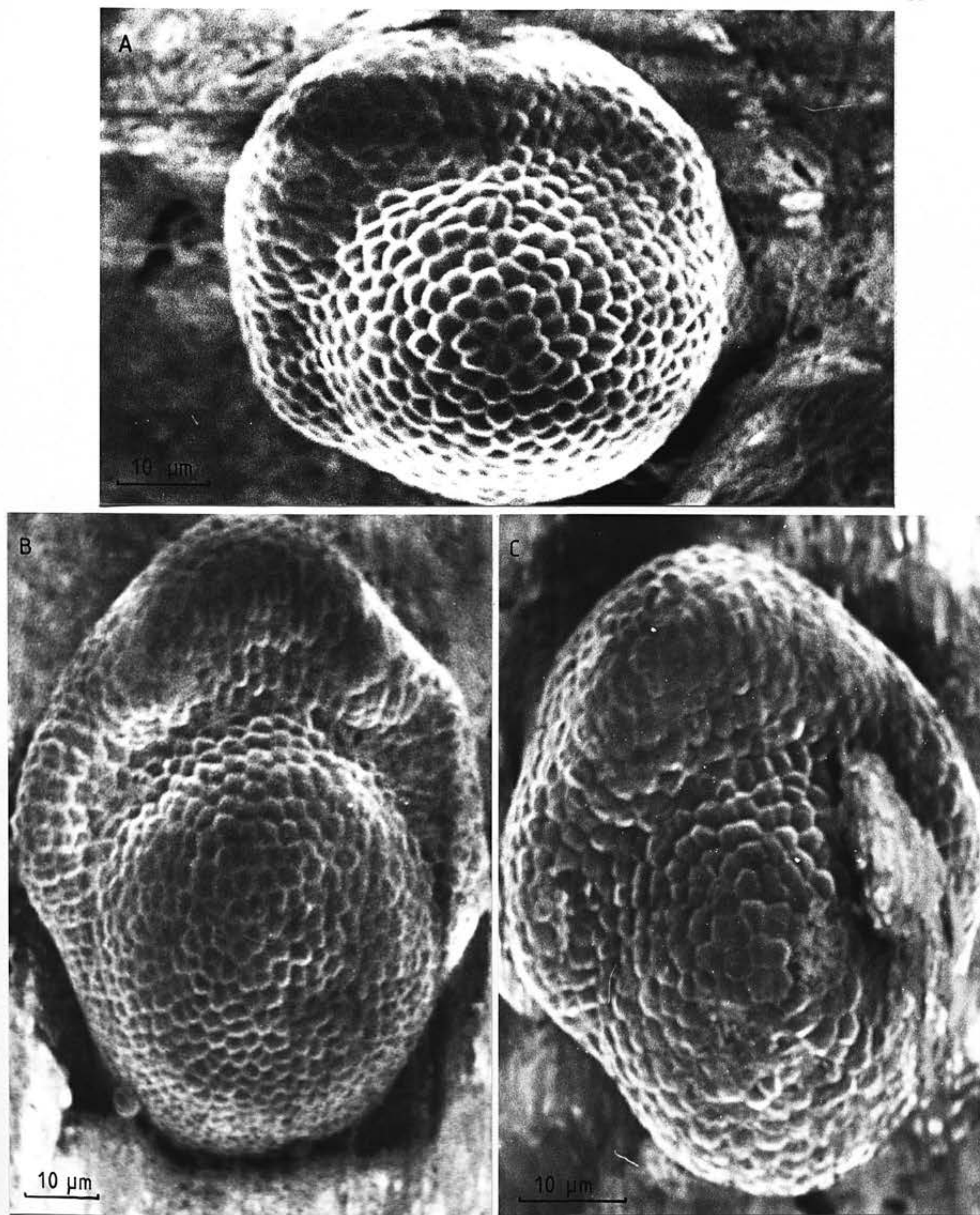


Figure VI. 7. Effect of 2,4-D on apical development of seedlings 48h (A) and 60h (B and C) from planting.
A and B - treated at 36h of imbibition
C - treated at 48h of imbibition
For further description see text.

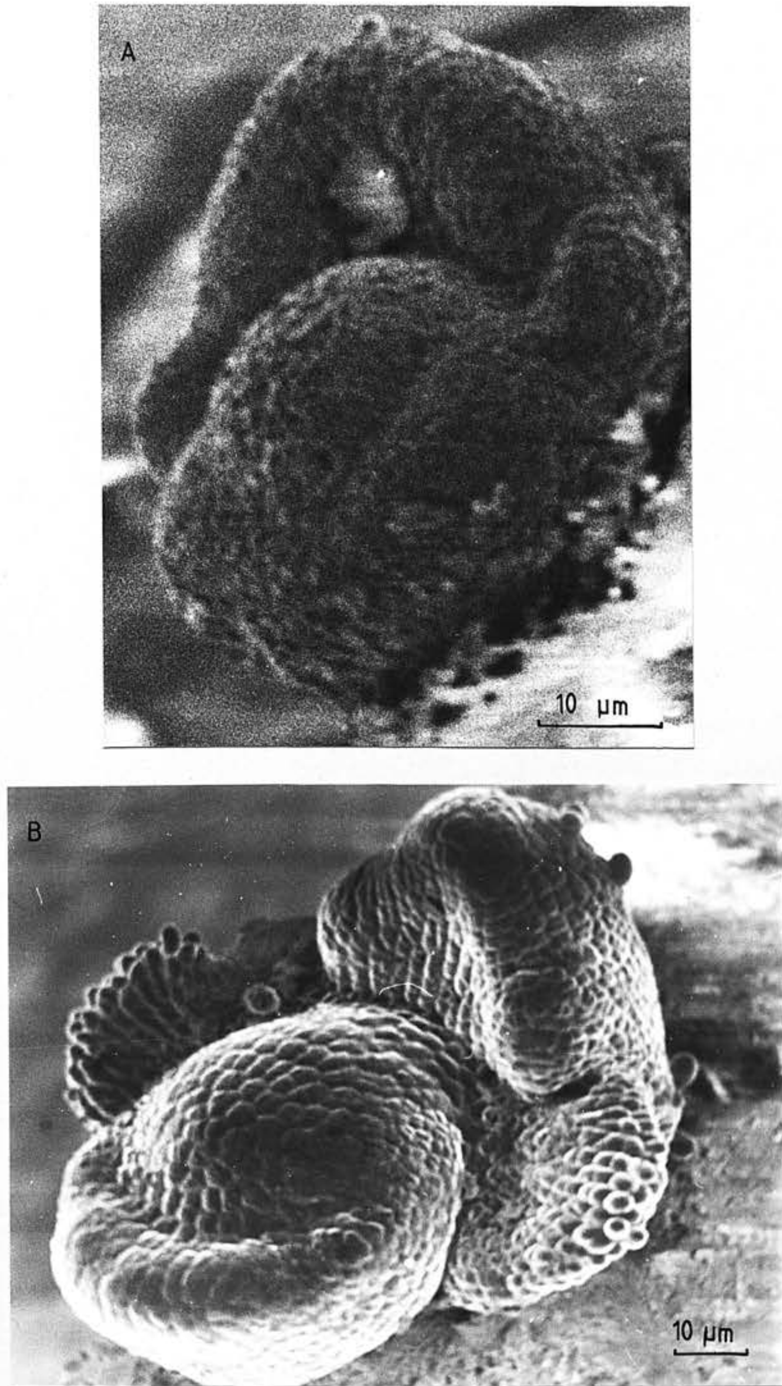


Figure VI. 8. Effect of 2,4-D on apical development of seedlings 72h from planting.
A - treated at 36h of imbibition
B - treated at 48h of imbibition
For further description see text.

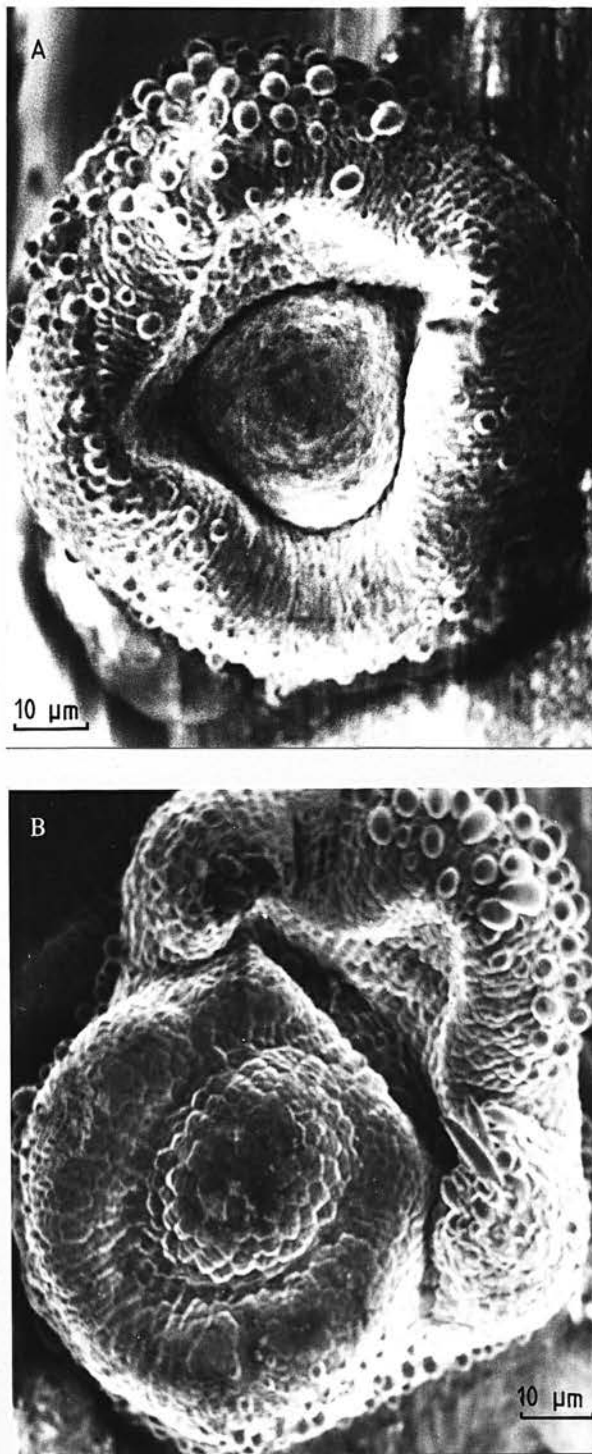


Figure VI. 9. Effect of 2,4-D on apical development of seedlings 96h from planting.
A - treated at 36h of imbibition
B - treated at 48h of imbibition
For further description see text.

The developmental stage of the first trifoliate leaf primordium at 48 h seems to be advanced enough to allow its inclusion in the ring formation and it developed separately. However its stage, by the time of treatment was not advanced enough to allow normal development, since lateral leaflets were totally inhibited.

2.3 Effects of 2,4-D on cell division and cell expansion

In the previous sections it was observed that 2,4-D can modify adult leaf form and shape in cv. Mont D'Or Golden Butter and that this effect starts very early during primordial development. The question which arises next is how 2,4-D exerts this effect. An attempt was made to answer this question in the next experiments, by examining changes in volume, cell number and cell volume on the whole apex and different regions of apices treated with 2,4-D at different developmental stages.

Seedling apices were treated with a droplet of 5.0 μl of 2,4-D 10^{-3}M after 24, 36 or 48 h of imbibition. Apices were harvested at intervals and examined after fixation, embedding and sectioning, as was previously described (Section 2.2.3, Chapter II).

2.3.1 Effects on the whole apex

In the first set of results effects on the whole apex were examined. The most striking effect of 2,4-D was the

inhibition of growth it caused in the whole apex. With respect to apical volume it seems that treatment after 48 h of imbibition is not so effective as at 24 or 36 h, especially in 72 and 96 h old seedlings. Although the difference is very small the curve resulting from treating seedlings at 36 h is always below the others (Figure VI. 10).

The curves in figure VI. 11, obtained for increase in cell number in the whole apex, follow closely the curves of increase in apical volume. In relation to cell number treatment at 36 h tended also to show lower values than treatment at 24 or 48 h of imbibition.

When these data are replotted as a percentage of the control value, against time after treatment some differences are shown (Figure VI. 12 - A, B). The reduction in apex sensitivity after 48 h of imbibition suggested for increase in volume and cell number is no longer apparent, at least for cell number, which was as inhibited in these apices as it was in those treated at 24 h or 36 h of imbibition (Figure VI. 12 - B). With respect to apical volume there was a tendency for apices treated at 48 h of imbibition to maintain constant the inhibitory effect of 2,4-D instead of the increasing inhibition with time observed in apices treated at 24 and 36 h of imbibition (Figure VI. 12 - A). These differences between the pattern of the curves for cell number and for apical volume suggest the involvement of minor effects of 2,4-D on cell expansion.

Confirming what was suggested in figures VI. 10 and VI. 11 again a tendency of apices treated at 36 h to be more

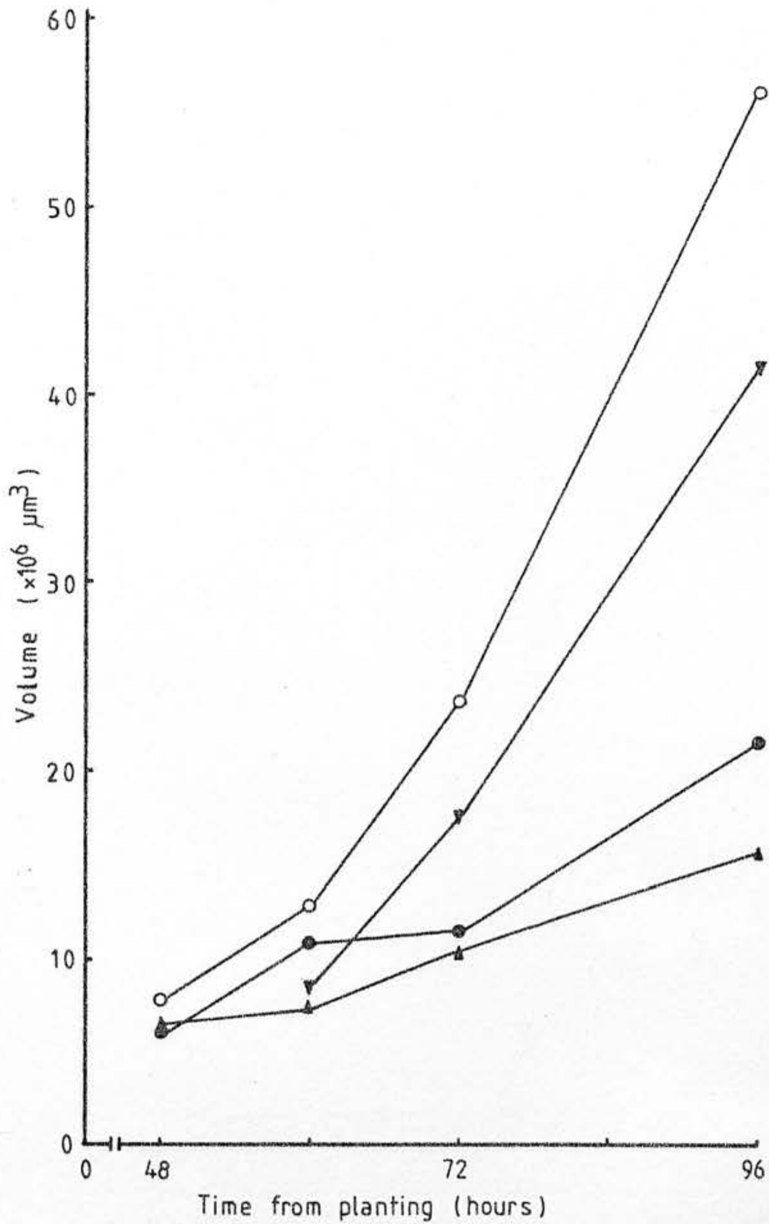


Figure VI. 10. Effect of 2,4-D on apical volume when treatment was applied after different periods of imbibition. Treatment at:
● - 24h ▲ - 36h
▼ - 48h ○ - control

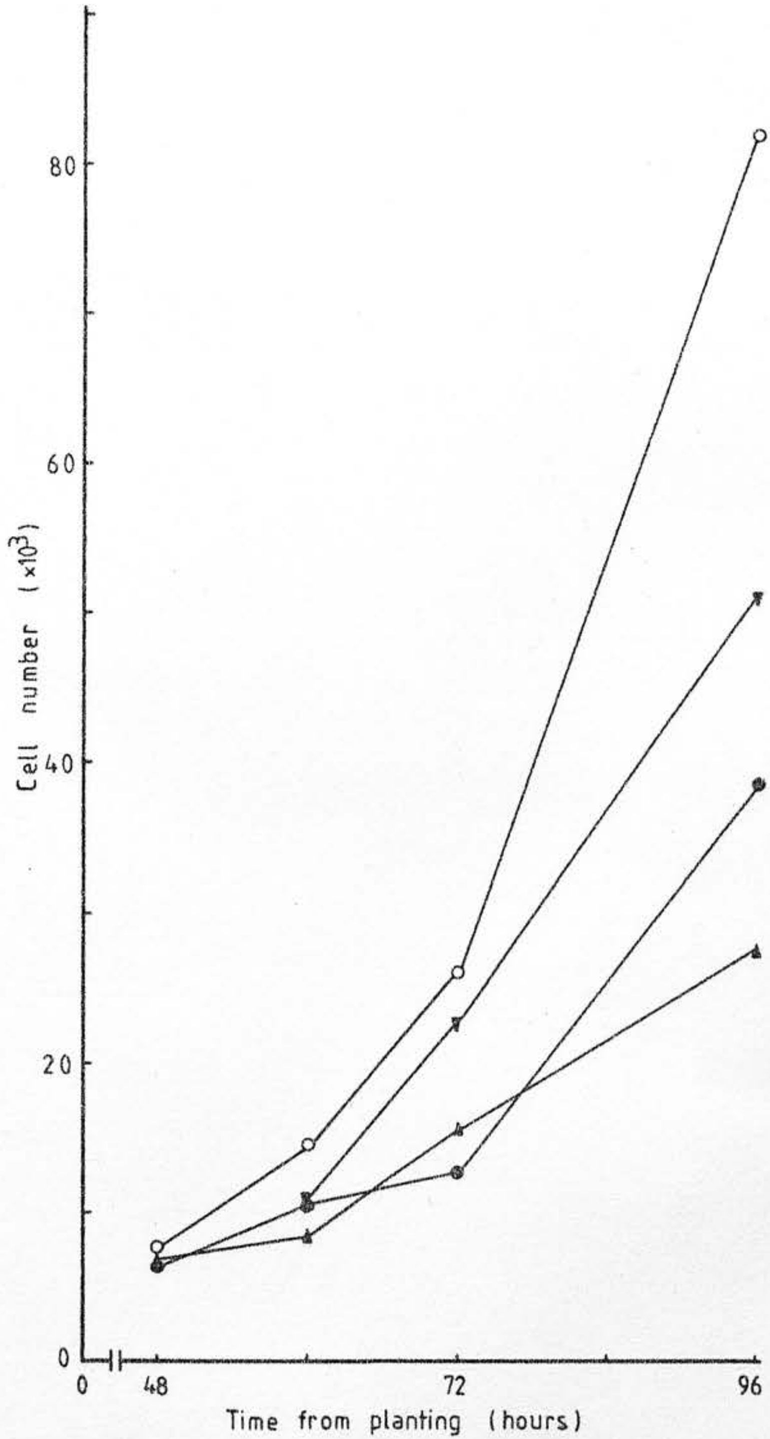


Figure VI. 11. Effect of 2,4-D on cell number of the whole apex when treatment was applied after different periods of imbibition.
Treatment at:
● - 24h ▲ - 36h
▼ - 48h ○ - control

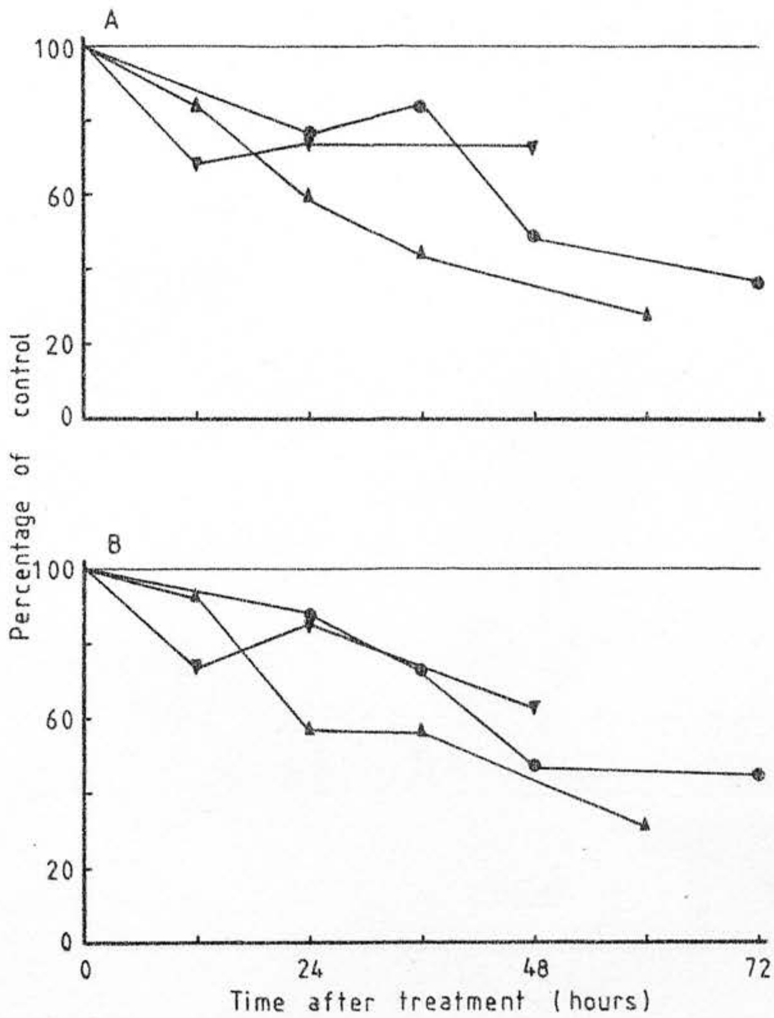


Figure VI. 12. Effect of 2,4-D on apical volume (A) and cell number (B) when data are expressed as percentage of control against time after treatment. Period of imbibition:
● - 24h ▲ - 36h ▼ - 48h

sensitive to treatment than at 24 or 48 h was noted (Figure VI. 12 - A, B).

These results suggest that 2,4-D inhibits apical growth by inhibiting mainly cell division, although cell expansion might be involved as well, since increase in volume and increase in cell number are not closely correlated at all points in time and treatment. It is also suggested that the apex is slightly more sensitive to 2,4-D treatment at 36 h of imbibition than at 24 h or 48 h.

2.3.2 Effects on different parts of the apex

Experiments to be described in this section were designed to examine whether the apex was uniformly affected by 2,4-D or if different regions were differently affected. For this purpose the outlines of the apical sections were divided into regions which were defined in Section 2.2.3, Chapter II.

Figures VI. 13 and VI. 14 show the increase in volume of the different regions of the apex, during the first 96 hours of seedling growth. Although minor differences could be noticed the major effect of 2,4-D was inhibition of growth in all the regions, no matter when it was applied. The pattern of growth found for axial regions (region 3 - Figure VI. 13 - A and region 4 - Figure VI. 14 - A) and for the first trifoliate leaf primordium (region 1 - Figure VI. 13 - B) was similar and similar also to the pattern of growth obtained for the whole apex (Figure VI. 10). This suggests that apical growth may be qualitatively if not quantitatively due to activity in those

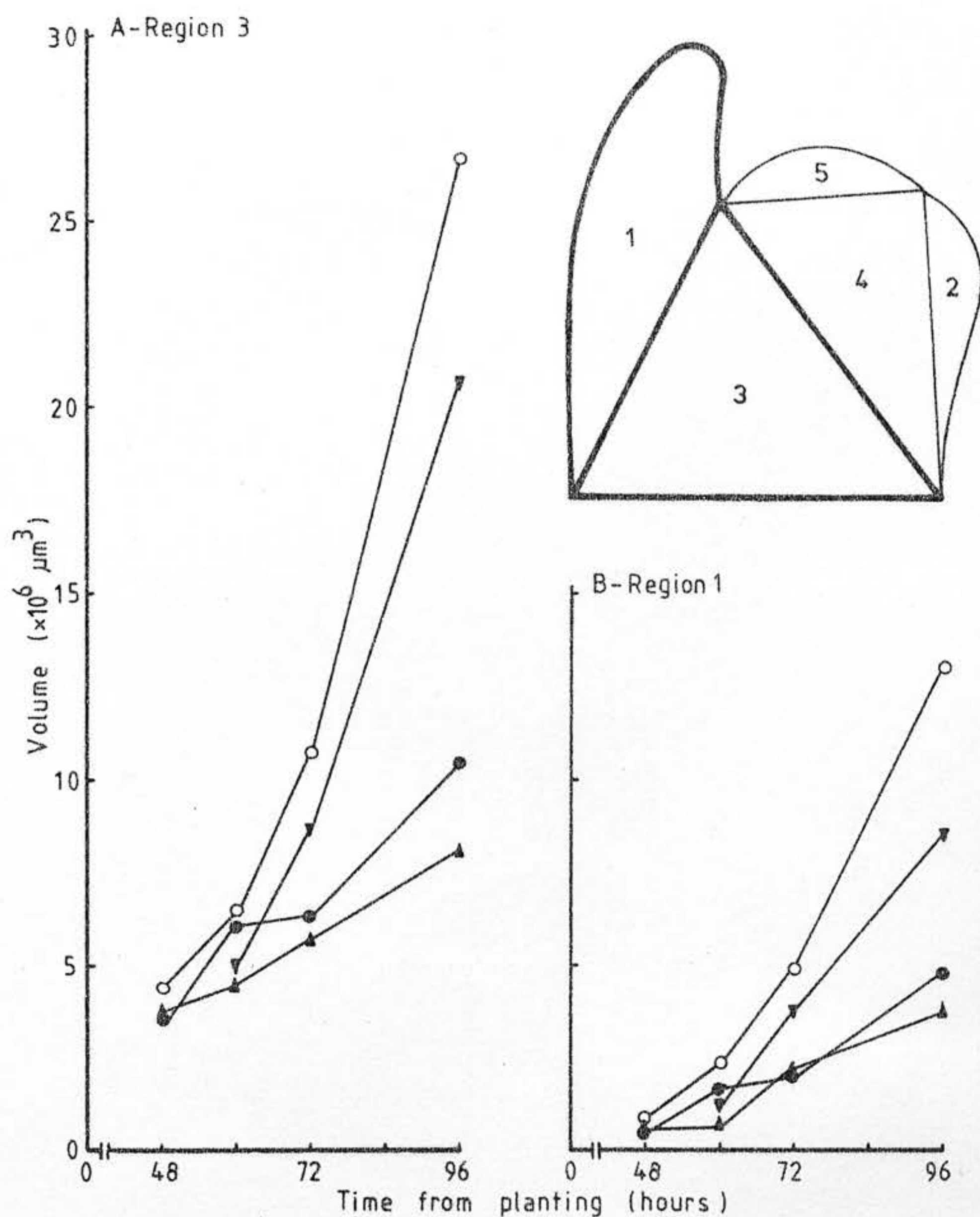


Figure VI. 13. Effect of 2,4-D on volume of regions 1 and 3 when treatment was applied after different periods of imbibition.
Treatment at:
● - 24h ▲ - 36h
▼ - 48h ○ - control

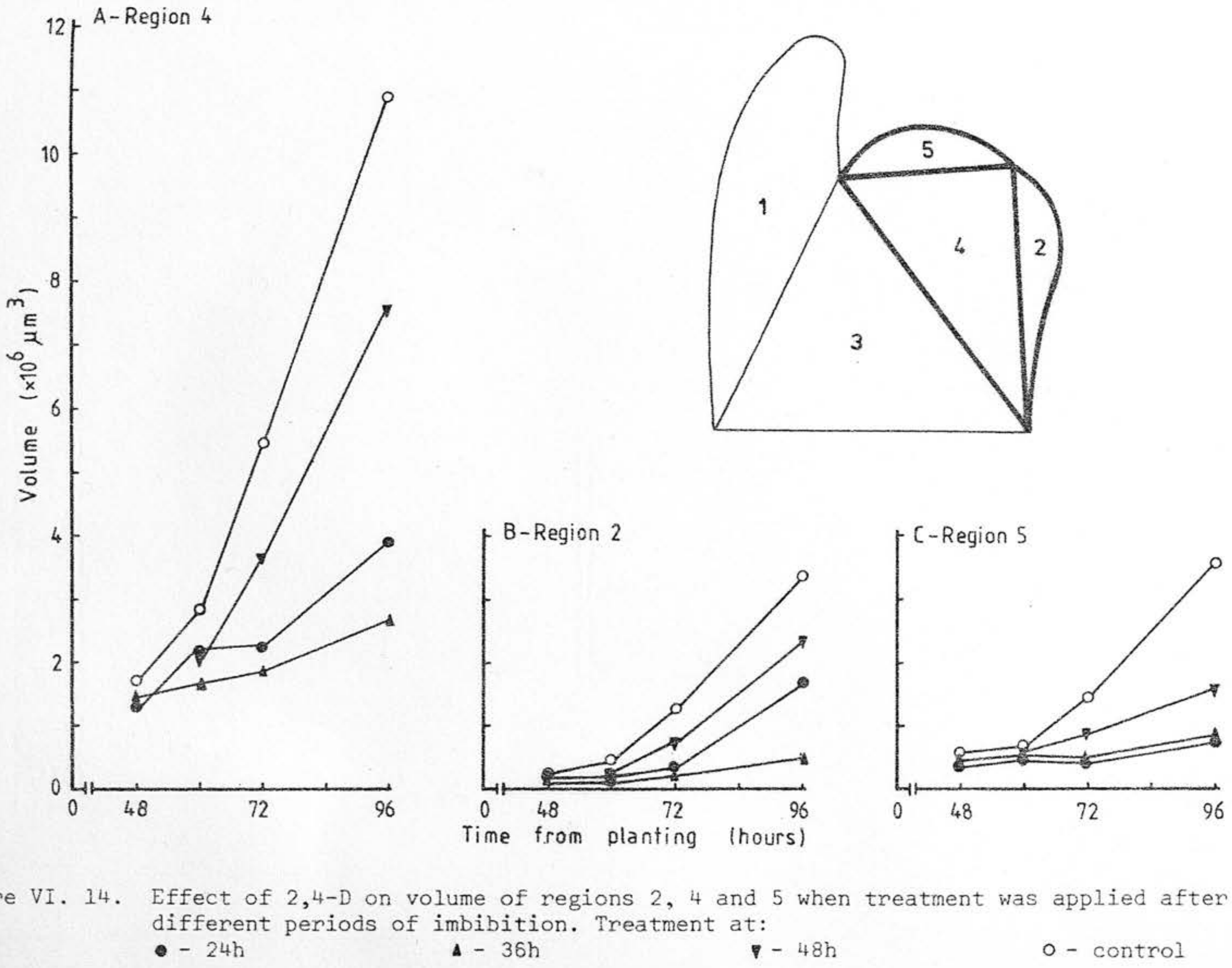


Figure VI. 14. Effect of 2,4-D on volume of regions 2, 4 and 5 when treatment was applied after different periods of imbibition. Treatment at:
● - 24h ▲ - 36h ▼ - 48h ○ - control

regions. A different pattern was obtained for growth of regions 2 (second trifoliate leaf primordium - Figure VI. 14 - B) and 5 (apical dome - Figure 14 - C) which were similar to each other.

Confirming what was observed for the whole apex, most of the regions appeared to be slightly more sensitive to 2,4-D treatment at 36 h from planting especially in the latest stages analysed. Treatment applied at 36 h from planting inhibited nearly completely growth of regions 2 and 5 during the 96 hours which this experiment lasted (Figure VI 14 - B, C). Region 2 of apices treated at 24 h from planting was totally inhibited up to 72 h from planting. After this time they partly overcame the effect of treatment and by 96 hours showed a volume very close to that of apices treated at 48 h from planting (Figure VI. 14 - B). Region 5 (Figure VI. 14 - C) was the most inhibited especially by treatment at 24 or 36 h from planting which completely stopped growth. Great inhibition, in this region, was observed even in apices treated at 48 h from planting, which in the other regions did not show a marked reduction of volume.

The curves for cell number, shown in Figures VI. 15 and VI. 16, followed very closely the pattern of increase in volume (Figures VI. 13 and VI. 14), suggesting, again, that cell division is a major process involved in increase in apical size. Regions 1, 3 (Figure VI. 15 - A, B) and 4 (Figure VI. 16 - A) presented a very similar pattern of increase in cell number and regions 2 and 5 (Figure VI. 16 - B, C) showed a slightly different pattern from that of the other regions. Despite the similarity of the curves for volume of the regions and cell

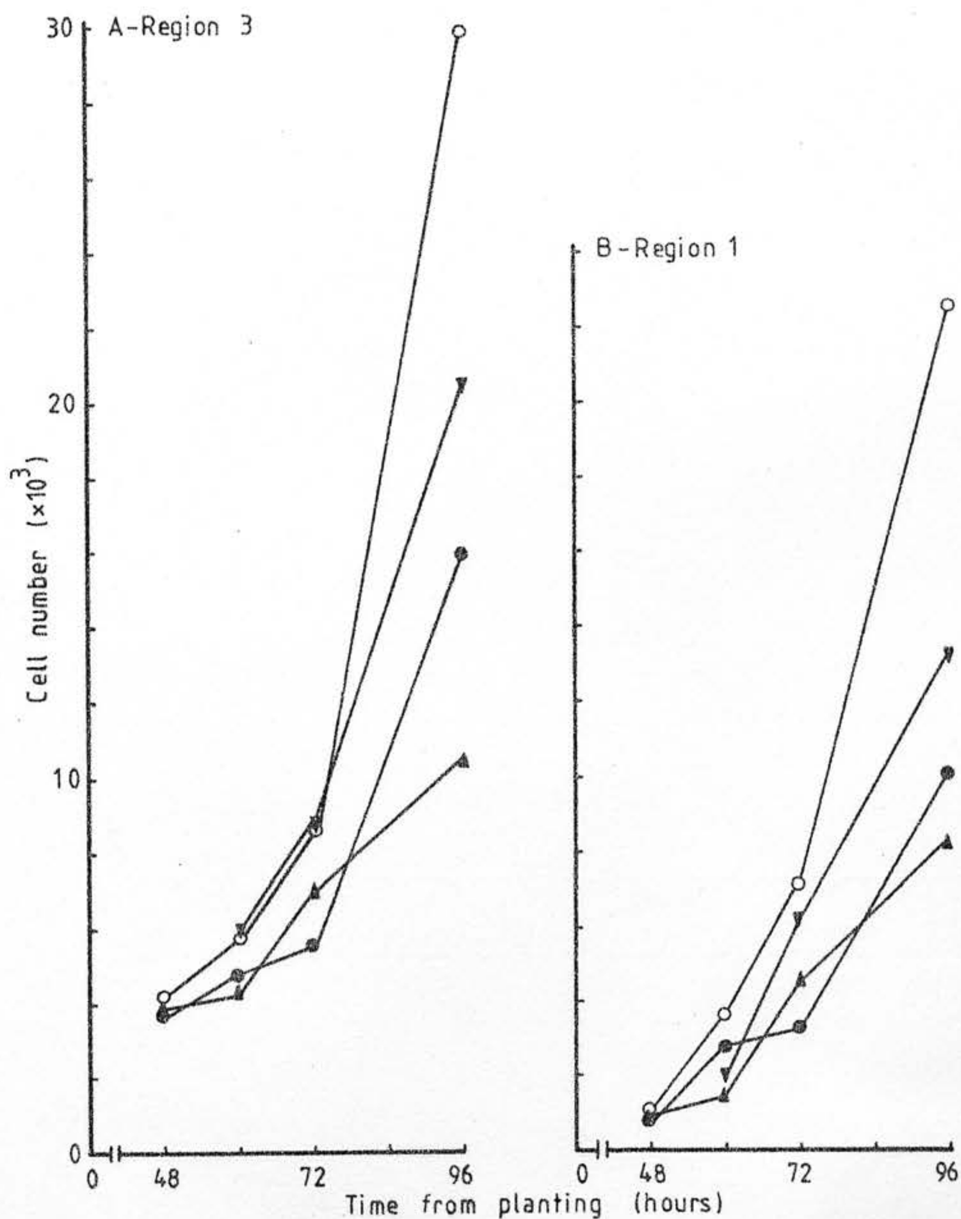


Figure VI. 15. Effect of 2,4-D on cell number of regions 1 and 3 when treatment was applied after different periods of imbibition.
Treatment at:
● - 24h ▲ - 36h
▼ - 48h ○ - control

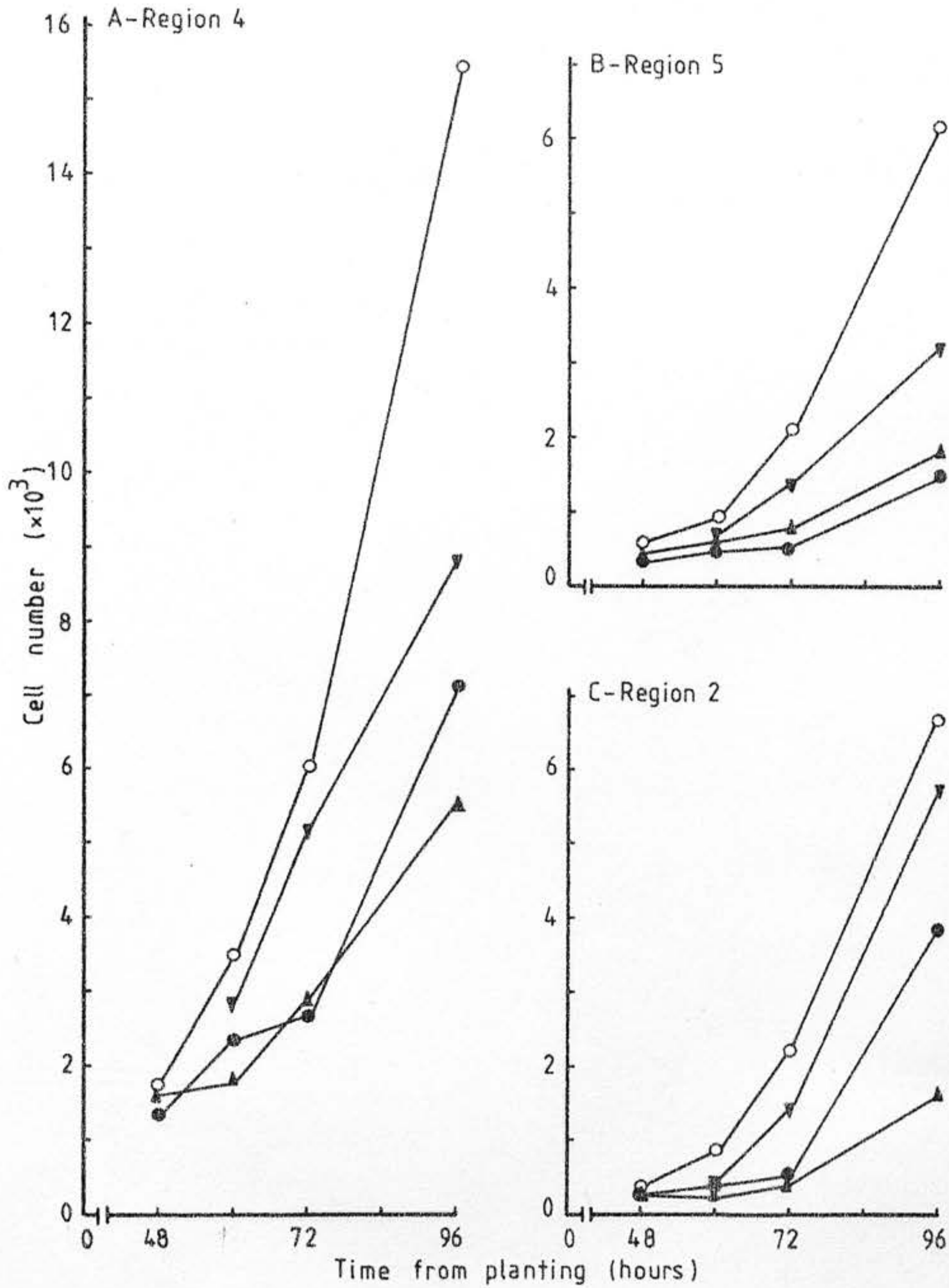


Figure VI. 16. Effect of 2,4-D on cell number of regions 2, 4 and 5 when treatment was applied after different periods of imbibition.
Treatment at:
● - 24h ▲ - 36h
▼ - 48h ○ - control

number some minor differences between these curves suggested that cell expansion might also be involved.

Figure VI. 17 shows mean cell volume of the different regions of apices at different ages. Cell volume is not uniform throughout the apex, and different regions have different mean cell volumes. This is also illustrated in Figure VI. 18 which shows the median section of control and treated apices 48 and 72 h after treatment. Internodal regions (regions 3 and 4) and apical dome (region 5) tended to have larger cells than leaf primordia (regions 1 and 2) in both treated and control apices. In control apices except for region 5, mean cell volume tended not to change with time in all regions. In region 5 mean cell volume changed from a value, which was similar to the cells of axial regions, at 48 h from planting, to a much smaller value 48 hours later. This was also found in treated apices with the difference that mean cell volume in control apices decreased gradually and in treated apices the decrease was abrupt in the last 24 hours (Figure VI. 17). The gradual decrease in mean cell volume in region 5 of control apices might be correlated with initiation and early development of new primordia, since this region, in the way it was defined, includes new primordia initiated after the second, and leaf primordia are shown to have the smallest cells in the apex (Figure VI. 17). The same explanation is not applicable for treated apices, since it was shown that no new primordium developed up to 96 h from planting, no matter when treatment was applied (Figure VI. 5 - B; VI. 9 - A, B). However it can be seen in Figure VI. 18 - D that cells in the flanks are

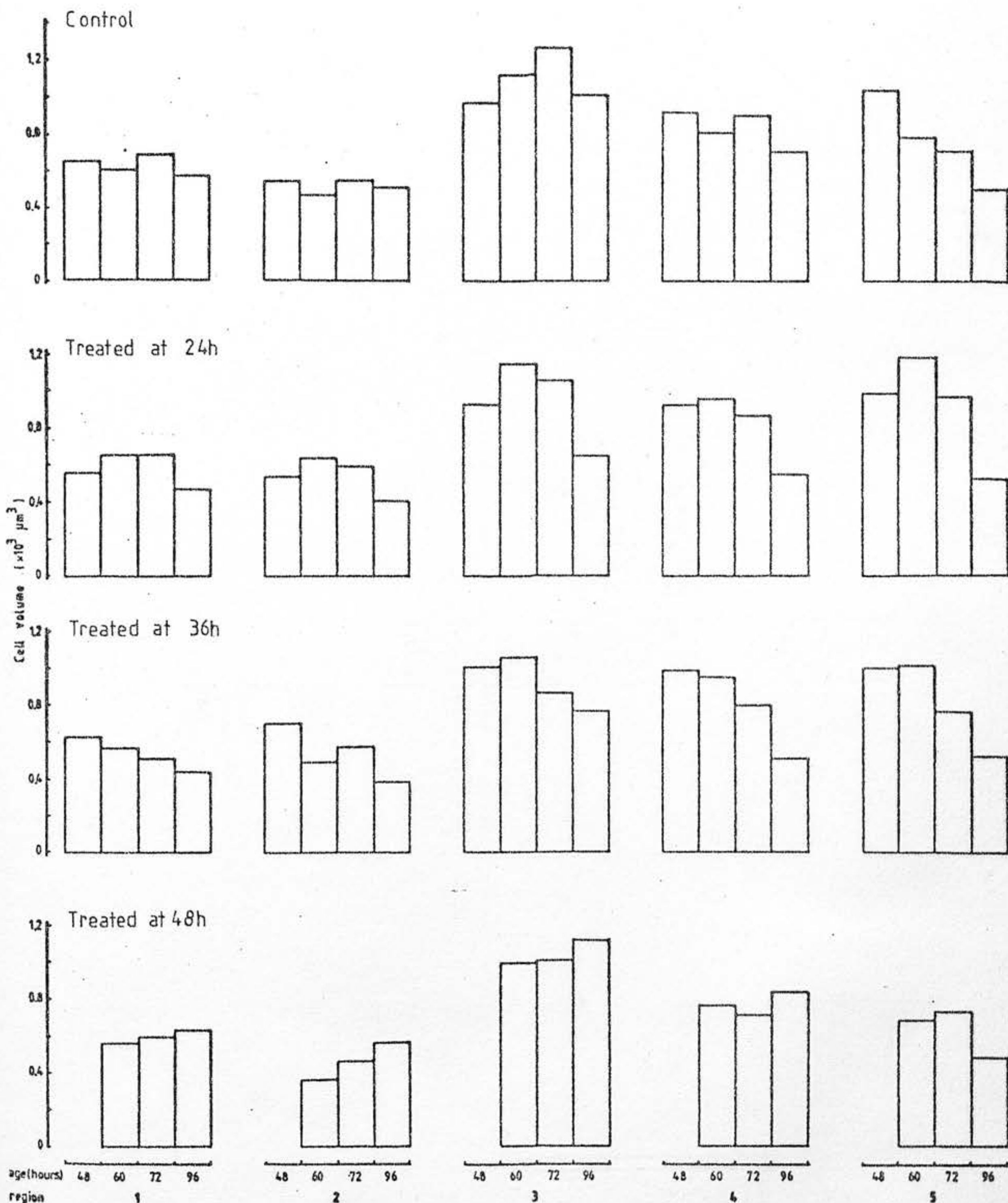


Figure VI. 17. Mean cell volume of different regions at different time from planting. Apices were treated after different periods of imbibition.

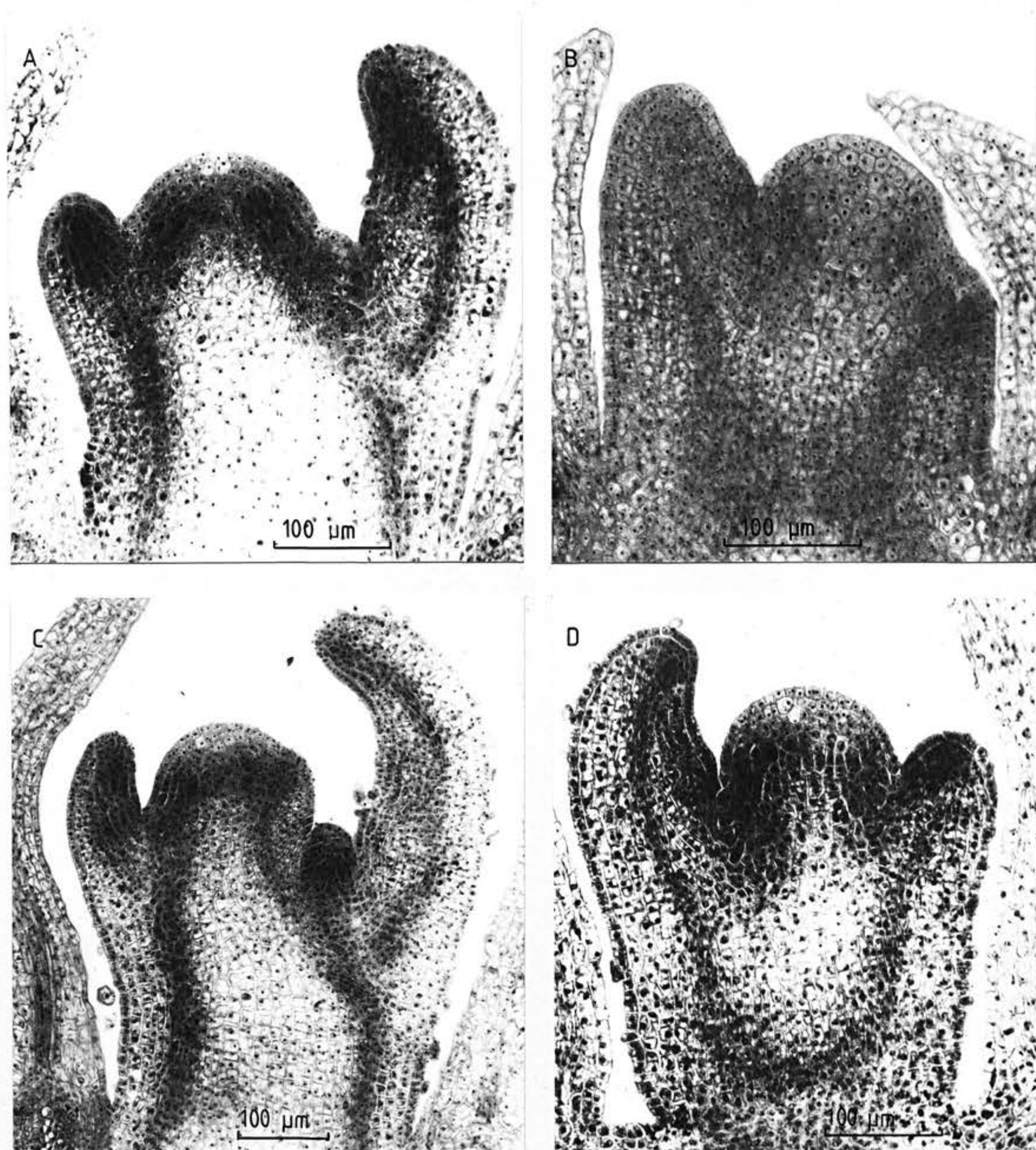


Figure VI. 18. Median sections of apices treated at 24h of imbibition and harvested at different periods after treatment.
A and B - 48h after treatment
C and D - 72h after treatment
A and C - control
B and D - treated with 2,4-D

smaller than at the very top of the apical dome, 72 hours after treatment. This might be related to the sudden increase in cell division which occurred in this region of treated apices during the last 24 hours of the experiments (Figure VI. 16 - C). This increase in cell division may reflect the involvement of this region 5 in the growth of the ring structure.

Decrease in mean cell volume also occurred in axial regions in apices treated at 24 and 36 h from planting. Apices treated at 48 h were more similar to control in relation to mean cell volume (Figure VI. 17). This might also be a reflection of the increase in cell number in the flanks of the apical dome, since part of those cells could have been counted in regions 3 and especially region 4.

In this section it has been shown that different regions of the apex grow at different rates. Next, the data were analysed differently to examine the contribution of each region to the growth of the whole apex and to determine whether this contribution was the same in control and treated apices. Using this approach the large inhibitory effects of 2,4-D were allowed for, since growth of the different regions was considered in relation to the growth of the whole apex, no matter how much it grew. The ratios used were defined in Section 2.2.7, Chapter II.

Figure VI. 19 shows the ratio Z between the COM of treated and control apices. Despite the large inhibitory effect of 2,4-D on the volume increase of different regions as shown previously, in some cases the contribution of a certain region for the total apical growth was seen to be higher in

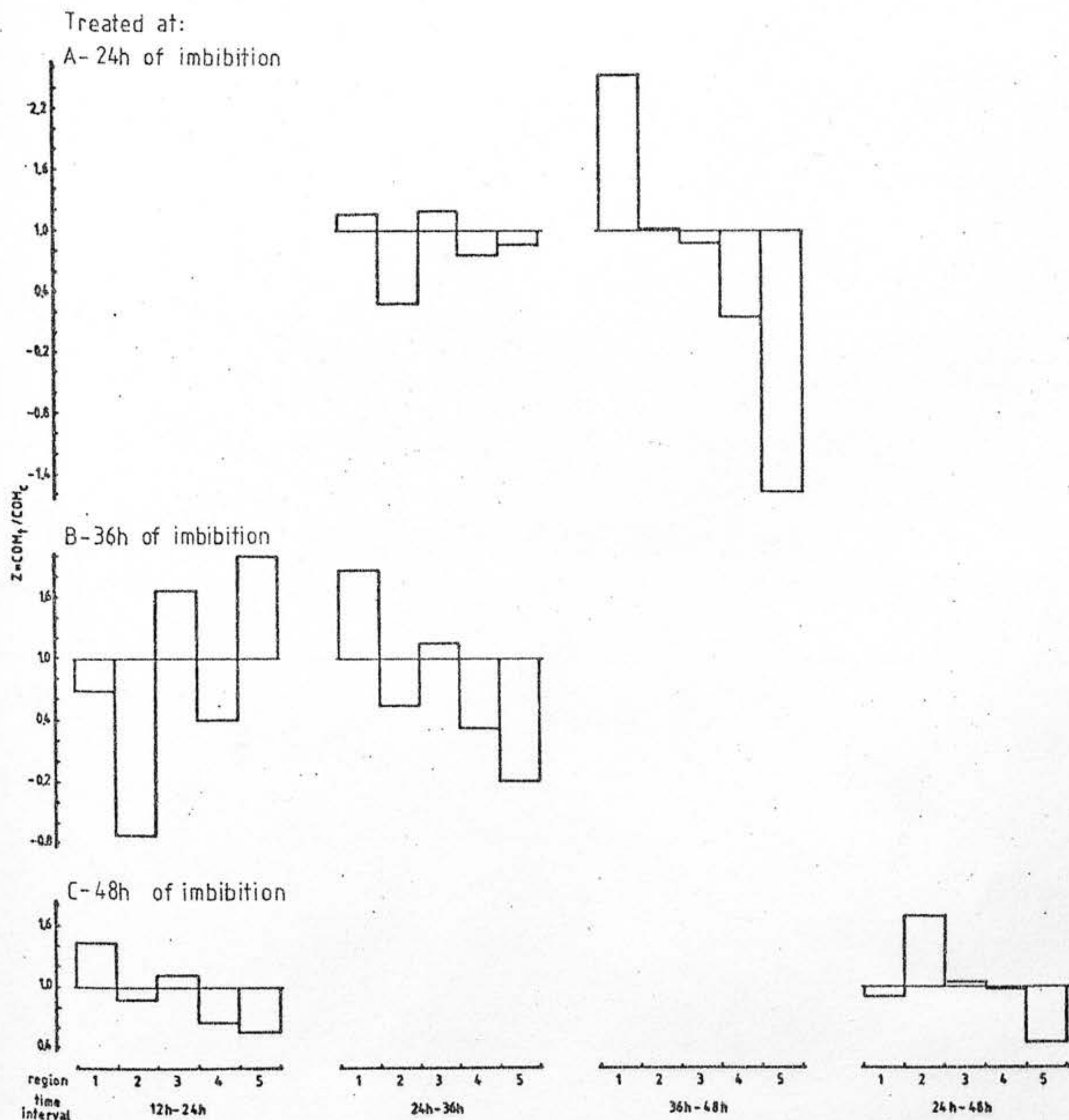


Figure VI. 19. Ratio (Z) of COM of treated and control apices for the volume of the different regions at different time intervals.

treated apices than in control. To simplify the discussion of the data this will be named promotion and the contrary will be termed inhibition. But the reader must be aware that this is a relative and not an absolute promotion or inhibition.

Corresponding regions of apices treated at 24 h from planting contributed to about the same extent to size in treated and control apices between 24 and 36 h after treatment, except for region 2 which was inhibited. During the interval between 36 and 48 h after treatment region 1 was promoted by comparison with control apices. Regions 4 and 5 were substantially inhibited in treated apices. Apices treated at 36 h from planting showed, during the first 12 hours after treatment, promotion of regions 3 and 5 and inhibition of the others, region 1 being only slightly inhibited. During the next time interval (24 - 36 h from treatment) region 1 was promoted and region 2, 4 and 5 inhibited (Figure VI. 19 - B). When treatment was applied at 48 h from planting only slight effects were noticed during the time interval between 12 and 24 h after treatment, but during the interval between 24 and 48 h after treatment region 2 was promoted and region 5 was inhibited.

If we remember that the establishment of the ring formation developed in apices treated with 2,4-D occurs between 36 and 48 h after treatment in seedlings given 2,4-D at 24 h from planting and between 24 and 36 h and 24 and 48 h respectively in apices treated at 36 h and 48 h from planting it seems that this promotion in leaf primordia and inhibition in apical dome could be associated with ring formation. The

promotion in region 2 in apices treated at 48 h from planting may be related to the fact that in those apices it is the second trifoliate leaf primordium that starts the ring formation, the first trifoliate leaf primordium developing separately.

The same ratios were calculated for increase in cell number and the results are shown in Figure VI. 20. It was verified that the main features are very similar to the ratios for increase in volume. However one striking difference could be noticed. Region 3 was contributing for apical growth to about the same proportion in treated and control apices, in relation to increase in volume. With respect to increase in cell number this region was promoted. This suggests that cells in region 3 are dividing faster in treated apices than in control, and cell expansion is not occurring at the same rate, since promotion in volume for that region was not observed.

3. EFFECTS OF OTHER AUXINS ON PRIMORDIAL DEVELOPMENT

As 2,4-D showed such marked effects on apical development experiments were designed to examine whether this was a specific effect of 2,4-D or whether the same effect would be obtained with other auxins.

3.1 Effects of indole-3-acetic acid (IAA)

Since the described effects on primordial development were obtained with a synthetic auxin it seemed to be of interest

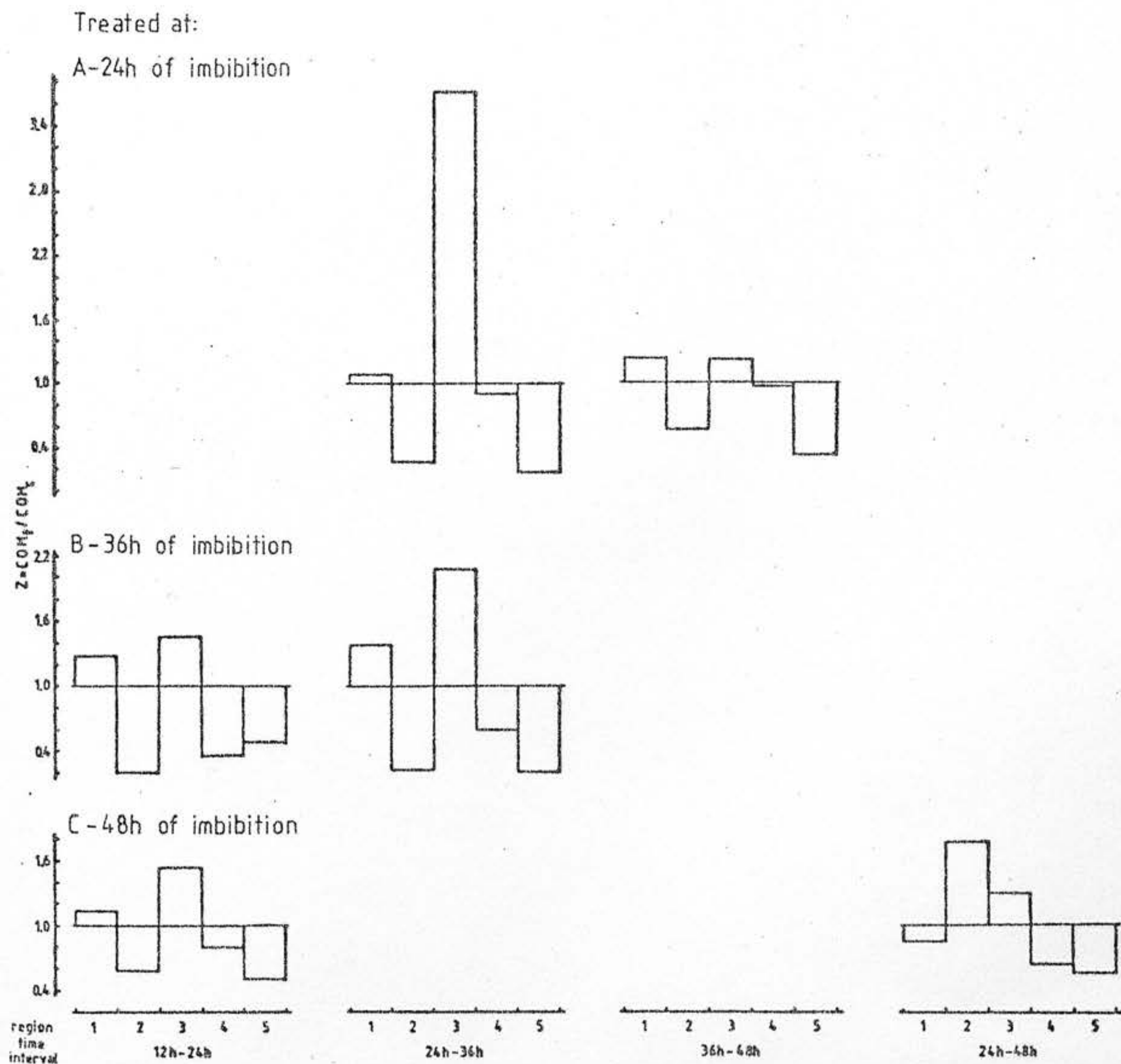


Figure VI. 20. Ratio (Ż) of COM of treated and control apices for cell number of the different regions at different time intervals.

to examine the effect of the naturally occurring auxin IAA, on primordial development. For this purpose three different treatments were applied to seedlings after 24 h of imbibition. In the first a single application of 0.6 $\mu\ell$ was made at the apex of concentrations varying from 10^{-5} to 10^{-3}M (experiment I). In the second the amount of IAA was increased by making three applications of 2.0 $\mu\ell$ each at 24 h intervals (experiment II) and in experiment III a single application of 5.0 $\mu\ell$ was made on the apex. In both experiments II and III IAA was used at the concentration of 10^{-3}M.

Table VI. 9 shows the results of the three experiments. In none of them did IAA treatment cause any modification of primordial development. Even the highest amount of IAA applied (1.0 μg per apex) was totally ineffective.

3.2 Effects of naphtalene-acetic acid (NAA)

One of the possible explanations for the total lack of effect of IAA in the previous experiments is that it might have been broken down in a stage prior to be effective. In this section the effect of a synthetic auxin on primordial development was then examined. A droplet of 5 $\mu\ell$ of NAA 10^{-3}M (0.9 μg) was applied to the apex of seedlings imbibed for 24 hours. Apices were examined 48 hours later.

NAA applied at a concentration equimolar to the effective concentration of 2,4-D delayed slightly but not significantly the rate of primordial initiation and the development of the later formed primordia (Table VI. 10).

TABLE VI. 9. Effect of IAA on primordial development (Experiment I and II-harvest: 72 h after treatment and experiment III-harvest: 48 h after treatment.)

IAA conc. (M)	Number of primordia	D.S. of each primordium				
		1	2	3	4	5
	Experiment I - 1 application of 0.6 μℓ					
0	4.0	4.0	4.0	3.4	2.3	0.0
10^{-5}	4.0	4.0	4.0	3.4	2.2	0.0
10^{-4}	4.1	4.0	4.0	3.9	2.4	0.1
10^{-3}	4.0	4.0	4.0	3.7	2.3	0.0
	Experiment II - 3 applications of 2.0 μℓ					
0	4.0	4.0	4.0	3.0	1.2	0.0
10^{-3}	4.0	4.0	4.0	3.0	1.0	0.0
	Experiment III - 1 application of 5.0 μℓ					
0	2.9	4.0	2.9	1.0	0.0	0.0
10^{-3}	3.0	4.0	3.0	1.1	0.0	0.0

TABLE VI. 10. Effect of NAA on primordial development.

	Number of primordia	D.S. of each primordium				
		1	2	3	4	5
H_2O	2.9	4.0	2.9	1.0	0.0	0.0
NAA $10^{-3}M$	2.6	4.0	2.6	0.6	0.0	0.0

There were no effects on morphology and ring formation was not observed.

4. EFFECTS OF 2,3,5-TRIIODOBENZOIC ACID (TIBA) ON PRIMORDIAL DEVELOPMENT

Abnormal apical development has been described for many species as a result of treatment with TIBA, as for example, in tomato, where TIBA caused development of ring fasciations and ceased leaf initiation (Gorter, 1951; Wardlaw, 1953; Bedesem, 1958). Modifications in phyllotaxis by changing shape of the apical dome were caused in Chrysanthemum (Schwabe, 1971). Experiments were designed to examine whether TIBA could modify primordial development in apices of seedlings of *Phaseolus vulgaris* imbibed for 24 hours.

Three different treatments were made. In experiments I and III a single application of respectively 1.0 $\mu\ell$ and 5.0 $\mu\ell$ was made on the seedling apex. In experiment II two applications of 2.0 $\mu\ell$ each were made at 24 h intervals. In all the experiments the concentration of TIBA was 10^{-4}M and apices were examined 72 h after treatment.

The rate of primordium initiation was delayed significantly by TIBA only when the largest amount was supplied (0.25 µg per apex). In this case development of primordia, except for the first, was also significantly delayed (Table VI. 11 - experiment III). The effect obtained was increased by increasing the amount of TIBA applied, but the formation of a trifoliate leaf primordium was not prevented nor was ring formation induced, even with the highest amount

TABLE VI. 11. Effect of TIBA 10^{-4}M on primordia development 72 hours after treatment.

	Number of primordia	D.S. of each primordium				
		1	2	3	4	5
	Experiment I - 1 application of 1.0 μℓ					
H_2O	4.0	4.0	4.0	3.9	1.8	0.0
TIBA	3.9	4.0	4.0	3.1*	1.5	0.0
	Experiment II - 2 applications of 2.0 μℓ each					
H_2O	4.0	4.0	4.0	3.6	2.0	0.0
TIBA	3.7	4.0	4.0	3.0*	0.7**	0.0
	Experiment III - 1 application of 5.0 μℓ					
H_2O	4.0	4.0	4.0	3.4	1.6	0.0
TIBA	3.0*	4.0	3.1*	1.7*	0.2**	0.0

* - significant at 5% level

** - significant at 1% level

of TIBA supplied.

The effect of TIBA on leaf primordia development could be a reflection of a general growth inhibition or a specific effect on leaf development. To examine this point growth of different parts of the seedling was measured by determining dry weight 72 hours after treatment. Treatment consisted in application of a droplet of 5.0 $\mu\ell$ of TIBA 10^{-4}M on apex of seedlings imbibed for 24 hours.

Table VI. 12 shows the results of this experiment. No significant difference was detected between growth of the different parts of control and treated seedlings.

Cotyledons in treated seedlings showed a slightly higher dry weight than in control, this representing 85% of the total weight in treated seedlings and 77% in control apices. These data may suggest that a general growth inhibition is more likely to occur as a response to TIBA treatment and in this case the delay in primordial initiation and early development would be a reflection of this inhibition. However a specific effect on primordial initiation and early development cannot be ruled out, since this effect was statistically significant and that general growth inhibition although suggested was not large enough to be significant.

5. DISCUSSION

In the experiments described in this Chapter, evidence was presented that 2,4-D can modify leaf form and growth in P. vulgaris cv. Mont D'Or Golden Butter. Soma's findings (1968)

TABLE VI. 12. Effect of TIBA 10^{-4}M on seedlings dry weight (mg) 72 h after treatment.

	H_2O	TIBA
cotyledon	82.3 $\pm$ 9.7[1]	98.6 $\pm$ 11.9
stem-root axis	22.5 $\pm$ 5.1	15.7 $\pm$ 3.6
primary leaves	2.3 $\pm$ 1.1	1.3 $\pm$ 0.9

(1) - 95% confidence limits are quoted.

were confirmed except that when applying treatment to the apex at different developmental stages no difference in sensitivity could be detected when adult plants were examined. Differences in apical sensitivity to 2,4-D treatment could not be detected even by treating material earlier during seed development. Seeds from 30 days onwards are sensitive at all stages with respect to response to 2,4-D. Direct application to the apex resulted in modified shape and reduced area for both primary and trifoliate leaves. In some cases treatment led to abnormalities such that similar shaped leaves were found in both primary and trifoliate leaf positions. Since primary leaf shape is already well defined in the embryonic axis treatment was effective in altering shape at a late stage in lamina expansion to result in this similarity.

20 days after anthesis seems to be the earliest stage for response of primary leaf shape to 2,4-D treatment. Treatment applied at this stage, but not earlier, modified primary leaf shape. This is not surprising especially if it is remembered that by this time the primary leaf blade is enlarging in the seed. The first trifoliate leaf primordium was not initiated at that time and the shape of the adult leaf was not modified by 2,4-D. Treatment applied earlier than this time although ineffective in modifying leaf shape, reduced leaf area. This means that at least some of the 2,4-D applied on the pod wall reached the embryo, and this occurred at a developmental stage not advanced enough for any modification of leaf shape to be produced. The fact that leaf growth, but not leaf shape, was affected when the very young seeds were

treated, suggests that two different effects of 2,4-D can be separated. Firstly 2,4-D can cause growth inhibition when applied to seedlings at very early stages of development, and secondly major morphogenetic effects which can be caused only later on.

2,4-D caused reduction in leaf area in both primary and trifoliate leaves when pods 15 days were treated but only the primary leaves were affected when pods 20 days were treated. Since the same amount of 2,4-D was applied in both cases, this effect can possibly be related to the size of the pods at the time of treatment and consequently to the amount of 2,4-D which reached the embryo, this amount being smaller in embryos 20 days than in 15 days after anthesis. The delay in primordial development observed when treatment was applied at day 15 of pod development can be a reflection of the growth inhibition caused by 2,4-D treatment, although Bedesem (1958) found 2,4-D to inhibit normal growth without affecting leaf primordia initiation on shoot apices of tomato.

In conclusion it is suggested that critical amounts of 2,4-D can inhibit growth at very early stages of seedling development but specific morphogenetic effects can be caused only later during embryo development.

The developmental basis of the abnormalities caused by 2,4-D on leaf development was examined using the SEM. Small amounts of 2,4-D up to 0.2 μg per apex were totally ineffective in modifying either primordial development or the rate of primordial initiation. However larger amounts as 0.4 μg per apex modified drastically the morphology of the apex. Although

the amounts of 2,4-D required for those large effects to be obtained are high this cannot be considered as a toxic or herbicidal effect since this amount can still be increased, at least, up to 1.0 μg per apex without either being lethal or increasing the effects obtained with smaller amounts. The requirement of high doses is more likely to be a consequence of a low penetration of the 2,4-D into the apex. The observed response to 2,4-D is an organised one in the sense that the ring formation is regular and the apical dome itself appears not to suffer anatomical or histological disorganization.

Evidence was presented that 2,4-D inhibits apical growth and this is mainly caused by inhibition of cell division. 2,4-D has been shown to inhibit growth through inhibition of cell division in the hook region of etiolated pea seedlings (Apelbaum and Burg, 1972). The authors explain these results as being mediated by auxin-induced ethylene formation.

The apex being an heterogeneous structure one might expect different parts to be differently affected by chemical treatments. The ring formation caused by 2,4-D on apices of mature seedlings seems to be related to a specific effect on leaf primordia (promotion) and apical dome (inhibition). Although our data suggest that cell division is much involved in this effect, conclusive evidence could not be provided to show whether this effect is due to a modification in cell number or cell volume or both, or whether it is solely due to a change in the orientation of the growth in those specific regions. The great importance of a change of direction of

growth for leaf primordia initiation has been discussed by Lyndon (1976). He regards the change in the plane of growth as the primary process and changes in the rate of growth being a secondary process for primordia initiation. This discussion is largely on the basis of his work with *Pisum* (Lyndon, 1970a; 1970b). Related to this could be mentioned the work of Kaufman (1955) who showed that in rice 2,4-D treatment inhibits cell division and cell elongation but at the same time promotes the radial enlargement of cells in the sub-apical region.

It was found that different parts of the apex grow at different rates. Increase in cell number followed very closely the increase in volume of the different parts of the apex. Lyndon (1976) has reviewed the literature about rates of cell division in different parts of the apex. He concluded that in all the studied species the rate of cell division in the apical dome was about half or a third of that on the flanks of the apex. Our data show that increase in cell number in the apical dome can be compared to that of the second leaf primordia. All the other regions show much greater rates of cell number increase.

Other auxins as IAA or NAA were ineffective in modifying primordial development. Two points can be advanced to explain this lack of effect. Firstly it is possible that they do not have the potential to modify primordial development. This is not to be expected, since in other species they have been shown to be effective. In *Tropaeolum* they caused leaf primordia to be produced out of their normal positions on the shoot apex. The formation of multiple leaves was observed as

a result of treatment (Ball, 1944). Varnell and Vasil (1978) found IAA, applied in lanolin to exposed apical meristems of *Lupinus albus*, to increase variation in the placement of primordia. The second point to explain the inefficiency of these auxins is that they might be effective but the amount which reached the apex was not enough to modify primordial development. Even considering that they were applied in equimolar concentrations to the effective concentration of 2,4-D, this explanation is not unlikely, since in aseptic culture of bean embryos 2,4-D has been shown to be about 10 times more active than IAA in the production of callus when parts of embryos were cultured (Furuya and Soma, 1957).

TIBA, known as an anti-auxin could delay primordial development but did not modify the shape of primordia nor did treatment induce the development of the ring structure. Its effect is more likely to be a general growth inhibition than a specific effect on leaf development, although the possibility that TIBA reached the apex at a concentration too low to modify the shape of leaf primordia cannot be ruled out. In this case 2,4-D might have been acting as an anti-auxin. Lockhart and Weintraub (1957) showed that 2,4-D reduced the endogenous level of auxins and this reduction preceded a decrease in growth rate. One of the most well known effects of TIBA is the inhibition it causes in auxin transport (Dela Fuente and Leopold, 1972). This effect was found to be caused either by TIBA or 2,4-D treatment in excised sections of stems and roots of bean seedlings (Hay, 1956).

CHAPTER VII

DISCUSSION

1. UPTAKE, PENETRATION AND MOVEMENT OF EXTERNALLY APPLIED SUBSTANCES

Since the seedling apex is such a small system the question arises as to why modification of early primordial development, if it is to occur at all, requires such large amounts of the applied substances (table VII. 1). Failure of the compound to penetrate must be envisaged as an explanation. The requirement for large amounts of IAA to modify apical growth in *Tropaeolum* was mentioned by Ball (1944). The author justified this necessity by pointing out that intact, unwounded shoot apices were used. However, at the same time he mentioned that the cuticle over the shoot apex was thinner than that over the mature epidermal cells and would probably not constitute an effective barrier to the entrance of the applied growth substances. In general, penetration into immature stem tissue is assumed to follow closely the characteristics of foliar penetration (Bukovac, 1976). Foliar penetration must also be considered in our experiments with *P. vulgaris* cv. Mont D'Or Golden Butter, since in most cases described here treatment was applied to the base of the primary leaves. As, these leaves cover the apex it must be considered that, at least, some of the substance penetrated through the leaves. Studies using mature leaf disks of *Phaseolus* showed that 2,4-D can penetrate the leaves but that penetration rate is much affected by

TABLE VII. 1. Effect and amount of the different substances supplied in solution to the apex, when leaf form or shape was examined.

substance	amount (μg per apex)	effect
BUDR	0.92	ineffective[1]
2-TU	0.64	shape
2,4-D	0.4 to 1.0	shape and form

(1) This compound in lanolin did affect shape (See Table IV. 5).

environmental conditions (Sargent and Blackman, 1962). Similar results were obtained for penetration of NAA in leaf disks of Pyrus communis (Greene and Bukovac, 1972). If penetration into the apex is similar to penetration through the leaves then another explanation for the high quantities required must be found.

Once penetrated substances must move within the apex, and if penetration is through the leaves then this movement must occur from the leaves to the apex. Movement can occur from cell to cell through the plasmodesmata, in this case without meeting the cell walls as barriers (Hay, 1976).

Studies with 2,4-D showed that when treatment was applied to the leaves it accumulated in the young leaves and apex and relatively little moved to the roots (Hay and Thimann, 1956; Hallmen and Eliasson, 1972). It was suggested that this accumulation in immature tissue might be associated with the inability of these organs to export substances (Hay, 1976).

Clearly more data are required on both penetration and movement of substances at the apex. Autoradiographic methods appropriate to soluble compounds could be used to answer these questions.

2. PRIMORDIAL INITIATION AND CONTROL OF LEAF FORM

Leaf primordia are formed at the shoot apex in regular arrangement such that the position of a primordium can be exactly predicted. Researchers have long been puzzled

by this aspect of plant morphogenesis. Resulting from this interest a number of theories have been proposed to explain the regular inception of leaf primordia at the shoot apex. In this work some of our results are relevant to one of them, namely the field theory of phyllotaxis and leaf origin. This theory originated with the work of Schoute which was described by Wardlaw (1965) as involving inhibitory substances coming from the incipient primordia and from the apex. The substances coming from the primordia and from the apex are not necessarily the same. The field theory has very recently been revived in the work of Thornley (1975, 1976) and Veen and Lindenmayer (1977) who have produced computer models by simulating leaf origin and arrangement. It has been assumed that primordia and the apical dome itself produce a diffusible morphogen which inhibits initiation and outgrowth of new primordia. This morphogen diffuses downwards from the apical dome and diffuses isotropically from the produced primordia. The field theory states that a new leaf primordium is initiated at the point where the concentration of the inhibitory morphogen is at the minimum. In other words a leaf primordium develops where there is a failure of an inhibitory field to exert its effect.

Although the main support for this theory comes from the computer simulation through which was possible to generate phyllotactic arrangements compatible with the ones found in nature by making very simple assumptions, other kinds of evidence are provided as when exogenous application of growth substances is able to change the original phyllotactic arrangement. Such a change was obtained as a result of IAA treatment in <u>Tropaeolum</u>

(Ball, 1944) and in Lupinus (Varnell and Vasil, 1978). Modification in phyllotaxis was also obtained after treatment with TIBA in Hydrocharis (Cutter, 1965b) and Chrysanthemum (Schwabe, 1971). In this case elongation of the apex and subapical region was considered to be the primary effect of TIBA. GA_3 has been shown to modify leaf arrangement in Xanthium (Maksymowych and Erickson, 1977).

There are also arguments to be set against the field theory especially in connection with the theoretical models. The first important point is that a single controlling morphogen is very unlikely. It seems more realistic to assume that more than one morphogen is involved. This is also the opinion of Shabde and Murashige (1977) who working with excised apical meristem of Dianthus caryophyllus proposed that a balance of hormonal substances, synthesized in young leaves and relocated to the meristem, regulates new leaf initiation at the shoot apex. The other point is that no one has isolated or obtained direct evidence for the existence of any such morphogen. This is not surprising considering the minute size of the apex. As a consequence of this there is no evidence for the nature of the inhibitory morphogen(s).

Some versions of the field theory assume that leaf primordia and apical dome produce the inhibitory morphogen (e.g. Veen and Lindenmayer, 1977), whilst others do not mention the apical dome itself as a inhibitor producer (Richards, 1951; Thornley, 1975; 1976). There is no evidence that growth regulators are synthesized at the apical dome. However there is evidence for this production in young leaves (e.g. auxin,

Moore, 1969; gibberellins, Jones and Phillips, 1966; cytokinins, Shabde and Murashige, 1977). So one possibility which cannot be ruled out is that the apical dome is not involved in the production of the inhibitory morphogen and this (or these) is produced by the young primordia. Support for this view is found in the work of Smith and Murashige (1970) with excised apical meristems of Coleus. They found that IAA was the only hormonal requirement for growth and organogenesis; it had to be supplied to the medium if young leaf primordia were absent from the explant. The work of Shabde and Murashige (1977) with excised apical meristems of Dianthus caryophyllus can also be taken as evidence for this aspect. They found that in this species IAA and K were the hormonal requirements for organogenesis. Two pairs of leaf primordia and one pair of expanding leaves could substitute the hormonal supply to the medium. Our results can be set against this background.

Auxin is likely to be a candidate for the inhibitory morphogen. However we have shown that the auxin-like 2,4-D promotes rather than inhibits growth of the subapical annulus and when distribution of growth within the apex was examined growth of leaf primordia was promoted rather than inhibited; also application of 2,4-D does not prevent further development of the primordium, although it continues in a modified form. In some cases treatment led to production of multiple leaves. So it seems that our results can be taken as indirect evidence against an auxin being the leaf inhibitory morphogen. The alternative is that 2,4-D in this case might have been acting

as an anti-auxin. There is some evidence in the literature that at least in some cases 2,4-D can exibit an anti-auxin effect, either inhibiting auxin transport (Hay, 1956) or reducing the endogenous levels of auxin (Lockhart and Weintraub, 1957). There is no report in the literature that formation of the subapical annulus, that we have found with 2,4-D, was obtained with another auxin application, but there are some reports where a similar structure has been obtained as a result of TIBA treatment, although some differences can be pointed out between the effects of the two substances. Usually TIBA treatment appears to result in the formation of an almost leafless stem (Gorter, 1949; 1951; Wardlaw, 1953) and in *Phaseolus* we have not found leaf formation to be stopped by 2,4-D. From our work it was not possible to show the formation of the ring structure with TIBA. But the question remains as to whether if a higher amount of TIBA could be induced to penetrate the apex would show a modified pattern of growth. Taking these views into account further observations are fundamental to decide conclusively whether in the case of *Phaseolus*, 2,4-D modified apical growth by acting as an auxin or as an anti-auxin.

The changes in phyllotaxis following treatment with GA_3 reported by Cutter (1963, 1965b) for *Hydrocharis* and by Maksymowych and Erickson (1977) for *Xanthium* have not been observed for *Phaseolus*. Nor do the data show any major effect of treatment on primordial initiation rate in contrast to results with other species (Njoku, 1958; Williams, 1961; Davis, 1967; Maksymowych and Maksymowych, 1973). There is no evidence for

the involvement of gibberellin in determining the position or rate at which primordia are initiated in Phaseolus, even though it is clear from other work (Gray, 1957; Felippe, 1967) that mitotic activity in the subapical meristem is affected by treatment with GA_3, as is also internodal cell expansion.

Nucleic acid and protein synthesis inhibitors have been shown to affect rate of primordial initiation but we have concluded that this effect is more likely to be a consequence of reduced growth rate than a specific effect on leaf initiation, since the growth of all the parts of the seedling was reduced.

A key point in the development of trifoliate leaves in Phaseolus is when lateral enlargement of the primordial base is followed by formation of lateral leaflet and stipular initials. For a compound leaf to develop marginal growth occurs at localized centres from each of which a separate leaflet develops (Esau, 1977). This stage of development is effectively prevented by treatment with 2,4-D which promotes general equatorial expansion from the primordial flanks, such that enlargement of the initials is either prevented or masked by the enhanced growth associated with annulus production. As a result, unifoliate leaves are produced since development of the main primordial axis is not prevented, even though leaflet shape is modified. It is regarded as significant that 2,4-D treatment leads to at least some plants where leaves in the primary and first trifoliate positions are of broadly similar shape. It may also be worth pointing out that of all the substances applied to the apex only 2,4-D has prevented development of lateral leaflet initials.

Some conditions which do not involve directly growth substances are thought to be important in the control of leaf

form. Allsopp (1953a, 1953b, 1954, 1965, 1967) attributes heteroblastic development to nutritional conditions in Marsilea, these conditions being important by regulating the size of the apex. Related to this view our data show that although use of low nutritional levels does not modify leaf form, the apex is much smaller when entire primary leaves are initiated then when the first trifoliate leaf primordium is formed. It is of interest to mention that the only treatment (i.e. with 2,4-D), in this work, which led to production of entire leaves instead of the normal trifoliate led also to the development of a smaller apex. However conclusions based on the correlation of these effects must be considered cautiously and a causal connection cannot be regarded as certain.

Besides the importance of the size of the apex to the development of a simple or a complex leaf form another aspect seems to have some importance. This is the time factor. Allsopp (1954) suggested that a complex leaf needs a longer period of meristematic growth than a simple leaf. This idea is supported by the work of Hageman (in Allsopp, 1967) with Adiantum in which primordia of the first-formed leaves develop within few days whilst the adult leaves require several months to develop. Evidence for this point is also given by Cutter (1957) who working with Nymphaeaceae showed a relatively more rapid maturation of juvenile leaves compared to adult on the same plant. Feldman and Cutter (1970b) working with excised leaf primordia of Centaurea solstitialis cultured in aseptic conditions verified that in order to develop an adult leaf the primordium needed to remain at the apex for a minimum period of time. More recently Franck (1976) found that in Darlingtonia

californica the juvenile leaf differentiates much faster than the adult leaf. As indirect evidence of the importance of this factor between the initiation of the entire primary leaves primordia and a well advanced lamina expansion of these leaves there is an interval of a few days. However, despite the initiation of the first trifoliate leaf primordium occurs early in embryo development, its trifoliate condition is not visible during the remaining period of embryogenesis.

3. THE CONTROL OF LEAF SHAPE

Modifications in leaf shape, as the term was defined here, involve much slighter changes than when form is affected.

Gibberellic acid is known to affect leaf shape in a large number of species (see references in Chapter V). The effects are varied but most usually treatment results in the development of narrower entire or lanceolate leaves, with the production of lobed leaves as in Hedera (Robbins, 1960; Rogler and Hackett, 1975) being the exception. With Phaseolus the general effect of leaf narrowing is found. Two related questions can now be asked: firstly, by what mechanism is leaf shape changed to become narrower, and secondly, why does the final effect on leaflet vary with time of treatment? It has been suggested that treatment with gibberellin affects activity of the marginal and plate meristems (Maksymowych and Maksymowych, 1973). Narrowing of the blade could be the result of decreased activity in these meristems but so far there is little evidence for this and data of Felippe (1967) indicate

that GA_3-treated leaflets do not contain fewer cells than the untreated controls despite marked differences in blade shape.

The results from GA_3 treatment at different times suggest that it is the part of the primordium which is youngest at the time of treatment that is most sensitive to the compound and that this sensitivity is lost with time. There is a need for more detailed analyses of the effects of gibberellin treatment on rates and directions of cell division and expansion in the developing primordium.

Is there a role of endogenous gibberellin in controlling leaf shape or form? Work with *Hedera* suggests that there may be. In this species the juvenile condition, where leaves are lobed, is associated with abundant production of adventitious roots and high levels of gibberellin thought to originate in these (Frydman and Wareing, 1973a; b; 1974). In *Phaseolus* treatment with the growth retardant CCC leads to a reduction in gibberellin content and the broadening of the base of the trifoliate leaflets in contrast to the narrowing that follows treatment with GA_3 (Felippe, 1967). Enlargement of the leaf base has also been found by Dale (Unpublished) for derooted plants together with a substantial reduction in area. High endogenous levels of GA during seed development might be related to the development of the entire primary leaves in *Phaseolus*, since immature seeds were shown to have higher endogenous GA levels than mature seeds (Skene and Carr, 1961; Felippe, 1967; Skene, 1970). These observations are consistent with the view that endogenous gibberellin can modify shape, rather than bring about changes in form of leaves in this species.

Nucleic acid synthesis seems to be scarcely related to leaf shape in this species. Application of inhibitors of either DNA or RNA synthesis modified slightly the base of the primary leaves. More detailed observations are required to provide evidence of whether this effect resulted from inhibition of cell division or cell expansion or both in that specific part of the leaves. Heslop-Harrison (1960, 1962) found 2-TU to inhibit cell division in leaves of Cannabis sativa, this effect being especially remarkable in the palisade layer and less evident in the upper epidermis of the basal parts of expanding leaves.

4. CONCLUSIONS AND FUTURE WORK

To sum up three points at which growth substances may affect leaf development can now be identified. Firstly, primordial initiation may be affected by morphogens at the apex although this notion remains speculative and with only indirect evidence to favour it. Secondly, early primordial development may be controlled by morphogens which determine the pattern and directions of growth and thus the general nature of the final form, for instance whether the leaf is uni or trifoliate. Thirdly, there may be long-term effects of growth substances acting during the whole phase of lamina growth and expansion to modify the final shape without affecting initial form.

The field theory of primordial origin seems to be the best explanation we have so far of how primordia are initiated. However this is not generally accepted and Halperin (1978) considers that instead of involving growth substances with the organ initiation sites it might be more fruitful to consider

these sites as regions where physical parameters are altered in such a way as to shift the direction of cell enlargement.

The only treatment with growth substances which, for *Phaseolus vulgaris*, changed the pattern of apical growth appeared to affect mainly cell division with smaller effects on cell expansion. An important question arises from this, namely: Is the whole apex involved in this apical modification? Although definite evidence could not be presented the answer to this question is likely to be no. It is suggested that leaf primordia and apical dome are specifically involved in these modifications.

Modifications in leaf shape and especially in leaf form were particularly difficult to obtain. Even where immature seeds were treated the shift from unifoliate to trifoliate leaves habit was not affected. The nearest we came to changing leaf form was to produce such modifications that in both primary and trifoliate leaves the final shape was similar. This difficulty suggests that in *Phaseolus* this morphogenetic leaf pattern is a very stable one and in this aspect it probably would have been more fruitful to have used a different system to study the control of leaf form and shape. Most aspects of primordial initiation and further development up to production of the adult leaf are still open to research. In particular the role of endogenous growth substances remains to be demonstrated. Experiments with exogenously-applied compounds such as 2,4-D now need to utilise more elaborate techniques to identify amounts penetrating the apex and the sites at which the compound accumulates and exerts effects.

This is a fascinating area but because of the technical difficulties the apex and primordia are not always attractive experimental material. It is a challenge to the physiologist to increase the understanding of developmental processes which occur in the apex despite these difficulties.

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